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Syphilis testing algorithms: A review

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Abstract

The methods and strategies used to screen for syphilis and to confirm initially reactive results can vary significantly across clinical laboratories. While the performance characteristics of these different approaches have been evaluated by multiple studies, there is not, as of yet, a single, universally recommended

algorithm for syphilis testing. To clarify the currently available options for syphilis testing, this update will summarize the clinical challenges to diagnosis, review the specific performance characteristics of treponemal and non-treponemal tests, and finally, summarize select studies published over the past decade which have evaluated these approaches. Specifically, this review will discuss the traditional and reverse sequence syphilis screening algorithms commonly used in the United States, alongside a discussion of the European Centre for Disease Prevention and Control syphilis algorithm. Ultimately, in the United States, the decision of which algorithm to use is largely dependent on laboratory resources, the local incidence of syphilis and patient demographics.

Key words: Syphilis; Treponemal infection; Immunoassay; Reverse sequence screening; Rapid plasma regain; *Treponema pallidum* particle agglutination test; Automation; Algorithm; Primary infection; Late latent infection

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Core tip: Many laboratories have adapted automated immunoassay methods for syphilis screening in the past decade. Since measurement of antibodies to *Treponema pallidum* (treponemal) antigens cannot readily distinguish current from past infection, additional tests, including traditional non-treponemal tests, are required to further clarify the disease state. As the incidence of syphilis and population demographics influence test performance, and due to local differences in the way clinical follow-up is offered, there is no single approach to syphilis testing that is universally applicable.

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INTRODUCTION

Over the past decade, the strategies and algorithms used by clinical laboratories to screen for and confirm syphilis have changed significantly. Among the three algorithms currently used for syphilis testing, there is not one that is universally recommended by medical or public health agencies. Two of these approaches, the traditional and reverse syphilis screening algorithms, were detailed in a point/counterpoint commentary in a recent issue of the *Journal of Clinical Microbiology*^[1].

Readers lacking familiarity with syphilis testing might be surprised to learn that assays with suboptimal sensitivity and specificity are used to confirm considerably more sensitive and specific tests. These readers might also be surprised to hear that for the reverse screening algorithm, initial syphilis screening assays are often fully automated, whereas confirmatory tests are performed manually and can be subjective to interpret. To understand the options available for syphilis testing today, this update will summarize the clinical challenges to diagnosis and the specific performance characteristics of treponemal and non-treponemal tests. Additionally, select studies evaluating the various approaches to syphilis screening over the past decade will be summarized.

CLINICAL CHARACTERISTICS OF SYPHILIS INFECTION

Primary syphilis infections, caused by the spirochete *Treponema pallidum* (*T. pallidum*), can be identified visually by the presence of a characteristic sore with a raised border, referred to as a chancre, typically observed on the genitalia. While lacking in sensitivity due to the painless nature of the lesion, visual diagnosis offers good specificity. This is particularly true in resource poor nations where syphilis continues to be commonly encountered and readily recognizable by local physicians, with reported prevalence rates approaching 10% in some countries^[2,3]. In industrialized nations, widespread treatment with potent antibiotics has had a dramatic effect on the prevalence of syphilis, as have safe sex initiatives and the availability of barrier protection methods. At one time there was hope that syphilis would be completely eliminated in industrialized countries, however, the opposite has occurred and syphilis has been on the rise for the past two decades, particularly among men having sex with men^[4]. Primary syphilis is uncommonly encountered by physicians who practice in resource rich nations, and therefore the infection may not be included in the differential diagnosis for genital lesions or other, more chronic disease manifestations.

Without treatment of primary syphilitic infections, patients may progress to secondary syphilis, which can manifest with a wide range of systemic symptoms, including maculopapular rash, characteristically affecting

the palms and soles in addition to the torso and extremities, fever, malaise and diffuse lymphadenopathy. Notably, these symptoms are significantly more severe among immunocompromised individuals, particularly human immunodeficiency virus (HIV) positive patients^[5]. Due to the low prevalence of syphilis in some regions, and the lack of medical care in others, secondary syphilis infections may be misdiagnosed or not diagnosed at all, in both cases leading to a lack of curative treatment.

Following secondary syphilis (several months to a year after infection), patients enter a latent stage of infection that is often entirely asymptomatic. Many years later, 15%-40% of infected individuals may develop late or tertiary syphilis^[3], which can present with a variety of serious health problems, including central nervous system, cardiovascular or ocular involvement.

PERFORMANCE OF TREPONEMAL AND NON-TREPONEMAL ASSAYS FOR DETECTION OF SYPHILIS

Currently, two different types of serologic tests are available to screen for syphilis and both approaches measure antibodies in serum or plasma. Non-treponemal assays, including the rapid plasma reagin (RPR) and Venereal Disease Research Laboratories (VDRL) tests, measure antibodies to lipoidal products (*i.e.*, cardiolipin), which are released from host cell membranes following injury or infection with *T. pallidum*. These assays have been used for over a century for syphilis screening^[2,3,6]. Since the 1960's, methods that measure antibodies specific to *T. pallidum* proteins (*e.g.*, R17, R15, R44.5 and R47), referred to as treponemal tests, have been available and are increasingly used as laboratories migrate from microscopic testing methods to fully automated immunoassay systems^[7].

Over the past decade multiple new treponemal tests have been developed. These methods incorporate highly purified, recombinant *T. pallidum* protein antigens, as well as polyvalent conjugates able to detect both IgM- and IgG-class antibodies to syphilis and are marketed as "total syphilis antibody" assays. Comparison studies between these methods, when used for screening, generally demonstrate very high concordance and excellent specificity (generally > 99%)^[8-12].

Both non-treponemal and treponemal methods will fail to detect some cases of primary syphilis, since the antibody response to infection is not immediate^[13]. Humoral antibodies generally appear and become detectable within 4 wk following chancre formation^[2]. There is some evidence that treponemal assays are more sensitive in cases of primary syphilis. In a large study, 9137 patients who were initially positive by an enzyme immunoassay (EIA) and RPR negative were recalled 8 wk after initial screening; 0.59% of these patients seroconverted to RPR positive at the second draw date, consistent with a diagnosis of primary

syphilis^[14].

Another challenge to detection of early syphilis, regardless of the method, is the host's immunostatus. Immunosuppressed individuals, whether due to infection (e.g., HIV) or immunomodulatory therapy can have a delayed or suppressed serological response^[15]. In general, the sensitivity of the common non-treponemal tests (e.g., RPR and VDRL) during primary syphilis infections ranges from 74% to 100%, depending on the time of sample collection compared to the initial exposure. A similar sensitivity, ranging between 70% and 100%, has been documented for the traditional treponemal assays, including the *T. pallidum* particle agglutination test (TPPA) and the *T. pallidum* microhemagglutination assay^[2]. Importantly, the sensitivity of both treponemal and non-treponemal assays improves to nearly 100% during secondary and latent infection. However, while treponemal assays will remain highly reactive during tertiary manifestation of disease, non-treponemal assays lose some sensitivity during this stage (range 71% to 73%).

Multiple factors can influence the specificity of both treponemal and non-treponemal tests. While false positive results are generally less common with treponemal assays, certain host conditions have been associated with inaccurate results, including systemic lupus erythematosus, the presence of high levels of heterophile antibody in patients with infectious mononucleosis and in some individuals with leprosy, collagen disease and drug addiction^[16]. Regarding non-treponemal assays, the most common cause of non-specific reactions is due to biological interference. The lipoidal products released during syphilis infection are also produced in many other infectious and non-infectious conditions, including Epstein-Barr, hepatitis, varicella, measles, tuberculosis, malaria, lymphoma and certain autoimmune diseases^[7]. Notably, none of these diseases produce false positive results by treponemal methods, thus making treponemal assays significantly more specific for syphilis. Additionally, infections caused by other *Treponema* species, including *T. pertenue* the causative agent of yaws, can lead to false positive results by either method^[17]. Finally, due to an increase in immunological activity and maternal antibody production, pregnancy can lead to inaccurate results by either method, posing an interpretive challenge to syphilis testing results.

Patients tested by non-treponemal methods may sometimes yield falsely negative results when extremely high levels of antibody are present, a result of the "prozone effect". Best practice requires that patient sera are analyzed at two distinct dilutions to identify this potentially confounding reaction. The sensitivity of non-treponemal tests is also suboptimal in latent disease, as the destructive activity of the bacteria at this stage is minimal leading to the absence of circulating lipoidal antigens. In contrast, treponemal antibodies continue to

circulate and are detectable for decades. Furthermore sera from patients who are successfully treated for syphilis will demonstrate decreased non-treponemal antibody titers, and 12-24 mo post-infection may be negative by these assays, while treponemal antibodies will continue to be positive^[2,6]. Patients who are re-infected with syphilis, years after successful treatment for their initial infection, will seroconvert from RPR negative to RPR positive status^[18].

Separate testing for IgM and IgG antibodies to *T. pallidum* using treponemal assays, is currently performed in many countries. IgM assays are considered less specific than IgG tests and concordance between the different available IgM tests is low, however a positive IgM result may be useful to provide supplemental information when an IgG screening assay is reactive^[11]. In the United States, tests detecting only IgM-class antibodies to syphilis are not routinely available and no assay supporting separate measurement of IgM has been FDA-cleared in the last twenty years.

THE SYPHILIS TESTING ALGORITHMS AND ASSOCIATED CLINICAL DILEMMAS

The traditional and reverse sequence syphilis screening algorithms

The reverse sequence syphilis screening (RSSS) algorithm first uses a treponemal assay as a screen, followed by confirmatory testing of reactive samples with a non-treponemal test. The term "reverse" is appropriate, because for many decades the traditional syphilis screening algorithm involved initial screening using a non-treponemal test, followed by confirmation of reactive results using a treponemal test. A distinct advantage of the RSSS is that initial screening with a fully automated, treponemal test solves the workflow challenge faced by hospital and reference laboratories performing thousands of tests every month. However, as with any assay, when a treponemal test is used as the screen in an environment where the prevalence of syphilis is low, many of the reactive results will be false. The advantage of the RSSS for laboratories however, is that the number of required manual confirmatory tests will be significantly decreased, particularly in industrialized countries where reactive rates of treponemal assays range from 1%-3%.

Since non-treponemal tests generally become non-reactive 12-24 mo after treatment of early disease, a negative non-treponemal confirmatory test does not definitively indicate that the original treponemal result was falsely reactive. A treponemal reactive/non-treponemal non-reactive result suggests that the patient is unlikely to have active disease, however this result does not rule out acute infection or late/latent disease. To conclusively determine whether the initial treponemal result was falsely reactive, the RSSS algorithm requires that a second treponemal assay,

different from the initial screening treponemal assay, be performed. If this assay is reactive, the initial screening result is confirmed indicating that the patient has been exposed to syphilis at some time in the past and further clinical evaluation is required to stage the infection. If the second treponemal assay is non-reactive, this suggests that the initial treponemal test was likely a false screening result and the patient is unlikely to have been exposed to syphilis^[19-21].

Many different treponemal assays can be used as the “third test” for the RSTS, including the TPPA test, the Fluorescent Treponemal Antibody Absorption test (FTA-ABS), multi-parameter line immunoassays and western blots. However, the ability of different treponemal tests to detect late untreated syphilis has not been evaluated in great detail and some studies suggest that there can be discrepancies^[22-24]. The prevalence of syphilis in specific populations (such as in prisoners) may also affect the development of an algorithm for retesting results when treponemal and non-treponemal results do not agree^[25].

The 2008 Center for Disease Control and Prevention guidance on syphilis^[26] proposed that if a patient has “not been previously treated, patients with reactive results from treponemal tests and nonreactive results from non-treponemal tests should be treated for late latent syphilis”. Hence the next intervention may be a tense conversation between doctor and patient. However, because medical records are often incomplete and because patients can be forgetful or less than fully truthful, determining whether a patient was previously treated for syphilis is not straightforward^[27,28]. Additionally, in an era when exposure to β -lactam antibiotics, tetracyclines, and macrolides for indications other than syphilis is widespread, there may be no practical way to identify a history of “non-observed treated” syphilis^[27]. For a physician, given discordant syphilis results, treatment with an inexpensive and highly effective drug may be a straightforward option; it is a low-risk and inexpensive choice that essentially eliminates the risk of complications from a serious infection. However, treatment in some cases will be unnecessary and possibly disturbing to the patient and the patient’s family.

Finally, in many regions of the world, treatment is only possible when the patient returns to the clinic. While a confirmatory test on a new specimen may increase laboratory confidence in the result, patients may not return for a second visit. Losing patients to follow up can be a significant concern in testing centers and geographic areas where syphilis is commonly encountered^[29].

The European Centre for Disease Prevention and Control syphilis algorithm

The 2008 European Guidelines on the Management of Syphilis [European Centre for Disease Prevention and Control (ECDC) guidelines] took a large step away

from the traditional syphilis algorithm^[30]. The authors recommended a treponemal test for screening, with an added IgM test when primary syphilis is suspected, followed by the use of a different treponemal test, preferably performed on second specimen, for confirmation. A treponemal EIA, TPPA or FTA-ABS assay (or multiple tests) may be used for confirmation following completion of the two treponemal tests. An IgG immunoblot is recommended as a supplementary confirmatory test if the first two antibody-based tests provide discrepant results. The ECDC guidance document recommends the use of RPR testing to monitor the response to therapy and greatly limits the situations where it may be used for screening. This algorithm offers two clear advantages compared to RSTS: Only two methods will be required to perform routine screening and confirmation, instead of three in cases of discordant results, and both of these methods can be fully automated.

Regarding treatment, since the ECDC does not mandate a non-treponemal test, patients tested according to this guideline are likely to be treated, or retreated, unless there are records to support prior therapy^[30].

Other international guidelines published since 2008 maintain a role for non-treponemal testing. A proposed Eastern European guideline called for treponemal and non-treponemal testing, but did not specify the order in which they should be performed^[31]. With regards to other international countries, most recently a guideline from Canada proposed the use of either the traditional or RSTS algorithm depending on laboratory needs and word flow restrictions^[32].

The three most common syphilis testing algorithms are illustrated in Figure 1.

COMPARISON OF THE THREE SYPHILIS SCREENING ALGORITHMS

Since the introduction of the RSTS and ECDC algorithms, numerous studies have compared these new methods to the traditional testing algorithm. Performing such comparison studies however is hampered by several significant challenges, including: (1) in countries and regions where syphilis is uncommon, a large number of sera must be tested to identify clinically positive patients; (2) blood bank laboratories perform a high volume of tests for syphilis, however any patient with syphilis, has been removed from the donor and therefore, the usage of stored blood bank samples is unlikely to be helpful; (3) in regions with a high incidence of syphilis, the likelihood of a true positive is high and therefore, results from assay evaluation in these regions may not be directly applicable to regions where syphilis is not as prevalent; (4) patients may not be available for clinical follow-up, or may not recall prior treatment for syphilis. As mentioned above, syphilis may have been treated inadvertently,



Figure 1 Comparison of the three common syphilis testing algorithms. A: Traditional algorithm; B: RSSS algorithm; C: ECDC algorithm. +: Positive result; -: Negative result; RPR: Rapid plasma reagin; TPPA: *Treponema pallidum* particle agglutination test; EIA: Enzyme immunoassay; CIA: Chemiluminescence immunoassay.

Table 1 Selected studies evaluating the reverse sequence syphilis screening and European Centre for Disease Prevention and control algorithms in comparison to the traditional algorithm for syphilis testing

Screening method	Confirmatory method	Third method	No. of samples screened	% reactive results in initial screen	% of screening results confirmed	% of discrepant results confirmed by third method	Ref.
EIA/CIA	RPR	TPPA, FTA-ABS	12774	14.5%	49%	86%	CDC ^[19]
TPPA	CIA	N/A	24124	11.4%	99%	N/A	Tong <i>et al</i> ^[28]
TPPA	RPR	CIA	24124	11.4%	76%	99%	Tong <i>et al</i> ^[28]
Multiple EIA methods	RPR	TPPA/FTA-ABS	116822	5.6%	44%	83%	CDC <i>et al</i> ^[26]
CIA	RPR	TPPA, FTA-ABS	28261	4.1%	31%	89%	Hunter <i>et al</i> ^[21]
EIA/CIA	RPR	TPPA, FTA-ABS	127402	2.3%	39%	59%	CDC ^[19]
CIA	RPR	TPPA	21623	2.2%	42%	78%	Park <i>et al</i> ^[33]
EIA	RPR	TPPA, FTA-ABS, TPHA	1037025	2.0%	30%	84%	Mishra <i>et al</i> ^[15]
CIA	RPR	TPPA	15713	1.7%	82%	82%	Lee <i>et al</i> ^[24]

RPR: Rapid plasma reagin; TPPA: *Treponema pallidum* particle agglutination test; EIA: Enzyme immunoassay; CIA: Chemiluminescence immunoassay; FTA-ABS: Fluorescent Treponemal Antibody Absorption test; CDC: Centers for Disease Control and Prevention.

without the patient's knowledge. Researchers are often unable to retrieve medical records for many patients. All of these factors make estimates of biological and analytical false positives increasingly difficult; (5) large prospective comparison studies are expensive and costly because two or more methods must be run in parallel for an extended period; and (6) while the cost of "over diagnosis" and "overtreatment" can be roughly quantified, it is much harder to determine the cost of a "missed diagnosis" and "under treatment". As a result, meaningful financial analyses are difficult to perform

unless follow-up is available for patients who have negative screens.

Table 1 summarizes results from large studies (> 10000 patients) that were performed with sera from patients evaluated at a hospital or clinic. Each study compared the RSSS or ECDC algorithm to the traditional approach. The studies are sorted by the observed positivity rate in the initial screen. Some important and valuable studies have been omitted because the authors did not identify the number of positive results obtain in their initial screen^[34,35] or used multiple confirmatory

methods at the same time^[36], rather than following the approaches proposed in the new confirmatory algorithms discussed above, or reported on a much smaller patient cohort^[37,38]. In the listed studies which evaluated the RSSS, it is clear that the confirmation rate, using a non-treponemal test, such as the RPR, varied widely, reflecting differences in the populations screened. However, the confirmation rate of an initially reactive treponemal assay by a second treponemal tests was more consistent, typically > 80% in most reports.

Three other review articles have included analyses of the utility of the new algorithms. Binnicker^[39] described studies comparing the traditional and reverse algorithm published by 2011. Soreng *et al.*^[40] focused on the workflow advantages associated with a fully automated technology in the initial screen. The authors also mention that the discrepancies between TPPA and other treponemal methods are often associated with patients who were co-infected with HIV. The problem of inconsistent serological results in immunocompromised patients has been previously documented^[41] and is well known; it likely plays a significant role in comparison studies of syphilis results. An extended review by Morshed *et al.*^[42] includes discussion of the new algorithms as well as the current status of neurosyphilis, prenatal and congenital syphilis screening.

CONCLUSION

The CDC's 2008 discussion of the RSSS proposed that "the best algorithm would depend on the population studied"^[26]. In patient cohorts with little syphilis positivity, the traditional algorithm will lead to less follow up testing, but at an increased risk of missing early and late/latent syphilis. Laboratories that see very little syphilis may choose a strategy that minimizes the expense of screening. While the reagent costs of immunoassays are generally higher than RPR cards for screening, the semi-automated ELISA methods used in the previous decade have now been widely replaced by fully automated systems, which provide labor savings that offset the difference in reagent costs.

In the urban sexually transmitted disease clinic, where higher rates of syphilis are observed, or in developing countries where access to testing is limited, the likelihood of encountering both early syphilis and late/latent syphilis (LSS) increases. The RSSS algorithm is likely to identify more cases of untreated disease in these environments, but cases of treated disease will also be detected, with potential follow-up costs and a requirement to manage the social impact associated with identification of past infection.

These observations summarized here reflect what has been learned from a decade of experience with the reverse algorithm. Today the physician should evaluate any patient with a confirmed reactive immunoassay screen and a discrepant RPR result as a possible case of latent syphilis or an undiagnosed syphilis infection that

has been successfully, but unintentionally, treated^[27,43]. Determining the need for additional treatment will remain a decision to be made by the physician on a case by case basis^[44]. Finally, for patients with HIV and other immunosuppressive diseases, repeat testing (or periodic testing) is essential due to the possibility of a delayed serological response (with a negative screen) or re-infection (with a positive treponemal screen).

The ECDC recommended algorithm, which was published several years ago, has not received much attention in the published literature. While it will not identify re-infection in sexually active individuals, it does offer a fully automated and more reproducible workflow than the RSSS and eliminates the positioning of a subjective test in a confirmatory role. It may be suitable in testing situations where patient information will be available to help a physician clarify the disease history.

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Role of tumor associated macrophages in regulating pancreatic cancer progression

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Abstract

Pancreatic cancer has an overall 5-year survival rate of less than 5%. Unfortunately, patient survival has not substantially improved in the last couple of decades despite advances in treatment modalities that have been successful in other cancer types. The poor

response of pancreatic cancer to therapy is a major obstacle faced by clinicians. Increasing attention is being paid to how tumor cells and non-tumor cells influence each other in the pancreatic tumor microenvironment. Tumor-associated macrophages (TAMs) are a highlight in this field because of their vast presence in the tumor microenvironment. TAMs promote angiogenesis, metastasis, and suppress the anti-tumor immune response. Here we review the current understanding of the role of TAMs in regulating the progression of pancreatic cancer.

Key words: Pancreatic cancer; Tumor-associated macrophages; Tumor microenvironment; Macrophages

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Core tip: Pancreatic cancer remains one of the most deadly cancers with dismal 5-year survival rates. Increasing importance is being given to the role of macrophages in pancreatic cancer. Tumor-associated promote angiogenesis, metastasis, and suppress the anti-tumor immune response. Targeting macrophages within the tumor microenvironment is an attractive novel therapeutic approach. Here we review the current understanding of the role of tumor-associated macrophages in the progression of pancreatic cancer.

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PANCREATIC CANCER

Pancreatic cancer is the eighth leading cause of death from cancer in men and the ninth leading cause of

death from cancer in women worldwide and is lethal in more than 95% of cases^[1]. The incidence of pancreatic cancer ranges from 1 to 10 cases per 100000 people, is generally higher in developed countries, and has remained stable for the past 30 years relative to the incidence of other common solid tumors^[2]. It is more common in the elderly and less than 20% of patients present with localized, potentially curable tumors^[3]. The overall 5-year survival rate among patients with pancreatic cancer is less than 5%^[4].

The causes of pancreatic cancer are not yet fully understood. Environmental factors have been implicated, yet evidence of a causative role only exists for tobacco use. The risk of pancreatic cancer in those that smoke is 2.5 to 3.6 times that in nonsmoking individuals^[5]. Several medical conditions are associated with an increased risk of pancreatic cancer, including diabetes, chronic pancreatitis, chronic cirrhosis, a high-fat diet, and a high-cholesterol diet^[3]. Additionally, it is estimated that 5% to 10% of cases are inherited, however currently there is no effective screening tool^[6].

Pancreatic ductal adenocarcinoma (PDAC) is the most common form of pancreatic cancer. The cancer arises in the ductal epithelium and evolves from premalignant lesions to fully invasive cancer. The lesion called pancreatic intraepithelial neoplasia (PanIN) is the best characterized histologic precursor of pancreatic cancer^[3]. The progression from dysplasia to invasive carcinoma is paralleled by the accumulation of mutations that include activation of the KRAS2 oncogene, inactivation of the tumor-suppressor gene CDKN2A, and inactivation of the tumor-suppressor genes TP53 and DPC4^[7].

In the past two decades, almost no progress has been made in the clinical management or outcomes of patients with pancreatic cancer^[1]. Approximately 70% of patients diagnosed with pancreatic cancer die from extensive metastatic disease while 30% present with limited metastasis, yet the majority of these patients have bulky primary tumors^[8]. The poor response of pancreatic cancer to therapy is a major obstacle faced by clinicians. Patient survival has not substantially improved despite advances in treatment modalities that have proven successful in other cancers (*i.e.*, breast, lung, colorectal, and melanoma)^[9-11]. Novel biologic therapies such as cancer vaccines and methodologies using therapeutic gene transfer have also been either ineffective or shown limited promise^[12]. The current standard chemotoxic drug, gemcitabine, has resulted in only a modest improvement in survival, and newer therapies show only marginal improvement^[13]. Surgical resection is ultimately the most effective for pancreatic cancer. However, only 15% to 20% of patients are considered candidates for surgical resection^[6] and outcomes of surgery alone are poor as less than 15% of resected patients are alive 5 years post-operatively^[14]. Therefore, the development of new treatment strategies for the vast majority of patients afflicted with pancreatic cancer is vital.

PANCREATIC TUMOR MICROENVIRONMENT

The development of PDAC is marked by increasing desmoplasia, which results in a vast stroma that often exceeds the epithelial component of the tumor. Unlike most adenocarcinomas whose volume is comprised primarily of transformed cells, a pancreatic tumor is comprised of fibro-inflammatory stromal elements and islands of neoplastic epithelium^[15,16]. Recent evidence suggests that far from being a passive observer, pancreatic tumor stroma affects both cancer progression and clinical outcome. In PDAC, activated pancreatic stellate cells have been shown to support tumor growth and immune dysfunction^[17]. In particular, by releasing nutrient growth factors, such as insulin-like growth factor 2 and PDGF, into the tumor microenvironment, the stromal component of pancreatic cancer has been closely linked to both tumor growth and invasiveness^[18-20]. In addition, chemotherapy resistance has been correlated with the extent of tumor desmoplasia as the stroma is thought to be a physical barrier to cytotoxic agents reaching the neoplastic epithelial cells^[21,22]. Consequently, increasing attention is being paid to how tumor cells and non-tumor cells influence each other in the tumor microenvironment. Tumor-associated macrophages (TAMs) are a highlight in this field. Here we review the recent evidence for the role TAMs possess in promoting pancreatic cancer progression.

MACROPHAGE POLARIZATION

The immune system plays a critical role in the response to infection and elimination of foreign pathogens. In the context of cancer, the immune system recognizes antigens produced by tumor cells; therefore the cancer cell is deemed a foreign pathogen. Histopathological analyses of human tumors have provided evidence that variable numbers of infiltrating immune cells, such as macrophages, mast cells, granulocytes and myeloid-derived suppressor cells (MDSCs), are found in most cases infiltrating or surrounding tumor beds both in the core and at the invasive front of the tumor^[23].

Macrophages play an important role in the innate immune system. They are essential in removing dead or dying cells and debris *via* phagocytosis, and activate the adaptive immune response^[24]. After tissue damage, macrophages organize immune defenses and coordinate the tissue repair process. Normally, this process is self-limiting, yet if it doesn't resolve it leads to chronic inflammation characterized by an alteration in the immune cell types involved, including an increase in infiltrating macrophages^[25].

Macrophages are activated in response to environmental signals, including microbial products and cytokines. Analogous to the dichotomous differentiation of T helper cells giving rise to T helper type 1 (Th1) and T helper type 2 (Th2) T cells with distinct cytokine

expression patterns and immunological functions, two fundamental macrophage phenotypes have been delineated. Activated macrophages are functionally divided into two subtypes: M1 (classical activated) and M2 (alternative activated)^[26]. Th1-related cytokines such as Interferon gamma (IFN- γ), and lipopolysaccharide (LPS), polarize macrophages to M1^[24]. M1 macrophages are characterized by IL-12^{hi}, IL-23^{hi}, tumor necrosis factor alpha (TNF α)^{hi}, IL-10^{low}, CXCL9^{hi}, CXCL10^{hi}, ROI^{hi}, RNI^{hi}, COX1^{low}, COX2^{hi} and iron uptake phenotype^[24,27]. M1 macrophages preferentially induce anti-tumor Th1 cells, whereas Th2 cytokines such as IL-4 and IL-13 induce M2 macrophages^[28]. These macrophages express IL-12^{low}, TNF α ^{low}, IL-10^{hi}, IL-1decoyR^{hi}, IL-1RA^{hi}, Arginase1^{hi}, CCL17^{hi}, CCL18^{hi}, CCL22^{hi}, CCL24^{hi}, COX1^{hi}, COX2^{low}, iron release, and increased phagocytic activity^[24]. M2 macrophages initiate pro-tumorigenic actions by promoting angiogenesis, tissue repair, and inducing immune suppression *via* induction of Th2 cells^[27]. It is worth noting that M1 and M2 macrophage designations are extreme ends of a fluid scale. In fact, there is ample literature describing the plasticity of macrophages. Macrophages receive signals from the microenvironment in which they reside^[27] and it is the integration of these signals that ultimately determines the macrophage subtypes.

MACROPHAGE RECRUITMENT

The development and progression of pancreatic cancer has been linked to inflammation^[29-31], with chronic inflammation being a risk factor for the development of PDAC^[32-35]. The inflammatory microenvironment that is characteristic of PDAC supports tumorigenesis through paracrine crosstalk between tumor cells and immune cells^[36]. Importantly, inflammatory conditions are not only prominent in the tumor microenvironment but also present in the peripheral blood of PDAC patients, thus highlighting a role for peripheral immune cells in disease progression^[37]. Once established, PDAC is characterized by marked leukocyte infiltration.

TAMs are the main population of inflammatory cells found in the tumor microenvironment of solid tumors^[38]. TAMs develop in response to tumor-induced cytokines, chemokines, and vascular endothelial growth factor (VEGF)^[26,39,40]. Additionally, it is also well documented that granulocyte macrophage colony-stimulating factor (GM-CSF), released from the tumor microenvironment, regulates TAM recruitment, maturation, and differentiation. The matricellular glycoprotein secreted protein acidic and rich in cysteine (SPARC), an intracellular matrix protein, may influence macrophage infiltration and distribution in murine pancreatic tumors. Murine macrophages expressing F4/80 are more plentiful in tumors from wild-type SPARC mice and are distributed at the tumor margins^[41]. Additionally, monocyte chemoattractants, such as the alarmins, may also play a role in the recruitment of monocytes and other macrophage precursors^[42]. A factor released by dying

tumor cells, the high mobility group box protein 1, is found in areas where TAMs reside^[43].

TAMS CAN PROMOTE TUMOR PROGRESSION

The role that TAMs play in the tumor microenvironment remains controversial. For instance, in colorectal tumors, TAMs are pro-inflammatory, and are anti-tumor, which has been associated with a good prognosis^[44]. However, in most tumors, the protumoral role of TAMs is supported by clinical studies that have reported a correlation between high macrophage content and poor prognosis^[45-49]. Epidemiological data suggests that a tumor microenvironment that is rich in macrophages will lead to a more aggressive tumor with the potential for metastasis^[50].

In pancreas cancer, there is evidence that TAMs infiltrate early lesions or PanINs and persist through invasive cancer^[35]. It is well documented that early and advanced lesions have significant increases in macrophage infiltration, compared to normal pancreatic tissue. Moreover, evidence suggests that TAMs may regulate PanIN development. TAMs produce IL-6 in PanIN lesions of Kras^{G12D}-expressing mice, therefore inducing STAT3 signaling and promoting cancer progression^[51]. Additionally, pancreatic cancer cell proliferation *via* sonic hedgehog production has been linked to NF κ B-activated monocytes^[52]. Liou *et al.*^[33] identified the macrophage-secreted inflammatory cytokines RANTES and TNF as mediators of acinar cell dedifferentiation and tumor initiation. These cytokines act *via* the activation of NF κ B and its target genes involved in regulating survival, proliferation, and degradation of extracellular matrix. Furthermore, the authors identified matrix metalloproteinases (MMPs) as targets that drive dedifferentiation and show that MMP inhibitors may be efficiently applied to block inflammation-induced dedifferentiation^[33]. Helm *et al.*^[53] conducted studies on the functional impact of TAMs from human PDAC tissues on premalignant and malignant pancreatic ductal epithelial cells. Both cell types acquired an elongated cell shape along with an increased expression of vimentin and a reduced expression of epithelial E-cadherin when indirectly cocultured with TAMs from PDAC tissues^[53]. When the cells were cocultured in the presence of polarized M1- and M2-macrophages, to elucidate whether pro- or anti-inflammatory properties account for these effects, similar to TAMs, both macrophage subsets induced epithelial-mesenchymal transition alterations and even greater invasiveness in the malignant epithelial cells^[53]. Similar findings have been reported by others, further elucidating the impact of pancreatic cancer cell interaction with macrophages on the differentiation and function of macrophages and behaviors of pancreatic cancer cells^[54]. Tumor growth inhibition following clodronate depletion of macrophages further clarifies the

pivotal role macrophages have in tumor progression^[55].

M2 macrophages

Macrophages often express an M2-phenotype at the tumor site^[56]. During the early stages of pancreas tumor initiation and development, M1-macrophages are more abundant^[57,58] and as the cancer progresses, macrophages switch to an M2-like phenotype^[27,59-61]. In human patients, more M2-macrophages were detected in the context of pancreatic cancer than in chronic pancreatitis^[62]. Furthermore, in pancreatic cancer patients a higher number of M2-macrophages was linked to larger tumor size, early liver recurrence, local recurrence, and reduced survival^[62]. Similar results have been reported by others^[63-65]. A higher percentage of M1-macrophages has been associated with longer survival^[66].

Interestingly, Tjomsland *et al.*^[67] found evidence that high gene expression levels of CD68 (general macrophage marker) might be associated with poor prognosis and thus decreased survival following tumor resection, whereas a CD163-dominating M2-macrophage phenotype conferred a survival advantage^[67]. Helm *et al.*^[68] have shown that human PDAC-associated TAMs concomitantly exhibit characteristics of pro-inflammatory M1-macrophages (e.g., HLA-DR, IL-1B, TNF- α) and anti-inflammatory M2-macrophages (e.g., CD163, IL-10). The authors also found HLA-DR and CD163 double positive cells by immunohistochemistry staining in pancreatic tissue of PDAC and chronic pancreatitis^[68], thus confirming that macrophages with a mixed phenotype sharing pro- and anti-inflammatory properties are abundant in PDAC and are already present in the setting of chronic pancreatitis. These findings challenge the dichotomous concept of M1- and M2-macrophages. Importantly, their location in the tumor and the tumor stage might affect their ability to contribute to tumor promotion^[27], which may explain conflicting findings.

Given the aforementioned findings, reprogramming TAMs towards a more anti-tumorigenic phenotype may prove promising. An agonist monoclonal antibody against CD40, a co-stimulatory protein found on professional antigen-presenting cells, has demonstrated efficacy in mouse models of PDAC^[69]. By reprogramming M2 TAMs into M1 TAMs using a CD40 agonist, tumor immune surveillance was restored^[69]. This increased the therapeutic efficacy of gemcitabine. These results show that tumor immunosurveillance can at times be governed strictly by innate immunity under the regulation of the CD40 pathway. A CD40 agonist has also demonstrated efficacy in human patients with PDAC^[70] when delivered in combination with the chemotherapeutic agent gemcitabine, ostensibly *via* the anti-tumor activities of macrophages^[71]. Low-dose tumor irradiation may enable recruitment of antitumor T cells^[72]. Using a mouse model, Klug *et al.*^[72] have shown that irradiation triggers the polarization of M2-macrophages toward M1-macrophages that express

iNOS. They further show that iNOS activity was responsible for vascular normalization and activation, T cell recruitment, and tumor rejection. The authors also obtained similar results when transferring irradiated macrophages and T cells, thus representing a promising adjuvant therapeutic strategy^[72]. These results hold great promises in the treatment of pancreatic cancer.

Immune suppressive activities of TAMs

Tumor immunosuppression is a recognized mechanism for regulating tumor growth and when it succeeds, tumor progression continues mostly unchecked. TAMs have been described as key players of the tumor microenvironment contributing to immunosuppression by secreting TGF- β , IL-10, and arginase 1^[73-75]. TGF- β promotes TAM polarization to an M2-phenotype, further promoting TGF- β release and immunosuppression^[56]. Additionally, TGF- β promotes Th2 cell differentiation, resulting in an inefficient antitumor response^[76]. Interestingly, Zhang *et al.*^[77] provide a novel model to explain the paradoxical role of TGF- β in the development of PDAC. Although TGF- β receptor signaling may inhibit cancer cell growth in the early stages of PDAC, its promoting role in angiogenesis may improve the long-term survival and progress of PDAC, thus it appears to be tumorigenesis-enhancing in the later stages of tumor progression^[77]. TAM-derived IL-10 suppresses IL-12 expression^[78], prevents *in situ* DC maturation^[74,79], and suppresses IFN- γ release^[80]. However, IL-10 may have a role in the antitumor immune response due to immunostimulating properties^[81-83]. Arginase 1, a marker for M2-macrophages that is expressed in tumors, causes dysregulation of the T cell receptor (TCR) signal resulting in a deficient CD8⁺ T cell response^[75,84]. Using a murine lung carcinoma model, Rodriguez *et al.*^[85] demonstrate that a subpopulation of mature tumor-associated myeloid cells express high levels of arginase 1. The authors also show that depletion of extracellular L-Arginine by tumor-associated myeloid cells blocked the expression of the TCR CD3 ζ and inhibited antigen-specific T cell proliferation^[85].

In human hepatocellular and ovarian carcinoma, CD14⁺ myeloid cells suppress autologous T cell proliferation and IFN- γ expression *in vitro* and nullify anti-tumor T cell activity during *in vivo* adoptive transfer experiments^[86,87]. In pancreatic cancer, TAMs have a significant immunosuppressive role. CCR2 or CSF1R blockade with gemcitabine reduced TAM numbers, increased cytotoxic T cells, and decreased FOXP3⁺ Treg infiltration compared to gemcitabine alone^[88]. These findings suggest that a reduction in macrophage infiltration at the tumor site resulted in an improved anti-tumor response in pancreatic cancer. More recently, using a retrospective cohort of patients with PDAC, Di Caro *et al.*^[89] report that the density of macrophages is a critical determinant of patient responsiveness to postsurgical conventional chemotherapy. *In vitro*, chemotherapy prevents the tumor-protective role of TAMs and reinstates their antitumor function, by a T-cell

independent mechanism^[89]. Targeting TAMs may be beneficial to tumor prognosis and in some cases be sufficient to ignite an effective antitumor action.

In addition to bona fide macrophages, there is extensive literature on abnormal accumulation of immature myeloid cells, known as MDSCs, which accumulate in the spleen and tumors as a consequence of tumor-associated changes in myelopoiesis and play a critical role in immunosuppression^[90]. MDSCs represent a heterogeneous population of cells and are Gr-1⁺CD11b⁺^[91]. Moreover, *in vitro*, Gr-1⁺CD11b⁺ MDSCs have the ability to impair T cell responses^[92]. Although the relationship between MDSCs and TAMs is not yet entirely clear, in the context of tumor-derived factors, it has been suggested that MDSCs have the ability to differentiate *in vitro* into macrophages with immunosuppressive characteristics^[93].

In pancreatic cancer, MDSCs may play a significant role in tumor progression. Using a murine model of PDAC, Bayne *et al.*^[94] demonstrate that GM-CSF, derived from the tumor, recruits Gr-1⁺CD11b⁺ myeloid cells that leads to the suppression of antitumor T cell response. *In vivo* abrogation of GM-CSF prevented the infiltration of MDSCs at the tumor site and blocked tumor progression^[94]. Similar findings have been recently reported^[95]. Interestingly, Pylayeva-Gupta *et al.*^[96] demonstrate that upregulation of GM-CSF in mouse pancreatic ductal epithelial cells is oncogenic Kras^{G12D}-dependent. Mutational activation of Kras triggers the production of GM-CSF, promoting the expansion of immunosuppressive Gr-1⁺CD11b⁺ myeloid cells and leading to the evasion of CD8⁺ T cell-driven antitumor immunity. The suppression of GM-CSF production, in turn, inhibits the *in vivo* growth of Kras^{G12D} tumors^[96]. These findings provide insights into the challenges for designing effective therapeutic modalities targeting pancreatic cancer.

Strategies to reduce the effects of myeloid cell populations within the tumor microenvironment may offer great therapeutic potential. Using a mouse model of PDAC, Zhu *et al.*^[97] demonstrate that CSF1R blockade may enhance macrophage antigen presentation and T cell responses. CSF1R blockade upregulated the T cell checkpoint molecules PDL1 and CTLA4^[97]. When PD1 and CTLA4 antagonists were combined with CSF1R blockade, this resulted in tumor regression. These findings provide a rationale to reprogram immunosuppressive myeloid cell populations in the tumor microenvironment under conditions that can significantly empower the therapeutic effects of checkpoint-based immunotherapeutics^[97].

TAMs promote angiogenesis

Angiogenesis is a requirement for tumor growth and spread. TAMs have the capacity to regulate the vascular programming of tumors^[27]. Upon activation, TAMs release multiple factors such as VEGF, PDGF, and TGF- β ^[98], all of which can promote angiogenesis. Vascular endothelial growth factor A (VEGFA) produced

by TAMs is critical for angiogenesis and reverses the effects of macrophage depletion^[99], whereas loss of VEGFA leads to vascular normalization^[100].

However, while increased vascularization during tumor progression is recognized as an important step for the majority of solid tumors, in the context of pancreatic cancer this is unclear. Hypovascularized regions are characteristic of PDAC and has served as a diagnostic tool^[101]. Moreover, this conflicts with the understanding that an angiogenic switch is required for tumor growth to occur^[102]. Interestingly, in several animal models Sunitinib, an anti-angiogenic drug that targets VEGF and PDGF receptor signaling, showed progressive blockade of tumor growth but did not inhibit PDAC progression in a murine model^[103]. In a murine model of pancreatic tumor of endocrine origin, which is highly vascularized and accounts for approximately 1% of pancreatic cancers in humans, Sunitinib reduced tumor formation^[104]. Schmid *et al.*^[105,106] suggest that tumor vascularization may be promoted by circulating macrophages. The authors have shown that the chemoattractants SDF-1 α and IL-1 β collaborate with myeloid cell integrin- α 4 β 1 to promote macrophage recruitment and adhesion to vascular endothelium resulting in tumor inflammation and growth. Inhibition of these molecules markedly decreased angiogenesis in pancreatic cancer models thus reducing tumor burden^[105,106].

TAMs and tumor metastasis

A hallmark determining the severity of cancer is tumor metastasis, which is the result of tumor cells traveling through blood and lymphatic vessels to form ectopic tumors. Pancreatic cancer commonly metastasizes to the liver, peritoneum, lungs, and bones^[104]. It has been suggested that TAMs have a role in tumor-cell migration, invasion, and metastasis^[107]. There is an abundance of evidence showing how TAMs influence tumor cell migration. Precursors of TAMs, blood monocytes, may also contribute to the formation of an invasive microenvironment in a manner dependent on the secretion of soluble mediators such as TNF^[108]. Conversely, pancreatic cancer cells induce differentiation of pro-tumor macrophages. Tumor-educated macrophages may promote pancreatic cancer cell invasion *in vitro*^[109], thus underscoring the crosstalk that occurs between tumor cells and TAMs resulting in disease progression. It has been suggested that EGF released by TAMs interacts with CSF-1 produced by tumor cells to induce tumor cell migration^[43]. TAMs also influence the tumor microenvironment by providing factors that promote tumor cell invasion, including proteases^[110]. Macrophage-derived microRNA have also been suggested to regulate tumor invasion^[111]. In pancreatic cancer of human origin, the macrophage inflammatory protein-3 α (MIP-3 α) has been linked to tumor cell invasion^[112,113]. Interactions between MIP-3 α and CCR6, the receptor expressed by PDAC cells, have been shown to induce PDAC cell proliferation, migration, and invasion in type IV collagen^[112,113].

Pharmacologic inhibition of macrophage infiltration at the tumor site using the CSF1R inhibitors decreased liver metastasis in pancreatic tumor mouse models^[88]. Focal adhesion kinase (FAK), which is a known regulator of cell migration, proliferation, apoptosis, and survival, has been linked to macrophage infiltration^[114]. Using an inhibitor of FAK (PF-562,271), Stokes *et al.*^[114] found diminished migration of tumor cells, cancer-associated fibroblasts, and macrophages. Treatment of mice with this inhibitor reduced tumor growth, invasion, and metastases^[114].

TAMs and cancer stem cells

Cancer stem cells (CSC) or cancer initiating cells are a specific subpopulation of cells with distinct stem cell properties, such as self-renewal and differentiation, with the ability to initiate tumorigenesis within tumors^[115,116]. The interaction between CSCs and TAMs promotes tumor growth, maintains the CSC population, and reduces therapeutic efficacy. Furthermore, the histological grade of the malignancy has been found to positively correlate with the number of infiltrating macrophages because TAMs have been found distributed around CSC populations^[117,118]. Wu *et al.*^[119] have shown that CSCs in glioma tissue recruit macrophages with an M2-phenotype that secrete IL-10 and TGF- β . The authors also found enhanced capacity of macrophages to inhibit T cell proliferation and therefore induce immunosuppression. In another study, blockade of macrophage infiltration lead to a reduction in the expression of ALDH, a CSC marker on pancreatic cancer cells^[88].

Recently, Hou *et al.*^[120] found elevated expression levels of the CSC markers CD44/CD133 and TAM marker CD204 in human PDAC tissues. High coexpression of CD44/CD133 and CD204 was associated with shorter overall survival and disease-free survival. These findings suggest that for patients with PDAC the coexpression of CD44/CD133 and CD204 may be useful for predicting survival^[120].

CONCLUSION

Pancreatic cancer remains one of the most common and deadly cancers. The limited success of chemotherapy in patients with pancreatic cancer is partly due to the complexity of the tumor microenvironment. Increasing attention is being given to the interactions of tumor cells and non-tumor cells found in the tumor microenvironment. In the pancreatic tumor microenvironment, TAMs are the most common cell population encountered. Accumulating evidence suggests that TAMs infiltrate early cancer lesions, subsequently promoting angiogenesis, tumor growth, spread, and immunosuppression. Additionally, the interaction between CSCs and TAMs promotes tumorigenicity, metastasis, maintains the CSC population, and impedes therapeutic efficacy. The success of studies inhibiting macrophage recruitments to the tumor microenvironment and reactivates their cancer cell killing activities provide evidence

of novel therapies for future tumor management. Altogether, these findings suggest that targeting TAMs with anticancer therapies may represent a novel strategy to treat pancreatic cancer.

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Maillard reaction and immunogenicity of protein therapeutics

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Abstract

The recombinant DNA technology enabled the production of a variety of human therapeutic proteins. Accumulated clinical experience, however, indicates that the formation of antibodies against such proteins is a general phenomenon rather than an exception. The immunogenicity of therapeutic proteins results in inefficient therapy and in the development of undesired, sometimes life-threatening, side reactions. The human proteins, designed for clinical application, usually have the same amino acid sequence as their native prototypes and it is not yet fully clear what the reasons for their immunogenicity are. In previous studies we have demonstrated for the first time that interferon- β (IFN- β) pharmaceuticals, used for treatment of patients with multiple sclerosis, do contain advanced glycation end products (AGEs) that contribute to IFN- β immunogenicity. AGEs are the final products of a chemical reaction known as the Maillard reaction or glycation, which implication in protein drugs' immunogenicity has been overlooked so far. Therefore, the aim of the present article is to provide a comprehensive overview on the Maillard reaction with emphasis on experimental data and theoretical consideration telling us why the Maillard reaction warrants special attention in the context of the well-documented protein drugs' immunogenicity.

Key words: Maillard reaction; Glycation; Advanced glycation end products; Protein therapeutics; Interferon- β ; Immunogenicity

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Core tip: The Maillard reaction occurs spontaneously in host cells and causes covalent modifications, proteolysis and crosslinking of therapeutic proteins. These are gross structural changes, which upon administration may provoke in patients classical type innate and adaptive

immune responses. The consequences of the Maillard reaction, however, reach far beyond. Specific and non-specific cellular receptors for the advanced products of the Maillard reaction may further enhance the immune response and elicit inflammation. All together the Maillard reaction actions are expected to result in drug neutralization and side effects in treated patients such as inflammatory and hypersensitivity reactions.

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INTRODUCTION

Protein pharmaceuticals are the major group of biopharmaceuticals used today in medicine for treatment of a large number of diseases. Accumulated clinical experience indicates that protein drugs can elicit an immune response especially when applied in multiple doses over a prolonged period. The antibodies (Abs) in treated patients are produced either by a classical immune response against non-self-antigens or by distortion of the immune tolerance^[1]. The development of anti-drug Abs is a slow process, in which the frequency of occurrence and the titer of the antibodies vary widely from low levels in most cases to very high, for example during treatment with some interferon- β pharmaceuticals (IFN- β)^[2]. The anti-drug Abs may have no clinical consequences^[3-5]. However, some studies show that Abs may neutralize drug activity and that of the endogenous drug counterparts thus provoking severe complications in patients^[6-8]. The most frequent side effects are flu-like symptoms and injection-site reactions^[9,10], although cases of allergy, anaphylaxis and serum sickness have also been reported^[11-16].

The amino acid sequence of therapeutic proteins may differ from that of the native human prototypes and such proteins are therefore recognized by the immune system as non-self. This was the case with porcine insulin used nearly 60 years (from 1920 to 1980) for treatment of diabetes. Porcine insulin differs by only one amino acid from human^[17] but all patients treated with porcine insulin developed anti-insulin Abs^[18]. To compensate for drug neutralization in such cases drug dosages are increased thus further boosting the immune response. The final result of such a vicious cycle is loss of therapeutic efficacy^[19,20]. The deviation from the native protein structure may explain why insulin and other therapeutic proteins of animal origin are immunogenic. However, the production of human insulin by the recombinant DNA technology did not fully solve the problem with the immunogenicity. Interestingly, the deletion of Tyr-19 in human insulin

resulted in reduced immunogenicity^[21]. We suppose that this Tyr-residue might be involved in covalent aggregation of insulin *via* the formation of dityrosine crosslinks.

To date, the reasons for the immunogenicity of human proteins are not fully understood. The dose and duration of treatment, the route of drug administration and patients' inborn characteristics may modulate the immune response^[22]. Excipient substances, used in drugs to stabilize proteins, may also be of particular relevance^[23-25]. However, the reasons for immunogenicity are mainly attributed to protein structural changes occurring during fermentation, purification, drug formulation and storage, including amino acid substitutions, non-native (or lack of) glycosylation, proteolysis, aggregation (both covalent and non-covalent), denaturation, deamination and oxidation. Some of these structural alterations (covalent aggregation and proteolysis) may occur during the Maillard reaction, which relation to drugs' immunogenicity is poorly studied and has inspired us to write the current review.

MAILLARD REACTION AND MAILLARD REACTION PRODUCTS

At the beginning of the past century the French chemist Louis Camille Maillard conducted research on peptide synthesis^[26]. At that time, when the mechanisms of protein biosynthesis were still a mystery, his far reaching goal was to understand the natural way of amino acid polymerization under mild physiological conditions. For this purpose Maillard used D-glucose, a widespread sugar in biological systems, as a soft condensing agent. Thus he realized that glucose, through its aldehyde group, is capable of reacting with amino acids^[26]. In this way Maillard did not only make a contribution to basic organic chemistry but ingeniously predicted that "The consequences of these facts appear... interesting in various fields of science: Not only in human physiology and pathology"^[26].

The significance of the Maillard reaction, known also as non-enzymatic browning reaction or carbonyl-amine reaction, for food chemistry was recognized soon. However, it took many years for biologists and physicians to grasp the physiological role of the Maillard reaction. Studies in the late 1960s revealed the existence of an abnormal fast-moving hemoglobin band in diabetic patients during routine electrophoretic screening for hemoglobin variants^[27,28]. In the same year, it has been shown that the fast-moving hemoglobin subfraction HbA_{1c} can be prepared *in vitro* by incubation of hemoglobin with glucose in the absence of enzyme catalysts^[29]. Later on, in the scientific lexicon were introduced the terms "non-enzymatic glycosylation" and "glycation" in order to distinguish the Maillard reaction, proceeding in biological systems, from

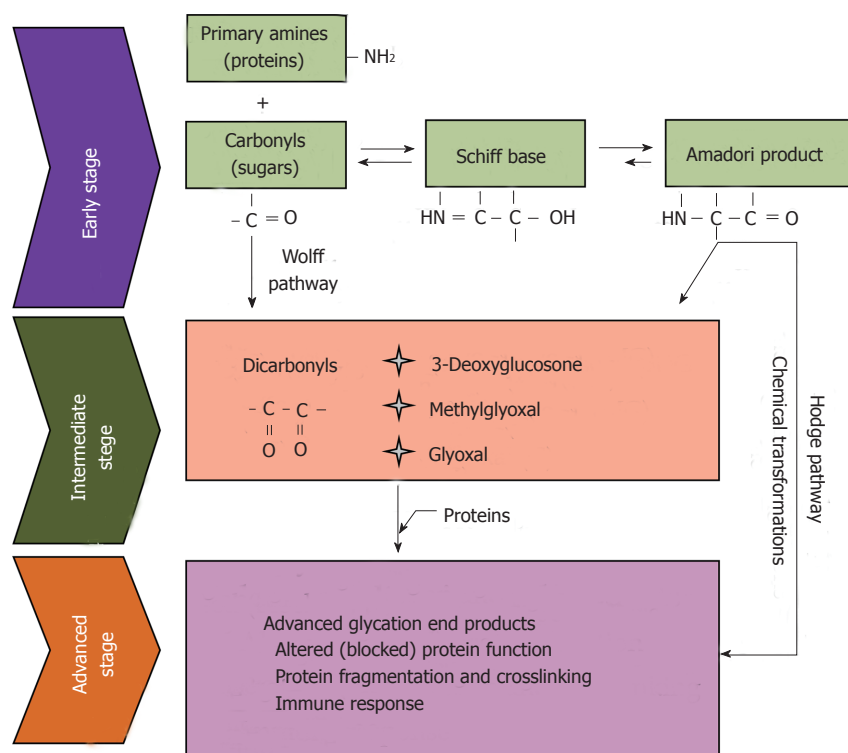


Figure 1 Maillard reaction.

the enzymatic glycosylation, which is a quite different, genetically programmed process. In the early 1980s, it has been hypothesized that glycation plays an important role in the pathogenesis of diabetic complications and aging^[30,31]. Subsequently, this hypothesis found a plethora of experimental support, and many reviews have been dedicated to the link between glycation, diabetes and aging^[32-38]. Although the carbonyl-amine reaction was discovered a century ago, its chemistry remains a still growing avenue of research. Two well defined stages can be distinguished in the Maillard reaction - early and advanced. The early stage includes reversible formation of Schiff bases between carbonyl and amino groups of the reactants, followed by a rearrangement of the Schiff bases to significantly more stable aldamines (Amadori products) or ketamine (Heyns products) (Figure 1).

The early stage of the Maillard reaction is relatively well understood^[39,40]. However, the same does not hold true for the chemical transformations of the Amadori and Heyns products into the so called advanced glycation end products (AGEs)^[41]. Generally, these are dehydrogenation, dehydration, cyclization, condensation, isomerization, oxidation, and fragmentation reactions, taking place in the advanced stage of Maillard reaction. Under anaerobic conditions, AGEs are derived directly from the Amadori product. However, under aerobic conditions, oxidative degradation of the Amadori products takes place, leading to the generation of highly reactive dicarbonyl compounds such as glyoxal (G), methylglyoxal (MG) and 3-deoxyglucosone (3DG)^[42,43].

This "interruption" between the early and the advanced stage is what we call the intermediate step of Maillard reaction. In turn, the intermediate products, which are small, diffusible and highly reactive species, again attack amino compounds, thus propagating the initial chemical burden. The formation of AGEs, either directly from the Amadori product or through its degradation intermediates, is known as the classical, or Hodge pathway^[40]. The Schiff base, formed early in the Maillard reaction, may also undergo non-enzymatic fragmentation to α -oxoaldehydes, which initiate another chain of chemical transformations known as the Namiki pathway^[44]. In addition, under physiological conditions free monosaccharides undergo a transition metal ions catalyzed autooxidation to H_2O_2 and the corresponding ketoaldehydes, which are precursors for AGEs formation in the Wolff pathway, also recognized as a separate reaction chain in the Maillard chemistry^[45-47]. The picture becomes increasingly colorful, pasting therein the carbonyl products released during lipid peroxidation, which also react with amines to form AGEs-like structures called Advanced Lipoxidation End Products (ALEs)^[48,49]. AGEs/ALEs are covalent adducts with diverse stability and physicochemical characteristics such as blue fluorescence, brown color and crosslinking properties. When accumulating in biological amines such as proteins, DNA and amino-lipids, AGEs may significantly impair their structure and physiological function. The chemical structure of a number of AGEs formed *in vitro* as well as in human plasma and tissues has been determined so far^[50-57].

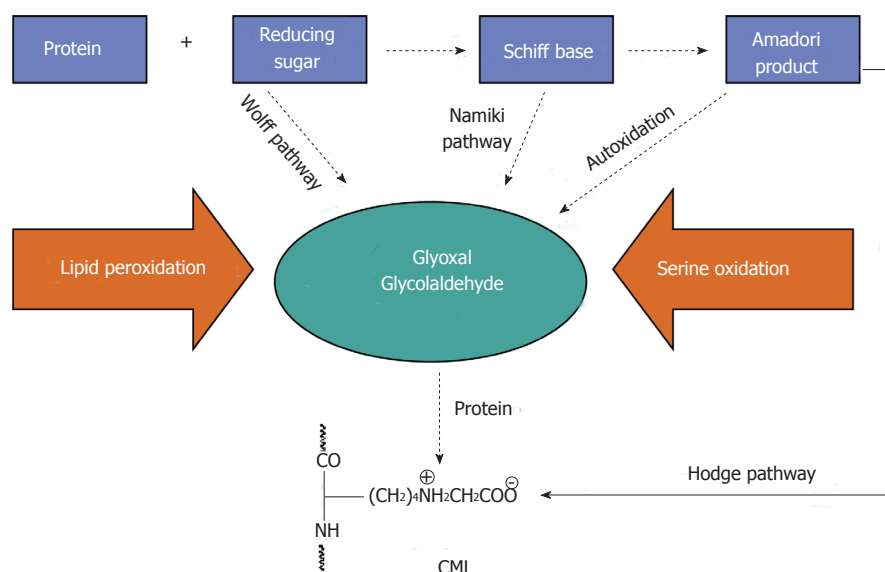


Figure 2 Pathways of N^ε-(carboxymethyl)lysine formation. CML: N^ε-(carboxymethyl)lysine.

N^ε-(carboxymethyl)lysine

N^ε-(carboxymethyl)lysine (CML) is one of the best-characterized AGEs^[58]. It is formed in the advanced stage of the Maillard reaction and always accompanies the formation of brown and fluorescent AGEs, although *per se* it is colorless and non-fluorescent. If proteins are glycated with either ribose or 3DG, then CML is formed only when the reaction is carried out under aerobic conditions^[52,59]. This is why the detection of CML is an indication that protein modifications have occurred in the presence of oxygen. CML is formed by different oxidation mechanisms *in vitro*, involving not only reducing sugars. It may also be a product of lipid peroxidation^[48] and serine oxidation^[60] (Figure 2), and because of that CML should not be considered a typical glycation marker. In most cases of *in vitro* glycation CML is the main product found in proteins^[61], and is also a dominant AGEs antigen in tissue proteins^[62]. Direct formation of CML from the Amadori product through its autoxidation has been reported by Miki Hayashi *et al*^[63]. The authors used as a model of Amadori compound glycated human serum albumin (HSA) and found that CML is formed by heat treatment of glycated HSA over 80 °C in a time-dependent manner. Further rise in the temperature to 100 °C resulted in the formation of the glycation intermediates G, MG and 3DG. The formation of CML in HSA was inhibited in the presence of a reducing agent (sodium borohydride), a chelator of transition metal ions (diethylenetriamine pentaacetic acid), or a trapping reagent for α-oxoaldehydes (amino-guanidine), which indicates that CML (AGEs) accumulation in proteins is manageable.

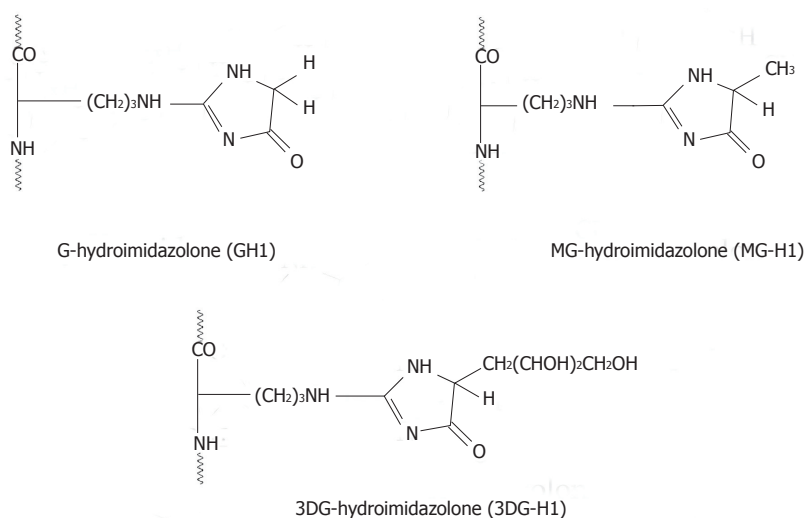
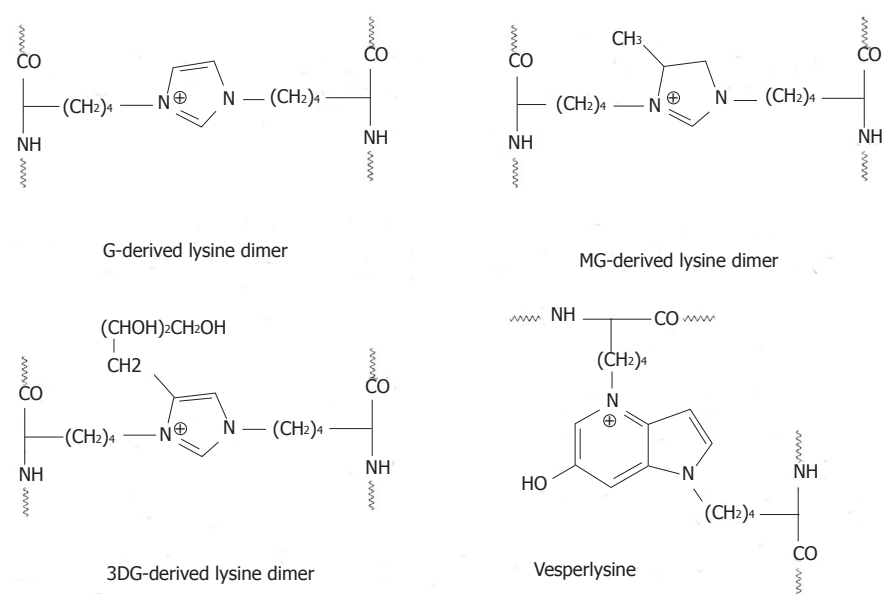
Hydroimidazolones

Hydroimidazolones are formed in a reaction of the guanidino group of arginine with G, MG and 3DG^[64-66]. The resulting products undergo structural and stereo-

chemical isomerization, which were extensively studied by Ahmed *et al*^[54,65]. The authors found that hydroimidazolones formed in the reaction of arginine with methylglyoxal (MG-H) are composed of three structural isomers designated as MG-H1, MG-H2 and MG-H3. The reaction of arginine with G and 3DG yielded similar structural isomers (Figure 3)^[67]. Due to racemization in the hydroimidazolone ring, each structural isomer may exist in two epimeric forms, which cannot be resolved without prior derivatization. As a rule, the stability of hydroimidazolones decreases with increasing pH and each structural isomer has a distinctive stability and half-life. The half-life of the individual stereoisomers of MG-H increases in the following order: MG-H1 > MG-H2 >> MG-H3. Under physiological conditions (pH 7.4, 37 °C) the half-life of MG-H1 is approximately 12 d. Ahmed *et al*^[65] suggested that in contrast to CML, hydroimidazolones are not accumulating in long-lived proteins over time *in vivo* but rather reflecting short episodes of enhanced protein glycation under conditions of abnormal rise in the concentration of α-oxoaldehydes. The authors also assumed that N_ω-(1-carboxyethyl)arginine and N_ω-(1-carboxymethyl)arginine, which are more stable and found in *in vivo* glycated proteins, are probable degradation products of hydroimidazolones.

AGEs crosslinks

The Maillard reaction not only yields sugar adducts on proteins but also results in gross structural changes such as proteolysis^[68-70], and intra- or inter-molecular crosslinking^[71,72]. Proteolysis of glycated proteins usually occurs at lysine and arginine residues^[73] but may also happen randomly as a result of transition metal ions catalyzed oxidation^[68,69]. In contrast, covalent crosslinking of polypeptide chains involves exclusively lysine and arginine residues. Lysine residues on the

Figure 3 Hydroimidazolones^[67].Figure 4 Lysine-lysine advanced glycation end products crosslinks^[67].

same or on different polypeptide chains become involved in the formation of shared AGEs structures such as G-derived lysine dimer (GOLD)^[74], MG-derived lysine dimer (MOLD)^[75], 3DG-derived lysine dimer (DOLD)^[76], G-derived lysine-lysine crosslinks GOLA and GALA^[77,78], glucose lysine dimer GLUCOLD^[79], crossline^[80,81] and vesperlysines^[82] (Figure 4)^[67]. AGEs crosslinks formed between lysine and arginine residues are G-derived arginine-lysine dimer (GODIC), MG-derived arginine-lysine dimer (MODIC)^[83], 3DG-derived arginine-lysine dimer (DOGDIC)^[84], pentosidine^[78] and glucosepane^[84]. To the best of our knowledge, to date crosslinks formed between two arginine residues or between arginine/lysine residues and other amino acids have not been detected neither *in vivo* nor in *in vitro* glycation reaction, although the possible involvement of the cysteine thiol group in the formation of such

crosslinks has been proposed^[85].

MAILLARD REACTION PRODUCTS IN THE HUMAN BODY

Endogenous formation of glycation products

Early and advanced glycation products are formed in the human body throughout its existence. Many glycation adducts, appearing on proteins in model reactions *in vitro*, have been detected also in physiological systems. Fructoselysine (FL), the Amadori product formed by glucose on the ϵ -amino group of lysine, has a relatively short half-life (2-6 wk)^[86]. As a rule, early glycation in healthy subjects is not age-dependent, although some rise in the physiological FL concentration has been observed during aging. For example, the concentration

of the collagen-bound FL increases from 5 mmol/mol Lys in 20-year-old individuals to 7 mmol/mol Lys in 70-year-old subjects^[87]. Short-lived proteins such as albumin (half-life *ca.* 20 d) and even hemoglobin (half-life *ca.* 120 d) accumulate *in vivo* predominantly early glycation products. Approximately 10% of HSA of healthy subjects is modified by FL at Lys-525^[88], and incubation of HSA with glucose under physiological conditions *in vitro* leads to glycation of the same Lys residue^[89]. Other Lys residues in HSA including Lys-439, Lys-199 and Lys-281 are also involved in FL formation *in vivo*^[90] and it has been reported that glycation impairs HSA binding to physiologically important ligands such as bilirubin and *cis*-parinaric acid^[89]. The historically crucial hemoglobin HbA_{1c} contains glucose-derived Amadori product at the amino group of the hemoglobin β -chain N-terminal valine (β Val-1) and amounts 4%-6% of the total hemoglobin in healthy individuals. Except the N-terminal valine, some internal Lys residues in HbA_{1c} also become glycated^[91]. Under hyperglycemia HbA_{1c} levels increase several times and correlate positively with the chronic diabetic pathology^[92].

Most AGEs are either not formed at physiological glucose concentrations (5 mmol/L) or their levels are quite lower (ten to hundred times) compared with hyperglycemic conditions. This makes AGEs relevant biomarkers of the glycation status of individuals, which changes with age and in disease state such as diabetes and kidney failure. Because of their great diversity, AGEs cover a wide concentration range *in vivo* (0.001 to 15 mmol/mol modified amino acid)^[86]. Physiologically important AGEs are those having long half-life (CML, CEL and pentosidine) and/or high concentration (hydroimidazolones). Stable AGEs accumulate on long-lived proteins such as skin and cartilage collagen^[93-96], and lens proteins^[97], although CML has also been detected in the short-lived HSA of diabetic patients^[98]. Hydroimidazolones, albeit relatively unstable, may also appear on long-lived proteins such as human lens proteins^[99].

Maillard reaction products (MRPs), which are found in the body, are classified into three groups - MRPs in proteins (> 12 kDa), MRPs in peptides (< 12 kDa) and free MRPs in amino acids. The term MRPs is more general than AGEs and applies to both early glycation products and AGEs. Peptide MRPs are detected mainly in the portal venous plasma and in the urine, and are most likely degradation products of glycated proteins. Free MRPs are formed during proteasome/lysosome degradation of glycated cellular proteins, then released into the bloodstream and excreted in the urine, which ensures efficient functioning of the cell proteome^[100,101]. Free MRPs may appear *in vivo* also as a result of amino acids glycation though this latter source of free MRPs is disputable^[55].

The impact of glycation on human physiology depends on many factors including the target amino compound and its half-life, the adduct stability and its location. Especially harmful are the consequences of

the formation of covalent cross-links in proteins that render them resistant to proteolysis^[102]. Both early glycation products and AGEs can cause damage *via* various mechanisms such as (1) release of oxygen radicals by early products^[103]; (2) altered (blocked) activity of enzymes^[104,105], receptors^[106] and regulatory proteins^[107,108]; (3) crosslinking of structural proteins^[78,109]; (4) damage of signaling pathways^[110,111], (5) damage of protein recycling^[112,113], and (6) induction of an immune response^[114].

Dietary intake of glycation products

Sugar-rich and processed foods are also a source of FL and AGEs in the human body^[115]. The biodistribution and metabolism of these products have not been systematically studied, except the dietary intake of FL in humans^[116]. Among foods rich in FL and AGEs are bread, biscuits, chocolate, breakfast cereals and hot milk. It has been reported that lactulose-lysine, an Amadori product formed during heat treatment of milk, is poorly digested in the gastrointestinal tract^[117]. Some microorganisms can hydrolyze FL in the intestine. For example, an enzyme has been discovered in *E. coli* that degrades N^ε-fructoselysine-6-phosphate to lysine and glucose-6-phosphate^[118]. FL ingested with food and resorbed by the intestine, enters the circulatory system and then penetrates liver and muscle cells by passive diffusion. It is not clear yet whether lysine and glucose can be recycled from FL, because no human enzymes have been identified so far catalyzing the breakdown of FL to free lysine and glucose. In rats, about 60% of dietary FL is excreted in urine^[119], whereas in humans this percentage is only 3%. In newborns, however, 16% of FL is found in the urine, and 55% is recovered in the feces^[116] (Figure 5).

Foods with high FL and AGEs content are poorly digestible, because glycated proteins are resistant to proteolysis. In addition, some AGEs inhibit intestinal proteases^[120]. It is thought that dietary AGEs are absorbed mainly in the form of free and peptide MRPs. The highest concentration of absorbed food AGEs is expected in the plasma of the portal vein, where MG-H1 is detectable mainly in peptides^[121]. Although CML is formed *in vivo*, it is hypothesized that urinary CML is mostly of exogenous nature^[122]. Studies with rats have shown that another product of the advanced glycation, 5-Hydroxymethyl-2-furaldehyde (HMF), administered *per os* or intravenously is present in the liver but is mostly in the kidney and the bladder. Also, HMF or its metabolites are rapidly eliminated in the urine with a recovery of 95%-100% after 24 h^[123]. Only 10% of dietary AGEs are absorbed, of which only 30% are excreted in the urine of healthy individuals with unimpaired renal function^[124]. Some dietary AGEs, after entering blood circulation, interact with low density lipoprotein (LDL) and tissue proteins (e.g., collagen) or bind to cellular receptors for AGEs to trigger intracellular oxidative stress, endocytosis and degradation of AGEs.

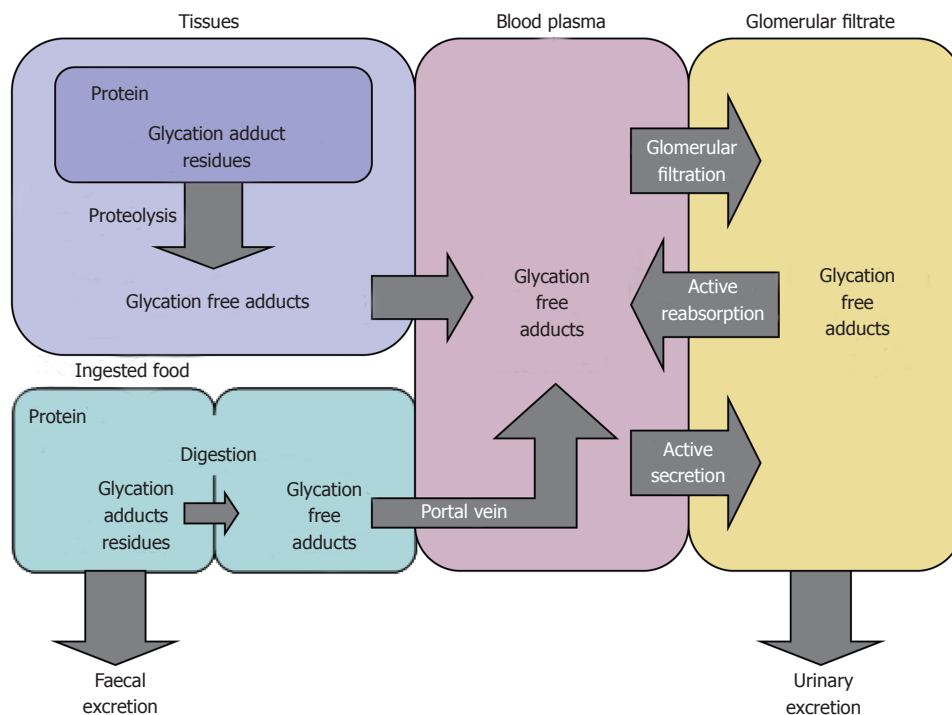


Figure 5 Biodistribution scheme illustrating flows of formation and removal of protein glycation free adducts^[86].

ROLE OF THE IMMUNE SYSTEM IN THE ANTI-GLYCATION DEFENSE

Cellular receptors for AGEs

Many cellular receptors are capable of binding AGEs. The AGE-receptor complex consists of AGE-R1 (oligosaccharyl transferase-48), AGE-R2 (80K-H phosphoprotein)^[125,126] and AGE-R3 (galectin-3)^[127]. Some receptors belonging to different classes of the scavenger receptor (SR) supergroup^[128,129] also bind AGEs. These are SR-A1 (SCARA1)^[130] and SR-A1.1 (SR-AII)^[131], SR-B1 (SR-BI)^[132] and SR-B2 (CD36)^[133], SR-E1 (LOX-1)^[134], SR-H1 (FEEL-1) and SR-H2 (FEEL-2)^[135]. These receptors are involved in detoxification of AGEs by intracellular degradation (endocytosis). Best characterized is the specific receptor for AGEs called Receptor for Advanced Glycation End Products (RAGE)^[136]. RAGE is a multi-ligand receptor of the immunoglobulin superfamily, which plays a key role in inflammatory responses. Its extracellular domain is composed of three immunoglobulin-like domains - one V (variable) type Ig domain followed by two C (constant) type Ig domains^[137]. RAGE also possesses a transmembrane domain and a cytoplasmic tail of 43 amino acids. Crucial for ligand binding is the V-domain, while the cytoplasmic tail is implicated in further transduction of captured signals^[138]. RAGE is expressed weakly on a number of cell types and tissues under physiological conditions, however, an increased expression is observed in sites of ligands' deposition. RAGE exists also in a soluble form (sRAGE)^[139], which is hypothesized to participate in detoxification of circulating ligands.

Apart from AGEs, ligands for RAGE are pro-inflammatory cytokine-like mediators from the S100/calgranulin family^[140], the β -amyloid peptide^[141] and amfoterin also known as High Mobility Group Box 1 (HMGB1) - a nuclear protein released during cell necrosis^[142-144]. AGEs and non-AGEs ligands bind to RAGE on endothelial cells, neurons, smooth muscle and immune cells to activate a variety of signaling pathways including expression of NF- κ B - a transcription factor, which plays a key role in regulation of the immune response^[145]. While expressed inducibly on T-cells of healthy subjects upon T-cell receptor activation, RAGE is constitutively synthesized in diabetics' T-cells, and a role for RAGE in the adaptive immunity has been proposed^[146,147]. It has been demonstrated that ovalbumin (OVA) modified with AGEs (pyrraline), but not native OVA, induces SR-A mediated uptake of the antigen by dendritic cells and enhances CD4⁺ T-cell immunogenicity and potential antigenicity of OVA^[148,149]. Characteristic for RAGE is that it recognizes tertiary structures rather than amino acid sequences. This feature endows RAGE with properties of the pattern recognition receptor (PRR), which recognizes repeated antigenic motifs of the type of pathogen-associated molecular patterns (PAMPs)^[150]. RAGE can also recruit immune cells in the sites of inflammation. For example, RAGE on endothelial cells can function as an adhesive receptor interacting with the leukocyte β 2-integrins^[151]. Intriguing findings in recent years suggest that RAGE's actions contribute to perpetuation of AGEs production by sustaining oxidative stress and inflammation, and by suppressing the detoxification of MG as one of the major AGEs precursors^[152,153].

Anti-AGEs antibodies

Apart from cellular immune responses, AGEs may elicit a humoral immunity. Excessive accumulation of AGEs with age and in pathology appears to correlate with elevated levels of anti-AGEs antibodies^[154]. Glycated proteins [histone H2A^[155], HSA^[156], poly-L-lysine (PLL)^[157], IgG^[158]], DNA^[159,160] and LDL^[161,162] have demonstrated higher immunogenicity in experimental animals than the non-glycated counterparts, and sera of diabetic patients were found to exhibit higher binding activity to glycated HSA^[163], PLL^[157], IgG^[158] and DNA^[159] than to the unmodified molecules. In addition, patients with renal failure of diabetic or non-diabetic etiology had higher autoantibody activity against CML (an AGE structure) than normal subjects or diabetics without renal failure^[164]. The injection of rats with *in vitro* glycated rat skin collagen leads to the formation of anti-collagen Abs, which do not cross-react with non-glycated collagen. The binding of the serum Abs to the glycated collagen is inhibited by 92% when the reaction is conducted in presence of glycated Lys as a competitor. This result indicates that glycated Lys residues on collagen are the most probable epitopes captured by the anti-collagen Abs^[165]. Of note, sera from streptozotocin-induced diabetic rats contained Abs that clearly bound glycated collagen.

Vay *et al.*^[166] have observed 289 diabetic patients and 120 healthy individuals for serum Abs against CML (anti-CML Abs). Although they have found an increased titer of anti-CML Abs (IgG-isotype) in sera of diabetic patients compared to controls ($P < 0.0001$), there was no correlation between the titer of the anti-CML Abs and patients' glycemic status. In a similar study including 58 children with type I diabetes, 19 children have been found to be anti-AGEs Abs positive^[167]. In contrast to the previous study, the authors have found that the titer of the anti-AGEs Abs correlates positively with some diabetic markers such as HbA_{1c}, microalbuminuria and retinopathy. While some studies show higher titer of circulating anti-AGEs Abs in diabetic patients than in healthy individuals^[164,166], others report on the reverse condition, *i.e.*, lower anti-AGEs Abs titers in diabetics than in normal controls^[168,169]. To explain this paradox the authors suggested that circulatory anti-AGEs Abs are either captured by tissue-bound AGEs^[168] or entrapped in immune complexes^[169] hampering their determination. If real, such events could explain the lack of a correlation between the titer of the serum anti-CML Abs and the patients' glycemic status in the above cited study^[166].

It is well known that some patients with rheumatoid arthritis (RA) develop auto-Abs against the IgG constant (Fc) region, which is designated as rheumatoid factor (RF). A pilot study with RA patients has shown that only RF-positive patients have serum IgM Abs binding to *in vitro* glycated IgG (IgG-AGEs), suggesting that the IgM anti-IgG-AGEs together with IgG-AGEs may contribute to the pathogenesis of RA^[170]. Further studies have

shown that immune complexes isolated from patients' sera indeed contain AGEs (CML and imidazolone) modified IgG. It is very likely that the anti-IgG-AGEs prevent the normal clearance of IgG-AGEs by AGEs receptors^[171]. Noteworthy, as with most biologics, some RA patients treated with antagonists of the human tumor necrosis factor (TNF) (infliximab, etanercept and adalimumab) develop anti-drug antibodies^[172]. The TNF-antagonists are in fact anti-TNF Abs of IgG isotype^[172] and in light of the current review two events could be proposed: (1) if the Fc regions of the therapeutic anti-TNF Abs are also glycated (anti-TNF-AGEs Abs), they could compete with patients' IgG-AGEs for binding RF in human plasma; and (2) if the patients' antibodies against the glycated biologic are specifically directed against the glycated moiety (AGEs) of the anti-TNF-AGEs Abs, apart from binding the drug, they would be also capable of interacting with patients' IgG-AGEs. The net result of this hypothetical and intertwined scenario is difficult to predict, but it should be taken into consideration by pharmacists, pharmacologists and clinicians. Intriguingly, subcutaneous immunization with AGEs modified LDL (AGEs-LDL) significantly inhibited atherosclerosis progression in hyperlipidemic diabetic mice possibly through activation of specific humoral and cell mediated immune responses^[173]. The anti-atherogenic effect of the anti-AGEs-LDL Abs, however, is disputable yet^[174] in so far as immune complexes containing such Abs are detected in human sera^[175] and shown to be important predictors of carotid intima-medial thickening in patients with type 1 diabetes^[176].

REASONS FOR THE IMMUNOGENICITY OF PROTEIN THERAPEUTICS

Aggregation of proteins

The most important structural change contributing to protein drugs' immunogenicity is aggregation, which may be both covalent and non-covalent. The non-covalent aggregation results from interaction between non-native transient protein conformers with partially preserved secondary structure called aggregation competent particles. Such particles accumulate progressively in the time course of protein storage due to structural fluctuations and beget intermolecular interactions resulting in protein aggregation. The aggregation process is often accompanied by protein precipitation and loss of biological activity^[177]. It is believed that the formation of aggregates proceeds by a cooperative mechanism, *i.e.*, the non-native conformers react with other native molecules to render them aggregation competent. The formation of dimers and higher order multimers in turn accelerates the aggregation process^[178,179].

Aggregates in protein drugs substantially contribute to immune response in treated patients^[23,180-185]. Studies with interferon- α have shown that the magnitude of the immune response does not depend on the size

of the aggregates but rather on the structure of the protein molecules involved in aggregation. Aggregates, formed by molecules with largely preserved secondary structure (native-like) are more immunogenic compared to aggregates composed of fully denatured molecules^[22,186]. This could explain why Purohit *et al.*^[187] have not observed an immune response against recombinant human factor VIII in model animals. Before injection into animals the authors heated the protein to stimulate aggregation, which perhaps caused its denaturing. The prevalent hypothesis, which explains the immunogenicity of the protein aggregates, is the "array" hypothesis^[188-194]. The human immune system is specialized to recognize proteins that are presented in an array format, as is the case with viral capsids and bacterial cell walls. In fact, aggregated proteins are arranged in similar "pathogen-like" structures and thus may be sensed by the immune system as foreign antigens.

Post-translational modifications of proteins

The enzymatic glycosylation is an inborn post-translational modification of many eukaryotic proteins and in most cases is indispensable for their biological activity and stability. Also, the lack of native polysaccharide residues may unmask potentially immunogenic epitopes in proteins. Increasing evidence in the last decade shows that glycoproteins are not a eukaryotic privilege. Competent to enzymatically glycosylate proteins are many prokaryotes including *Escherichia coli* - the "workhorse" of the current biotechnology^[195-198]. Prokaryotic glycosylation, however, differs significantly from that in eukaryotes. Therefore, it is preferable that naturally glycosylated human proteins are produced in eukaryotic cell lines. On the other hand, glycosylation in eukaryotes is species and cell specific^[199]. Polysaccharides may vary in composition, chain length, binding sites on proteins and points of chain branching. This means that for the production of human proteins with preserved glycosylation pattern appropriate host cells have to be selected performing identical or similar glycosylation to that of the natural proteins^[200].

While native glycosylation of human therapeutic proteins is desirable, other non-enzymatic post-translational modifications should be avoided^[201]. Spontaneous oxidation and deamination, sometimes followed by isomerization, are major causes of proteolysis during protein isolation, purification and storage. Deamination affects mainly asparagine residues and leads to their conversion into aspartate or iso-aspartate residues^[202,203]. Glutamine in proteins may undergo similar spontaneous deamination to glutamate. Studies with oxidized and non-oxidized forms of IFN β have shown that oxidized protein species are significantly more immunogenic^[204]. The oxidation may lead to covalent aggregation of therapeutic proteins through the formation of cysteine and/or tyrosine covalent cross-links^[54,205]. This is the reason why functionally unimportant Cys residues in

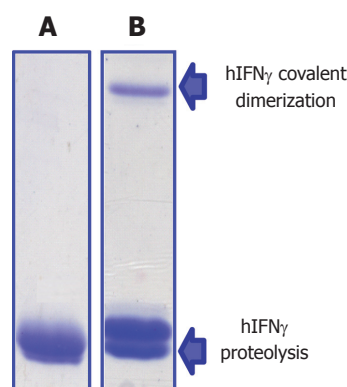


Figure 6 Covalent dimerization and proteolysis of *Escherichia coli*-derived hIFN γ (A) after storage for one month in solution (0.4 mol/L NaCl, Tris-HCl pH 8.2) at 4 °C under nitrogen (B). Protein species were separated on 15% denaturing (sodium dodecyl sulfate) polyacrylamide gel.

therapeutic proteins are often replaced by alternative amino acids. To our knowledge, no one has tried so far to reduce covalent cross-linking of therapeutic proteins through replacement of Tyr residues. Last but not least, the leitmotif of the current review, the Maillard reaction, is another non-enzymatic post-translational modification of proteins, which contribution to the immunogenicity of protein drugs will be discussed in detail below.

OUR FOCUS ON THE LINK BETWEEN THE MAILLARD REACTION AND THE IMMUNOGENICITY OF PROTEIN THERAPEUTICS

We entered the Maillard reaction field nearly 15 years ago when searching a reasonable explanation of the unexpected behavior of a cysteine-less variant of human interferon-gamma (hIFN γ) expressed in *E. coli*. Despite the lack of Cys residues, the protein underwent progressive covalent dimerization during storage under anaerobic conditions, which excluded oxidation (*i.e.*, formation of disulfide bridges and tyrosine dimers) as a cause of the observed phenomenon (Figure 6). Brief literature survey led us to suppose that the Maillard reaction may provide a rational explanation of hIFN γ covalent dimerization and proteolysis. At that time, however, it was not yet clear whether the Maillard reaction may occur in prokaryotes because of their short life span and intense protein turnover. We undertook relevant investigations in this direction and found that both endogenous and recombinant proteins^[73,206] as well as chromosomal DNA^[207] of *E. coli* are involved in glycation under normal physiological conditions. The short life span of *E. coli* does not disagree with these observations. During batch fermentation of *E. coli* a generation time of *ca.* 20 min and a few divisions are sufficient for the Maillard reaction to take off through the formation of Schiff bases in proteins. Then, the whole Maillard cascade of chemical

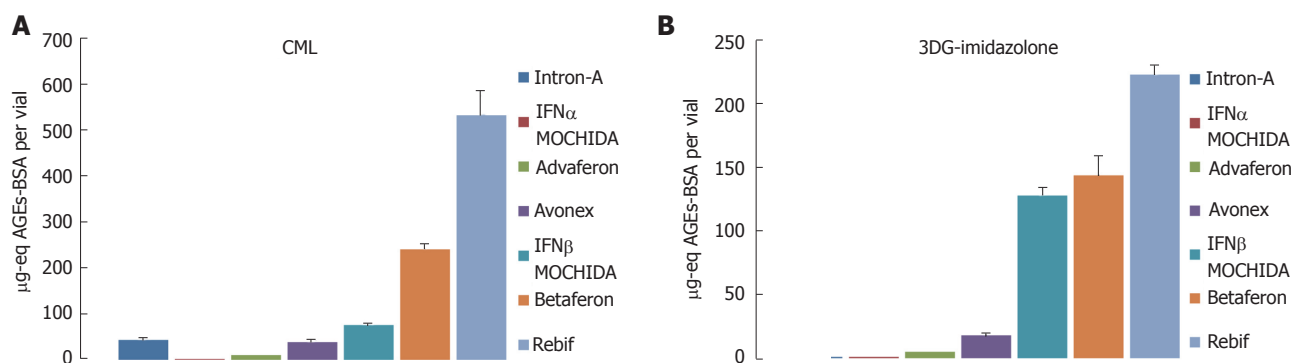


Figure 7 N ϵ -(carboxymethyl)lysine (A) and 3DG-imidazolone (B) in interferon pharmaceuticals. Three vials of each drug were analyzed by competitive enzyme-linked immunosorbent assay in duplicates, and the pooled results were presented as mean $\pm$ SD. One μ g-eq AGEs-BSA corresponds to the amount of CML or 3DG-imidazolone present in one microgram of the referent AGEs-BSA. The latter was prepared by incubating 10 mg/mL BSA with 0.5 mol/L glucose for 3 mo at 37 $^{\circ}$ C. AGEs: Advanced glycation endproducts; CML: N ϵ -(carboxymethyl)lysine; BSA: Bovine serum albumin.

transformations resulting in AGEs formation may happen *in vitro* during protein isolation, purification, formulation and storage. We indeed found that freshly isolated hIFN γ contains Amadri products but not AGEs, which accumulated in the protein over time accompanied by covalent aggregation, proteolysis and loss of biological activity^[73,208]. Interferons are basic proteins rich in lysine and arginine residues and because of that susceptible to glycation. On the other hand, human interferons Type I (α and β) are the active ingredients of a number of pharmaceuticals used today for treatment of various cancers, autoimmune and viral diseases. For this reason we decided to analyze interferon-based pharmaceuticals for the presence of AGEs.

AGEs in interferon pharmaceuticals

Type I interferon pharmaceuticals manufactured by different companies worldwide were tested: *E. coli*-derived hIFN α -2b (Intron-A, Schering-Plough Corp., Kenilworth, NJ, United States), natural (lymphoblast) nhIFN α (IFN α MOCHIDA1000, Mochida Pharmaceutical Co., Ltd, Tokyo, Japan), *E. coli*-derived hIFN α -con1 (Advaferon-1800, Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan), natural (fibroblast) nhIFN β (IFN β MOCHIDA600, Mochida Pharmaceutical Co., Ltd, Tokyo, Japan), *E. coli*-derived hIFN β -1b (Betaferon, Schering AG, Berlin, Germany), CHO-derived IFN β -1a (Rebif 44 mg, New Formulation, Merck Serono, Bari, Italy), and CHO-derived IFN β -1a (Avonex 30 mg, Biogen Idec Inc., Weston, MA, United States). All pharmaceuticals except Advaferon and Rebif were powders containing HSA as a stabilizer while Advaferon and Rebif were liquid formulations without protein excipients. We tested all drugs for AGEs by enzyme-linked immunosorbent assay (ELISA) using two monoclonal antibodies specific for CML and 3DG-derived imidazolone (Figure 7). Lymphoblast IFN α MOCHIDA was negative for both CML and 3DG-imidazolone, whereas the content of both AGEs in fibroblast IFN β MOCHIDA was higher than that in the *E. coli*-derived Intron-A and Advaferon. Also, Rebif produced in Chinese hamster ovary (CHO) cells proved to contain higher levels of AGEs compared

to the *E. coli*-derived Betaferon. Based on these observations we concluded that therapeutic proteins are affected by glycation independently of whether they are produced by native or recombinant (either pro- or eukaryotic) cells. This result is not surprising bearing in mind both pro- and eukaryotic cells are glycation-proficient. Whether there are any differences in the glycation power between pro- and eukaryotic cells is a largely unexplored issue and a direction for future investigations. We suggest that the variable AGEs content in the drugs reflects different manufacturing technologies rather than specific characteristics of the cell-producers.

Five of the pharmaceuticals are formulated with HSA at concentrations ten to hundred times higher than that of interferons. Thus the reasonable question emerged whether AGEs, we are measuring, are located in HSA or in interferons. The answer of this question is "in interferons" because of the following reasons: (1) a highly purified pharmaceutical grade HSA is used for drugs' formulation. Own mass-spectral analyses of all five HSA containing drugs confirmed the low glycation status of HSA^[209], although 50% of HSA proved to be cysteinylated as shown also by other authors^[210]. How HSA-Cys impacts drug stability is not yet clear and should be outlined as another direction for future investigations; (2) we have isolated an IFN β enriched fraction from Betaferon by size-exclusion HPLC, which demonstrated 15 times higher concentration of CML as compared to Betaferon's HSA^[211]; and (3) as seen in Figure 7, the HSA-free drug Rebif demonstrated the highest concentration of CML and 3DG-imidazolone among all seven drugs while the HSA containing IFN α MOCHIDA was deprived of these two AGEs.

AGEs and immunogenicity of IFN β pharmaceuticals

In most cases AGEs are bulky chemical moieties that may behave like haptens rendering self-proteins immunogenic. As seen in Figure 7, the four IFN β pharmaceuticals tested proved to contain more CML and 3DG-imidazolone than the three IFN α drugs. Also, a number of studies report on immunogenicity

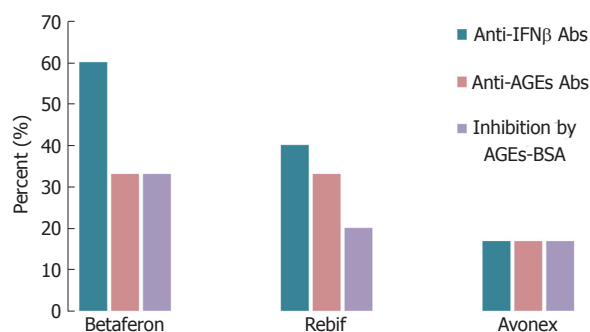


Figure 8 Percent sera of MS patients treated with Betaferon, Avoex and Rebif, containing anti-IFN β , anti-advanced glycation endproducts Abs, and responding to Advanced glycation endproducts-bovine serum albumin inhibition of binding to IFN β . For detailed information on the experimental conditions see ref. No. 211. AGEs-BSA: Advanced glycation endproducts-bovine serum albumin.

of IFN β ^[2,212-227]. Therefore, to either verify or reject the hypothesis that AGEs contribute to IFN β immunogenicity, we studied sera from patients suffering from relapsing-remitting multiple sclerosis (MS) treated with Avonex (12 patients), Betaferon (15 patients) or Rebif (15 patients)^[211]. Seventy four percent (31/42) of the patients enrolled in our study had flu-like symptoms, injection-site reactions, or both. Sera were tested for binding anti-IFN β and anti-AGEs Abs of IgG isotype by direct ELISA. The percentage of anti-IFN β Abs positive (Abs⁺) patients was highest in the Betaferon group (60%) followed by the Rebif (40%) and Avonex (17%) groups (Figure 8), which is consistent with data about the relative incidence of persistent neutralizing Abs (NAb) in patients treated with the same drugs^[228]. Although CML and 3DG-imidazolone levels were higher in Rebif (Figure 7), Betaferon demonstrated higher immunogenicity than Rebif. Note that besides AGEs-modifications, and in contrast to the CHO-derived IFN β 1a (Rebif), the *E. coli*-derived IFN β 1b (Betaferon) lacks glycosylation at Asn-80, has an amino acid substitution (Ser¹⁷ for Cys¹⁷) and is prone to non-covalent aggregation^[229], which could explain its higher immunogenicity. Seventeen percent (2/12) of the patients in the Avonex group were anti-AGEs Abs⁺, whereas for the other two groups (Betaferon and Rebif) the percentage of anti-AGEs Abs⁺ patients was 20% (3/15). All anti-AGEs Abs⁺ patients were anti-IFN β Abs⁺. This, however, does not necessarily mean that the formation of anti-AGEs Abs in these patients was provoked by the IFN β therapy. Convincing evidence for the link between the formation of anti-IFN β and anti-AGEs Abs was obtained by competitive ELISA. The addition of an external AGEs-competitor (AGEs-BSA) to sera inhibited binding to IFN β of two (Avonex), three (Rebif) and five (Betaferon) sera with inhibition ranging from 9% to 70%. The different degree of inhibition most likely reflects the relative contribution of AGEs to the overall IFN β immunogenicity in each particular patient. All ten sera responding to inhibition by AGEs-BSA were tested also for response to inhibition by mAbs

raised against CML and 3DG-imidazolone. Significant inhibition ($P < 0.05$) of sera reactivity to IFN β was obtained with one serum from the Avonex group and with two sera from the Rebif group. One can suggest that in the remaining seven anti-AGEs Abs⁺ sera, which did not respond to inhibition by the two mAbs, the anti-AGEs Abs were raised against other AGEs (not CML and 3-DG-imidazolone) in IFN β .

Whether the anti-AGEs Abs that cross-react with IFN β neutralize drug activity and/or contribute to the observed inflammatory side reactions in treated patients are questions to be addressed in the future. Also, besides injection-site reactions and flu-like symptoms, cases of allergy^[13-15] and anaphylaxis^[11,16] have been reported for MS patients on IFN β therapy. Therefore, it is worth to conduct case studies with such patients in order to answer the question of whether AGEs in IFN β have contributed to the observed hypersensitivity reactions.

POSSIBLE SCENARIOS UPON TREATMENT WITH IFN β -AGEs

AGEs are formed in the human body under normal and pathological conditions, and are consumed with food as well. Because of the negative impact of AGEs on human physiology, elaborate mechanisms do exist for anti-AGEs defense ranging from enzymatic detoxification of AGEs' precursors (*i.e.*, carbonyl compounds^[230-234], Schiff bases^[235] and Amadori products^[236]) going through intracellular proteasome/endosome degradation of protein AGEs, and ending up with renal clearance of free and peptide AGEs. Anti-AGEs antibodies have been detected in sera of healthy individuals as part of a homeostatic mechanism, which clears altered structures *via in situ* destruction or *via* opsonization^[237]. The formation of AGEs starts during embryonic development and one can assume that against some stable AGEs in long-lived proteins an immune tolerance might be developed. IFN β , however, is a short-lived protein (half-life of hours) and it is expected that upon administration in patients IFN β -AGEs would be recognized as non-self-antigen thus provoking a classical type immune response.

The adaptive immunity against IFN β may be preceded by activation of the innate immunity. The binding of IFN β -AGEs to non-specific (for AGEs) cellular receptors, such as the scavenger receptors and AGE-R3 on macrophages can result in IFN β -AGEs degradation by phagocytosis (Figure 9). On the other hand, it has been shown that the specific receptor for AGEs, RAGE, belongs to the immunoglobulin superfamily and has the properties of a pattern recognition receptor (PRR)^[137,238,239], which can sense recurring structures of the type of PAMPs^[150]. It is well-known that the interaction of PRR with PAMPs triggers an intracellular signaling cascade, which initiates an innate, inflammatory by nature, immune response^[240] resulting in degradation of the pathogen and in enhancement

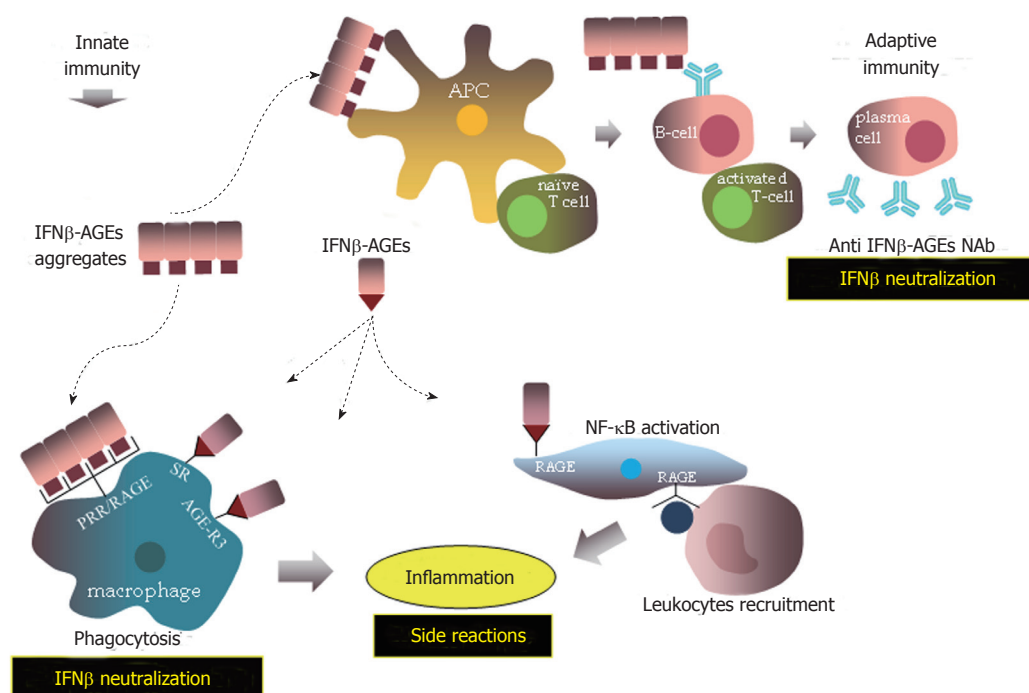


Figure 9 Proposed immune responses against advanced glycation end products modified IFN β (IFN β -advanced glycation endproducts). AGEs: Advanced glycation endproducts.

of the adaptive immune response. We have shown that Advaferon, Betaferon and Rebif contain covalent IFN β aggregates^[211,241], which are formed perhaps by glycooxidation *via* the mediator role of crosslinking AGEs like GOLD, MOLD, pentosidine *etc.* Such covalent aggregates may resemble PAMPs thus interacting with RAGE on macrophages, natural killer (NK) cells and other immune cells to elicit an innate immune response.

RAGE may play further role in inflammatory reactions^[151,242] during IFN β -AGEs therapy. It has been reported that CML^[243,244] and MG-derived hydroimidazolone^[245] are ligands for RAGE. Binding of RAGE with these and other AGEs and non-AGEs ligands such as amfoterin does not result in ligands' degradation but rather triggers different signaling pathways, including proinflammatory responses *via* the activation of transcription factor NF- κ B^[138,246,247]. Also, RAGE on endothelial cells may act as an adhesive receptor interacting with leukocyte β 2 integrins^[151] to recruit immune cells into sites of inflammation. Taken together the different pathways, in which the immune system may react against IFN β -AGEs, are expected to result in neutralization of IFN β activity (inefficient therapy), and in immune-mediated side effects (risky therapy).

CONCLUSION

Many proteins used today in medicine (erythropoietin, insulin, human growth hormone, clotting factors VIII and IX) have been shown to elicit varying incidence of antibody generation in treated patients^[5]. Proteins are susceptible to glycation and it is the protein structure that determines to what extent given protein will

accumulate AGEs. Scaling up the studies by including other therapeutic proteins will reveal the magnitude of AGEs as a causal factor of drugs' immunogenicity. On the other side, not only the protein structure but the whole manufacturing process may impact the glycation status of proteins. Therefore, specific anti-glycation strategies have to be applied to prevent protein glycooxidation. The choice and engineering of appropriate host cells are of particular importance. The glycation potential of host cells should be controlled at all stages of the Maillard reaction including its initiation, propagation and progression. Engineering host cells to overexpress enzymes that detoxify carbonyl compounds will block glycation initiation and its propagation. In a similar way, the overexpression of deglycases^[235,236] in host cells may interfere with early glycation stages. Also, a number of studies report on synthetic and natural compounds with anti-glycation activity^[248]. Such substances can be added to the fermentation media of host cells to inhibit protein glycation. Fermentation parameters such as glucose concentration^[249], oxygen supply (when applicable), temperature, and pH-control are also of particular relevance. If despite all these measures there are glycation adducts still left on proteins, additional techniques could be applied such as: (1) boronate affinity chromatography for extraction of protein species modified by early glycation products^[250]; and (2) use of AGEs-breakers^[251,252] for removal of AGEs. Although costly, isolation, purification, formulation and storage of proteins are best to be conducted under anaerobic conditions, thereby avoiding the use of carbonyl compounds and transition metal ions at each step. Finally, health care authorities

including the American Food and Drug Administration and the European Medical Agency are recommended to undertake relevant initiatives for AGEs assessment of protein drugs.

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Essential vitamins for an effective T cell response

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Abstract

Effective adaptive immune responses rely upon appropriate activation of T cells by antigenic peptide-major histocompatibility complex on the surface of

antigen presenting cells (APCs). Activation relies on additional signals including co-stimulatory molecules on the surface of the APCs that promote T cell expansion. The immune response is further sculpted by the cytokine environment. However, T cells also respond to other environmental signals including hormones, neurotransmitters, and vitamins. In this review, we summarize the mechanisms through which vitamins A and D impact immune responses, particularly in the context of T cell responses.

Key words: Vitamin D; Retinoic acid; T helper cells; Cytokines; Transcription factors; Allergy; Autoimmune diseases

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Core tip: Vitamins are organic compounds that are required in small quantities as nutrients. When used as dietary supplements, vitamins can be used to treat diseases and maintain physiological processes including bone and skin health. Vitamins also play important roles in immune system including lymphocyte activation, and T helper cell differentiation. Among all the vitamins, vitamin A and D have garnered more interest in clinical setting. In this review we have focused on how vitamin A and D regulate adaptive immunity especially how both the vitamins modulate T cell responses. We have highlighted the mechanisms how these vitamins affect T helper cell differentiation. How these vitamins affect inflammatory disorders including allergy and autoimmune diseases have also been described in this review. The purpose of this review is to suggest the potential of dietary supplements such as vitamin A and D can be used to treat inflammatory disorders.

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Vitamins are organic compounds required in small amounts in the human body. As humans cannot synthesize vitamins in sufficient quantity, essential vitamins are provided by diet or dietary supplements. In recent years vitamins have generated considerable clinical interest as they promote pivotal activities in the immune system. Vitamins modulate a wide range of immune responses including lymphocyte activation, homing, and T helper cell differentiation. An association of vitamin B6-deficiency and poor immune parameters has been observed, and a separate study has observed that vitamin B6 is important for normal T-cell function *in vivo*^[1,2]. Systemic administration of vitamin B2 is used to treat septic shock^[3]. Food supplementation of vitamin E is used for hepatitis B treatment^[4]. The maintenance of healthy bones in inflammatory bowel disease might be enhanced by vitamin K^[5]. Vitamins A and D modulate the immune system in very specific ways influencing both the adaptive and innate immune system. The bioactive metabolites of these vitamins have hormone-like properties, making them distinct from other vitamins. Vitamin D deficiency is recognized as a worldwide problem with health consequences including increased incidence of food allergy, while vitamin A deficiency has been acknowledged as a public health problem^[6,7]. This review is aimed at highlighting the mechanisms of vitamin A- and D-dependent regulation of an optimal T cell response.

VITAMIN D SYNTHESIS

The term vitamin D refers to both vitamin D3 (cholecalciferol) and vitamin D2 (ergocalciferol) (Figure 1A)^[8]. Vitamin D is acquired either from diet or from conversion of 7-dehydrocholesterol in the sebaceous gland of animal skin upon exposure to sunlight, though levels generally decrease with aging^[8,9]. To become biologically active, vitamin D undergoes several modification steps (Figure 2A). The enzymes CYP27A1, CYP2R1 among others are required for the formation of 1, 25-hydroxyvitamin D3 (1, 25D3), also known as calcitriol, the biologically active form of vitamin D^[8]. CYP27B1 (one of many cytochrome P450 vitamin D hydroxylases) converts 25-hydroxyvitamin D (25-D) to 1, 25-dihydroxy vitamin D3 by 1 α -hydroxylation^[8,10]. CYP27B1 is found in placenta, monocytes and macrophages^[11]. 1, 25D3 is released into circulation from kidney, an important organ of 1 α -hydroxylation reaction^[8,12]. Transport of vitamin D in blood is aided by vitamin D binding protein^[11]. This protein does not affect the amount of 1, 25D3 entering cells or the synthesis of vitamin D target proteins^[13]. Cultured alveolar macrophages can secrete 1, 25D3^[14]. The level of circulating 25-hydroxyvitamin D is the defining parameter of vitamin D sufficiency or insufficiency. There is considerable debate though as to what should be considered as the cut-off level of required vitamin D in the healthy body. Even though some studies recommend concentrations of 20-30 ng/mL of 25-hydroxyvitamin D as an indicator of vitamin

insufficiency, one study has argued that 20-50 ng/mL of 25-hydroxyvitamin D is normal^[15,16]. Toxicity induced by high concentrations (> 150 ng/mL) of vitamin D is rare^[17,18].

VITAMIN A SYNTHESIS

Metabolites of vitamin A include 11-cis-retinaldehyde and all-trans retinoic acid (*atRA*); also known as retinoic acid (RA) (Figure 1B)^[19]. The abundance of RA is regulated by retinol dehydrogenases and retinal dehydrogenases (RALDH), while degradation is controlled by CYP26^[20]. Though *Rdh5*-deficient mice are viable, *Rdh10* mutants exhibit characteristics similar to RA deficiency^[21]. Carotenoids and retinoids are converted to RA in the intestine, liver and finally in target cells^[22]. Proteins including cellular retinol-binding proteins (CRBP), cellular retinoic acid binding proteins (CRABP) and retinol-binding proteins play important role in the biochemical conversion of retinoic acid^[23]. The key retinoid in circulation binds to retinol-binding protein 4 (RBP4)^[24]. *STRA6*, a retinoic acid inducible gene, facilitates the uptake of RBP4 in tissues^[25]. Retinol can be obtained from animal retinyl esters by hydrolysis. Unesterified retinol gets converted to retinyl ester by lecithin-retinol acyltransferase enzyme^[22]. Free retinol binds to retinol-binding protein while in plasma it binds to transthyretin^[26]. Inside the target cell retinol binds to CRBP to give rise to retinal which subsequently gets converted to RA by RALDH^[27]. When RA binds to CRABP, RA translocates to nucleus where it can bind to different nuclear receptors. The synthesis of vitamin A is shown in Figure 2B.

VITAMIN D SIGNALING

1, 25D3, responsible for most of the biological actions of vitamin D binds to the vitamin D receptor (VDR), a member of the superfamily of the nuclear receptors^[28]. While expressed at low to moderate levels in most human tissues and cell-types, the highest expression of VDR is found in kidney, bone and intestine^[29]. The presence of a structurally conserved α -helical ligand-binding domain (LBD) and a DNA-binding domain are the hallmarks of the nuclear receptor superfamily^[30,31]. A molecule having roughly the size of cholesterol and at nanomolar concentrations can activate VDR^[32]. The LBD of ligand-binding receptors have a 400-1400 Å³ ligand binding pocket^[33]. VDR forms a heterodimer with retinoid X receptor (RXR) in the presence of vitamin D^[8]. This heterodimer is required for the recognition of a vitamin D response element (VDRE) that lies in the regulatory region of vitamin D target genes. The VDRE is found both in proximal promoter regions and in regions located far away from the promoter^[34,35]. VDRE is composed of direct repeats (DRs) of PuG(G/T)TCA motifs that are separated by a 3 bp (DR3) or 6 bp or 8 bp separation of everted repeats (ER6 or ER8)^[36-38]. The 3 bp spacing of DR3 allows a proper platform for VDR/

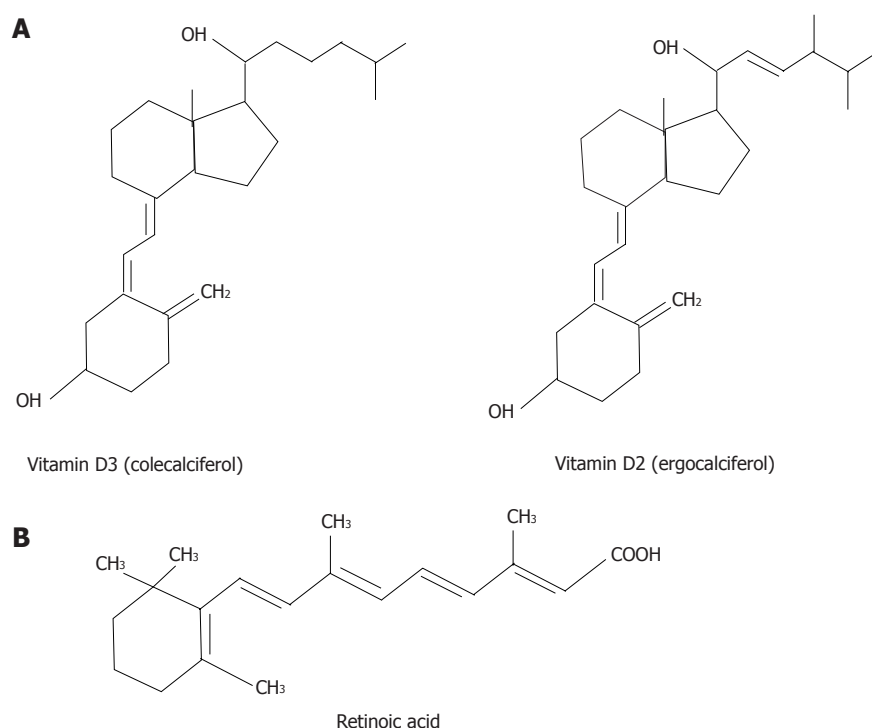


Figure 1 Structure of vitamin D and retinoic acid. A: The chemical structure of vitamin D3 and vitamin D2 are indicated by line diagrams; B: The chemical structure of retinoic acid is indicated by line diagrams.

RXR heterodimer formation that has been confirmed by genome-wide analyses for VDR binding sites^[39]. However, VDR binding to DR3 covers approximately one third of all genomic binding sites suggesting additional mechanisms for VDR to associate with the genomic loci. These mechanisms include binding with unrecognized proteins and tying up to other transcription factors^[39]. Therefore VDR would possibly recruit co-activators and co-repressors to gain access to its target genes. In addition to transactivation, VDR/RXR heterodimers can cause transrepression at their target genes^[40,41].

Chromatin immunoprecipitation (ChIP) has been used extensively to analyze genome-wide binding of VDR^[42]. The regulatory regions of VDR target genes including CYP24A1, CYP27B1 and CCNC were analyzed for VDR binding by ChIP^[43-45]. More than 2500 genomic VDR-binding sites were observed when human lymphoblastoid cells were treated with vitamin D^[46]. Similarly when human monocytes were stimulated with vitamin D, approximately 1200 VDR peaks were observed^[47]. After 3 h stimulation with vitamin D, human colorectal cells show 1674 VDR-binding sites that co-localize with RXR^[48]. ChIP-seq studies also confirm VDR-binding sites on vitamin D target gene including VDR and MYC^[49,50]. When different cell lines and differences in ligand stimulation time were taken into account it was observed that roughly 20% of the genomic sites are identical in assessing VDR-binding sites^[39]. It has been suggested that VDR may not bind regulatory genomic regions to open the gene loci as a "pioneer factor", but rather VDR would act as an "accessory factor", binding already accessible regions of a gene^[39].

VDR and other nuclear receptor interactions with chromatin have been well described^[51,52]. Recruitment of RNA pol II and chromatin modifying enzymes has been observed with VDR on human genes including CYP24A1 and MYC^[43,50]. VDR-dependent changes in the transcriptome have been assessed by both mRNA and miRNA microarrays as well as by ChIP-seq^[39,53]. ChIP-seq data suggested that VDR either has a very promiscuous DNA binding specificity, or that VDR interacts with other DNA-binding transcription factors that alter the DNA binding site^[39].

VITAMIN A SIGNALING

The biologically active form of vitamin A, *atRA* binds to RXR and retinoic acid receptor (RAR). Both RAR and RXR have three subtypes: α , β , and γ ; each encoded by separate gene^[54]. Each subunit of the receptor has multiple isoforms^[55]. RAR and RXR both belong to the retinoid receptor family. RAR α , RXR α and RXR β are constitutively expressed in mouse embryos^[19]. Functional redundancy is observed between RAR and RXR isotypes^[20]. The functional domains of retinoid receptor include the N-terminal DNA binding domain (DBD) and a C-terminal LBD separated by a central hinge region^[55]. The DBD, a highly conserved domain, contains two zinc finger modules while the activation function-2 (AF-2) domain is part of the LBD^[22]. RAR can be activated by *atRA*, while RXR is activated by 9-*cis* RA^[22]. Ligand binding to LBD causes a conformational change in the receptor complex^[22]. AF-2 also plays an important role in the conformational change^[22]. RAR-

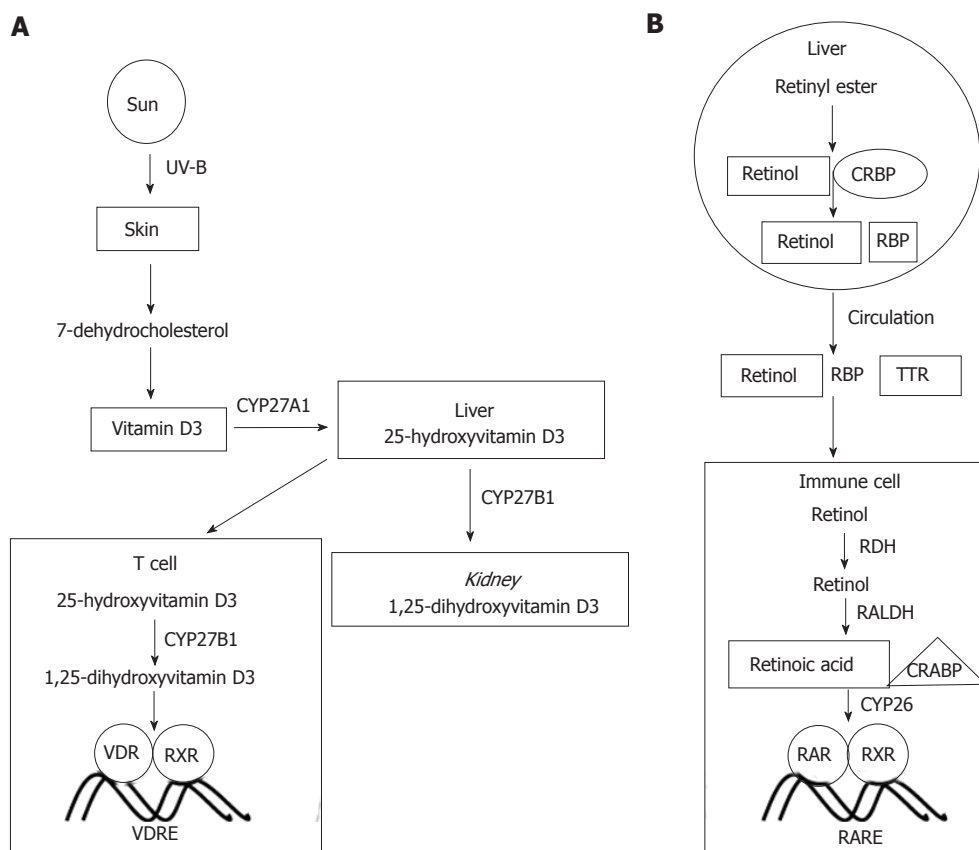


Figure 2 Vitamin D and retinoic acid biosynthesis and activity. A: Vitamin D biosynthetic pathways are indicated, showing critical enzymes in particular tissues. Binding of VDR to DNA with RXR is indicated in a T cell; B: Retinoic acid biosynthetic pathways are indicated. Binding of RAR to DNA with RXR is indicated in an immune cell. RXR: Retinoid X receptor; VDR: Vitamin D receptor; RAR: Retinoic acid receptor; CRBP: Cellular retinol-binding proteins; RDH: Retinol dehydrogenases; VDRE: Vitamin D response element; RARE: Retinoic acid response element; CRABP: Cellular retinoic acid binding proteins.

RXR heterodimer binds to the DNA region known as retinoic acid response elements (RAREs) located in the enhancer regions of RA target genes^[19]. Even though RA functions by binding to RAR, RA can also signal by binding to PPAR- β/γ , an orphan nuclear receptor^[56].

The availability of retinoic acid is governed in a spatiotemporal and tissue-specific fashion^[57]. Transcriptional regulation mediated by RA involves many co-activator and co-repressor proteins^[19]. In the absence of ligand, the RAR/RXR heterodimer binds to a co-repressor complex containing nuclear receptor co-repressor (NCoR) 1 and 2 (known as SMRT), polycomb repressive complex 2, and proteins having histone deacetylase activity^[19,58]. NCoR/SMRT contains two conserved consensus sequences^[54]. Once ligand binding occurs (such as RA), the RAR/RXR heterodimer interaction with the co-regulatory protein is altered with increased binding towards co-activator proteins and reduced interactions with the co-repressor proteins. Co-activator proteins have multiple LXXLL motifs that interact with ligand-activated RAR/RXR heterodimers. Proteins including SRC complexes and histone acetyltransferase such as p300 can interact with RAR/RXR heterodimers^[54,59]. In the presence of appropriate ligand, co-activators preferentially bind to RAR but not RXR. Functional RARE sites consist of hexameric direct

repeats-(A/G)G(T/G)TCA with spacing of either 2 bp (DR2) or 5 bp (DR5)^[19]. AGTTCA is the most efficient hexamer for RAR binding^[60]. *In vitro* studies have suggested a role of DR1 as a RARE; however, the *in vivo* significance is not completely understood^[61]. Protein poly (ADP-ribose) polymerase 1 (PARP-1), present on RAR-inducible promoters in cells, can bind directly to RAR and other mediators^[62]. The events that determine whether a specific RARE will have activation or repression function are also not well understood.

VITAMIN A AND D RECEPTOR POLYMORPHISMS

VDR is located at chromosome 12q13.1 in human and its locus covers more than 100 kb^[63]. The locus contains eight introns and nine exons^[63]. In the *VDR* gene four restriction enzyme polymorphisms have been identified: ApaI (rs7975232), BsmI (rs1544410), FokI (rs10735810), and TaqI (rs731236)^[63]. FokI is located in exon 2, ApaI and BsmI are located in intron between exon 8 and 9 and TaqI is located in exon 9^[63]. There is no structural change in the VDR protein because of the ApaI, BsmI and TaqI single nucleotide polymorphisms^[64]. The FokI polymorphism, however,

results in a second protein variant^[65]. Additionally, the FokI polymorphism has been associated with serum 1, 25D3 concentration in twins with multiple sclerosis^[66]. In systemic lupus erythematosus (SLE) patients, an association between serum 1, 25D3 level and FokI polymorphism has been observed^[67]. In contrast to vitamin D serum concentrations, there is no association between the FokI polymorphism and the clinical spectrum in SLE and multiple sclerosis (MS) patients^[67,68]. However, the BsmI and TaqI polymorphisms are associated with risk of autoimmune diseases including MS, SLE^[63]. VDR polymorphisms have also been associated with increased susceptibility to infections including human immunodeficiency virus-1, tuberculosis and hepatitis B virus^[69]. Single nucleotide polymorphism (SNPs) in vitamin A and D receptor genes in healthy Caucasian children and adults correlate with the efficacy of vaccine-mediated immunity^[70].

Vitamin A receptor allelic variations and haplotypes affect immune responses to measles vaccine^[69]. A specific *RARB* haplotype (resulting from SNPs rs6550978 and rs6777544) was associated with variations in measles antibody titers and measles-specific IL-2 and IFN- γ secretion, while the *RARB* AAC haplotype was concurrently associated with variations in measles antibody titers, and IL-10, IFN- α , and TNF- α secretion in Caucasians^[69]. Using ChIP-seq new RAR-binding loci has been identified along with diverse spacing and topology of DNA^[71,72].

VITAMIN D AND RETINOIC ACID: SIGNALING IN T CELLS

Nutrient status and metabolism affect CD4⁺ T cell development^[73]. Both vitamin A and D control the expression of key players of innate and adaptive immune responses^[74,75]. T cells are directly regulated by vitamin D *via* various mechanisms^[76]. VDR expressed in T cells has high affinity for 1, 25D3, and the concentration of free 25-hydroxyvitamin D to antigen presenting cells regulates the balance between inflammatory and regulatory T cell responses^[77,78]. Inhibitory responses has been observed *in vitro* on adaptive immune cells in the presence of vitamin D^[79]. Activated T cells have increased VDR expression compared to naïve T cells^[80]. Both increased and decreased VDR-dependent Foxp3 expression in murine model has been reported^[81]. Murine Th1, Th2, and Th17 cells express significant *Vdr* transcripts, and the defined functions of VDR in these Th subsets is summarized below^[81]. In the following sections we highlight the specific of vitamin D activity on each Th subset. The function of vitamin D in peripheral CD4⁺ T cells is summarized in Figure 3A.

Th1 cells

Th1 cells are characterized by the production of IFN- γ following differentiation stimulated by IL-12. IFN- γ activates and enhances the microbicidal activity of

macrophages, and as such Th1 cells provide immunity against intracellular bacteria, fungi and protozoa^[82,83]. T-bet is a master regulator of Th1 cell development^[84]. In addition, both STAT4 and STAT1, the signaling and transcription factors downstream of IL-12 and IFN- γ , respectively, promote Th1 cell differentiation^[85,86]. IL-12 signaling also induces the transcription factor IRF-1, required for Th1 cell differentiation^[87].

Several reports have demonstrated the effect of vitamin D in peripheral T cell responses. 1, 25D3 leads to an inhibitory effect on adaptive immune cells^[79]. Cell proliferation is inhibited and the expression of IL-2, a T cell growth factor is attenuated in the presence of 1, 25D3^[88,89]. Memory T cells that have abundant expression of VDR show profound inhibitory effect of 1, 25D3^[90]. Vitamin D suppresses the development of Th1 cells while promoting Th2 cell differentiation^[91]. 1, 25D3 directly inhibits IFN- γ production *in vitro*^[92,93]. *In vivo* Th1 cell development is impaired in the presence of vitamin D that correlates with protection from inflammatory bowel disease^[94]. Even though VDR-deficient animals have normal CD4⁺, and CD8⁺ cells; IFN- γ production by CD4⁺ T cells is significantly increased^[95]. Vitamin D attenuates the production of IL-12 and IFN- γ from human leukocytes infected with *Mycobacteria*^[96]. Mice lacking VDR augment IFN- γ responses and increase clearance rates of *L. major in vivo*^[97]. The effects of the vitamin D response on host response to *Candida* infections is not completely understood though vitamin D deficiency results in enhanced replication of *M. bovis*, while addition of vitamin D suppresses tuberculosis replication^[96,98,99]. In summary, vitamin D decreases IFN- γ production and IL-12 by DCs thereby attenuating Th1 responses.

Th2 cells

Naïve T cells activated by antigen in the presence of IL-4 differentiate into Th2 cells that secrete IL-4, IL-5 and IL-13. Th2 cells provide immunity against extracellular parasites^[100]. IL-4 acts as a positive feedback loop for Th2 differentiation, while IL-5 is eosinophil recruitment and development cytokine^[101,102]. IL-13 plays a key role against parasite infections^[103]. GATA3 acts as the master regulator of Th2 cells, while c-Maf expressed in Th2 cells is a potent transactivator of IL-4^[104,105]. IL-4-induced STAT6 is also required for the development of Th2 cells^[106,107].

In Th2 cells 1, 25D3 enhances the production of IL-4^[93]. When injected subcutaneously in mice in an allergic asthma model, vitamin D increases allergen-induced T-cell proliferation, IL-4 and IL-13 and IgE production^[108]. Surprisingly airway eosinophilia is attenuated following vitamin D administration^[108]. 1, 25D3 polarizes human T cells and murine T cells to a Th2 phenotype^[109]. With increased concentrations of vitamin D, STAT6 expression is up regulated in human PBMCs, and STAT6 is required for the therapeutic effects of 1, 25D3 in EAE^[109]. When pregnant rats are

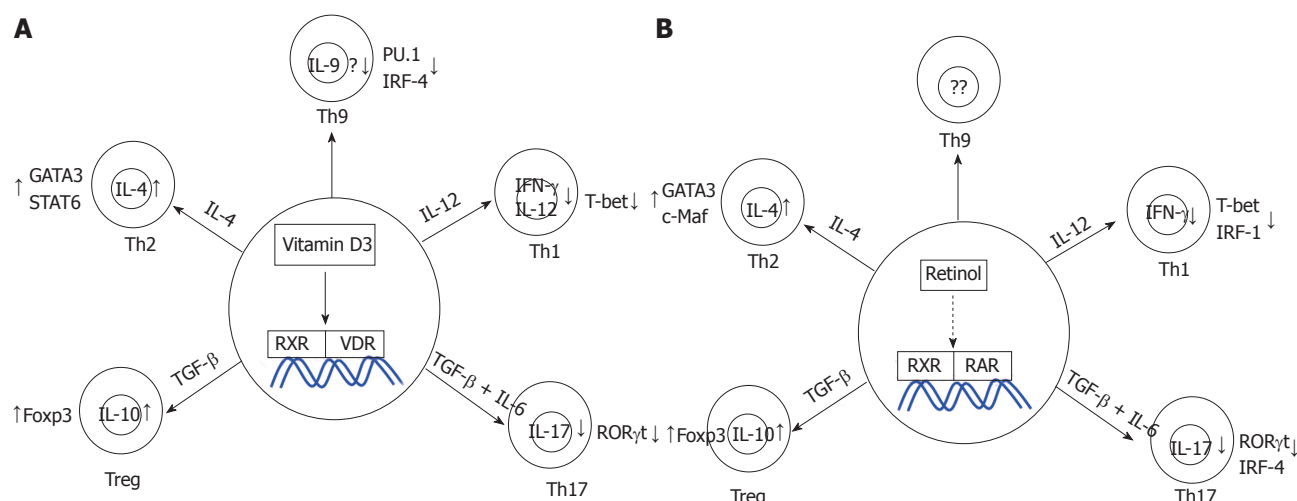


Figure 3 Effects of vitamin D and retinoic acid on T helper cell differentiation. A: The effects of vitamin D on each of the T helper subsets are indicated, detailing changes in expression of cytokines and transcription factors; B: The effects of retinoic acid on each of the T helper subsets is indicated, detailing changes in expression of cytokines and transcription factors. IL: Interleukin; TGF: Transforming growth factor; Treg: Regulatory T cell.

supplemented with high dose of vitamin D, the offspring develop allergic responses due to altered Th1/Th2 ratios^[110]. Patients with allergic bronchopulmonary aspergillosis have attenuated Aspergillosis-induced Th2 responses in the presence of vitamin D^[111]. However, when vitamin D-deficient healthy females undergo vitamin D supplementation there is significant difference in the expression of IL-4, IFN-γ, GATA-3, T-bet, STAT6 and STAT4^[112]. Overall, vitamin D augments Th2 differentiation by increasing the secretion of IL-4, IL-5, IL-10 and Th2-specific transcription factors.

Th17 cells

In the presence of TGF-β, IL-6 and IL-21, naïve CD4⁺ T cells differentiate into Th17 cells that produce IL-17, IL-21 and IL-22^[113-115]. Th17 cells provide immunity to several extracellular pathogens including defense against infections from *Candida*, *Citrobacter* and *Klebsiella*^[116,117]. The transcription factor RORγt is required for Th17 differentiation^[118]. IRF4, an inducer of GATA3 in Th2 cells is also required for Th17 development^[119].

Vitamin D suppresses Th17 cell responses partly by inhibiting IL-6 and IL-23 production, cytokines required for Th17 cell differentiation and commitment^[120-123]. One report suggested that vitamin D can prevent experimental autoimmune uveitis owing in part to direct suppression of Th17 cells^[124]. *In vitro* 1, 25D3 inhibits Th17 cytokine production *via* VDR but does not suppress *Th17* gene transcription^[125]. Mechanistically, 1, 25D3 induces the expression of C/EBP homologous protein that suppresses the production of Th17 cytokines^[125]. The inhibitory effect of 1, 25D3 on IL-17A transcription has been attributed to competition with NFAT at the level of DNA binding, recruitment of histone deacetylase to the *IL17A* promoter and sequestration of Runx1 by 1, 25D3^[126]. The same study has also demonstrated that 1, 25D3 represses *mIL17a* transcription by attenuating recruitment of the

transcription factor Runx1 to the *IL17a* promoter^[126]. 1, 25D3 also inhibits Th17 differentiation in both young asthmatic patients and healthy controls through a dendritic cell-dependent mechanism, inhibiting the expression of IL-17, IL-23R, RORC, and CCR6^[127]. Overall, studies suggest Th17 cell polarization is attenuated by vitamin D.

Treg cells

Regulatory T cells (Tregs) control pro-inflammatory responses of effector Th cells and promote self-tolerance^[128]. Naïve CD4⁺ T cells exposed to TGF-β develop into inducible Tregs in the periphery, and these cells have similar properties to natural Tregs, derived from the thymus including having suppressive functions^[129,130]. The transcription factor Foxp3 has been identified as a reliable marker for Treg cells in both human and mice^[131,132]. IL-10 signaling in Tregs maintains the expression of Foxp3 for immune regulation^[133].

1, 25D3 induces the differentiation and expansion of Foxp3⁺ Treg cells through several mechanisms^[122,134,135]. Immunoglobulin-like transcript 3, (a surface molecule expressed on DCs and macrophages) up-regulation by 1, 25D3 in DCs could induce the production of Treg cells, though this is likely dispensable for Treg induction^[134]. When applied topically, vitamin D augments the suppressive capacity of CD4⁺CD25⁺ cells and CD4⁺ T cells from skin draining lymph nodes have reduced ability to proliferate *in vitro*^[135]. With the help of IL-2, 1, 25D3 increases the frequency of activation-induced human Foxp3⁺ T cells^[136]. There is increased number of CD4⁺CD25⁺Foxp3⁺ Treg cells and increased CTLA4 expression when CD4⁺CD25⁺ T cells are cultured in the presence of vitamin D *in vitro*^[136,137]. Vitamin D supplementation is associated with increased percentage of circulating CD4⁺ Treg cells in healthy individuals^[138]. When administered

orally, calcitriol induces Tregs and immature DCs *via* intestinal exposure while decreasing atherosclerosis in mice^[139]. There is an *in vivo* correlation between vitamin D status and Treg suppressive function in relapsing-remitting multiple sclerosis patients^[140]. However, there is a negative correlation between the Th1/Th2 ratio and 25-D level^[140]. A VDRE is present in the human *FOXP3* conserved non-coding sequence (CNS) that is homologous to the enhancer 1 region of the mouse *Foxp3* gene and there is enhanced *FOXP3* promoter activity in the presence of 1, 25D3^[141]. Vitamin D also induces the development of IL-10-producing Treg cells; although, vitamin D does not affect IL-10R expression^[142-144]. Monocyte-derived DCs pretreated with 1, 25D3 up-regulate the expression of PD-L1 and when co-cultured these DCs convert CD4⁺ T cells to IL-10-secreting Treg cells^[145]. 1, 25D3 in the presence of dexamethasone induces the population of IL-10-secreting Treg cells in both murine and human CD4⁺ T cells^[146]. Overall, Treg cell development is augmented in the presence of vitamin D.

Th9 cells

Naïve CD4⁺ T cells in the presence of TGF- β and IL-4 differentiate into Th9 cells producing IL-9^[147,148]. The transcription factors PU.1 and IRF4 are required for the development of Th9 cells^[149,150]. The effect of vitamin D has also been ascertained in IL-9-secreting Th9 cells. 1, 25D3 inhibits the development of Th9 cells in mice concomitant with increased IL-10^[151]. Vitamin D attenuates the production of IL-9 in purified memory T cells from healthy controls and patients with asthma^[152]. However, vitamin D-mediated inhibition of IL-9 production by Th9 cells is not possible with anti-IL-10 treatment^[152].

Additional mechanisms

Vitamin D might regulate Th cell development and function through additional mechanisms that include direct regulation of gene expression by vitamin receptors, regulation of miRNAs, and by altering epigenetic regulatory mechanisms. miRNAs control effector T cell differentiation and function^[153]. Vitamin D-mediated regulation of T helper cells *via* modulating miRNA has not been studied in great detail. Vitamin D induces the expression of miR-22 in colon cancer cells that can potentially be therapeutic^[154]. In addition, vitamin D-mediated regulation of miRNA has been established in bone^[155]. T helper cell differentiation is also regulated by epigenetic modifications^[156]. Vitamin D-mediated signaling *via* VDR entails both histone acetyltransferase and histone deacetylase (HDAC)^[157]. Histone and DNA methylation are also regulated by vitamin D^[158]. Vitamin D prevents the migration of T helper cells into CNS thereby attenuating experimental autoimmune encephalomyelitis (EAE)^[159].

Retinoic acid and peripheral T cell responses

Retinoic acid affects specific adaptive immune responses

and elicits effector CD4⁺ T cell responses^[79,160,161]. Retinoic acid augments T-cell proliferation by inducing IL-2 secretion^[162]. RA induces gut-homing receptors on T cells^[163]. Depleting vitamin A significantly attenuates the number of IgA-secreting cells in rat ileum^[164]. Vitamin A-deficient mice demonstrate defective Th-cell activity^[165]. Retinoic acid augments the expression of gut-homing molecules $\alpha 4\beta 7$ and CCR9 on CD4⁺ T cells maintaining intestinal homeostasis^[166]. In the following sections we highlight the specific of retinoic acid on each Th subset. The function of retinoic acid on T helper cells is summarized in Figure 3B.

Th1 cells: Vitamin A deficiency leads to enhanced production of IFN- γ -secreting Th1 cells^[167]. Retinoic acid inhibits Th1 cell development by attenuating IFN- γ production directly *via* RAR but not RXR^[168]. In RA-treated immunized mice there is reduced expression of Th1-specific transcription factors T-bet and IRF-1^[169]. RA also attenuates the expression of STAT4 and T-bet in a mouse model of autoimmune insulinitis^[170]. Whether *atRA* can regulate Th1/Th2 balance in a strong Th1 environment has been assessed^[171]. In Gata3-deficient CD4⁺ T cells, *atRA* induces the expression of IL-4 while reducing the secretion of IFN- γ ^[171]. *atRA* abrogates the induction of Th1 cells in spleen and lymph nodes *in vivo*^[172]. In RAR α -transfected Jurkat cells, *atRA* significantly down-regulates *Ifng* promoter activity^[173]. However, a report published this year argues RA is important for Th1 cell stability while preventing a switch to Th17 cells^[174]. RA is required for STAT4-dependent T-bet expression in Th1 cells^[174]. At Th1 lineage associated loci, RA-RAR α regulates enhancer activity^[174]. Overall, retinoic acid inhibits the production of Th1 cytokines both *in vitro* and *in vivo*.

Th2 cells: Vitamin A-deficient mice demonstrate poor antibody responses owing to significant decreases in Th2-cell frequency^[165]. In the absence of exogenous cytokines, *atRA* significantly attenuates IFN- γ production but does not change IL-4 production^[175]. When Th2-polarizing cytokines are present, *atRA* significantly enhances IL-4 secretion in the presence of antigen-presenting cells^[175]. *atRA* enhances the transcription of Th2-associated genes including *Gata-3*, *c-Maf* and *IL-4R α* in murine CD4⁺ T cells^[168]. *atRA* has also been shown to have a similar Th2-promoting effect on human MBP-specific T cell lines *in vitro*^[176]. A recent report suggested that in vitamin A-deficient mice, mesenteric lymph node DCs induce a Th subset producing high IL-13 and TNF- α ^[177]. Induction of this specific subset was mediated by B220-CD8 α -CD103-CD11b⁺ DCs and was dependent on IL-6 and OX40L but inhibited by RA^[177]. Both *atRA* and 9-*cis* RA are equally potent in promoting human Th2 cell development^[178]. The role of specific retinoic acid receptors in either Th1 or Th2-mediated disease pathology has remained unclear. RAR α induces human Th2 cytokine production that correlates with the expression of CD38 and CD69^[179].

Similarly, RAR agonists, Am80 and Tp80 mimic the effect of RA^[168]. However, one study reported that Am80 did not have any effect on IFN- γ production by Th1 clones^[180]. *atRA* enhances the percentage of IL-5+ Th2 cells while RAR α antagonist Ro415253 reduces the percentage of IL-5+ Th2 cells from house dust mite specific short-term cell lines^[181]. Therefore, the studies indicate that retinoic acid promotes Th2 differentiation and maintains Th1-Th2 balance.

Th17 cells: *atRA* and RAR α suppress the formation of Th17 cells and induce the production of Foxp3⁺ Treg cells^[182,183]. The *atRA*-mediated inhibition of Th17 cells is independent of IL-2, STAT3 and STAT5 to overcome the effect of IL-6; however some initial IL-2 signaling may be required for the regulatory function of *atRA*^[182,183]. In Th17 cells, RA attenuates the expression of TGF- β -induced IL-6R α and the transcription factor IRF4^[184]. RA also suppresses Th17 cell development from Th1 precursors^[174]. In the absence of RA signaling there is increased expression of Th17-specific genes including *Rorc*, *Il23r*, *Il22*, *Il21*^[174]. Overall, retinoic acid blocks the development of Th17 cells by down-regulating ROR γ t expression.

Treg cells: *atRA* plays an important role in immune tolerance *via* induction of Treg cells. In the presence of RA there is an induction of Foxp3⁺ Treg cells in murine CD4⁺ T cells^[184]. 1, 25D3 augments dexamethasone-induced expression of IL-10 by Tregs^[185]. There is increased expression and phosphorylation of the TGF- β -induced molecule Smad3 but not Smad2 in the presence of RA^[184]. RA can override the negative effect of co-stimulatory molecules and specific secreted cytokines in enhancing the conversion of naïve T cells to Treg cells^[186]. Even though there is RA-mediated induction of Smad3, enhanced Treg conversion is Smad3-independent^[186]. The same study demonstrated that RAR α , but not RAR α 1 deficiency blocks Treg cell conversion^[186]. TGF- β also induces ERK1/2-signaling pathway and Smad3 and ERK1/2 have complex interaction that is cell type-specific^[187]. In human Tregs generated from cord blood ERK1/2 signaling is impaired, while in relapsing-remitting multiple sclerosis patients the activity of ERK1/2 is altered^[188,189]. In contrast, ERK1/2 signaling is activated by *atRA* leading to enhanced Foxp3 expression^[190]. *atRA* induces CTLA-4, part of the T-cell co-stimulatory signal that differentially regulates ERK signaling pathway and is required by TGF- β to generate CD4⁺CD25⁺ Tregs^[191-193]. For the development of human inducible Tregs (iTregs), the addition of *atRA* is required, even though *atRA* does not affect proliferation or survival of human CD4⁺ T cells^[194,195]. After *atRA* treatment, human natural Tregs (nTregs) become resistant to conversion to Th1 and Th17 cells with sustained expression of Foxp3^[191]. *atRA* induces the expression of Foxp3⁺Tregs from naïve CD4⁺ T cells but not from memory CD4⁺

T cells^[195]. Maintenance of Foxp3 expression during nTreg expansion is mediated by *atRA*^[196]. Even in the presence of inflammatory cytokines, *atRA*-pretreated Tregs maintain their suppressive capabilities^[191]. Mechanistically *atRA* increases histone acetylation and methylation of *Foxp3* promoter as well as conserved non-coding sites at the gene promoter^[190]. In nTregs, *atRA* can inhibit methylation of Foxp3 in the presence of inflammatory cytokines^[191]. In contrast to Th17 cells, retinoic acid induces the development of Foxp3⁺ Tregs indicating the reciprocal regulation between Th17 and Treg cells. Overall, the potential role of retinoic acid in peripheral T cells is described in Figure 3.

Retinoic acid has been shown to mediate epigenetic changes in embryonic stem cells^[197]. Retinoic acid induces the expression of specific microRNAs and their precursors in human AML and in neuroblastoma cells^[198,199]. miR-10a, which can act as tumor suppressor in breast cancer is also induced by retinoic acid and TGF- β altering the plasticity of T helper cells in process^[200,201].

Therefore, both vitamin D and retinoic acid could potentially regulate T helper cell differentiation *via* epigenetic modification and miRNA.

ROLES OF VITAMINS IN ALLERGY AND AUTOIMMUNE DISEASES

Both vitamin D and retinoic acid have been implicated in allergy and autoimmune diseases^[81,202]. This section highlights the role of both vitamins in allergy and autoimmune diseases such as EAE and type 1 diabetes (T1D).

Allergy

As both the prevalence of asthma and vitamin D deficiency has been increased it has been hypothesized that both of these parameters might be linked^[203,204]. A cross-sectional study from the Third National Health and Nutrition Examination Survey suggests that serum vitamin D level is positively associated with FEV-1, the maximum amount of air to be expelled during the first second of forced expiration^[205]. In Chinese adults with asthma, vitamin D deficiency has been associated with reduced lung function^[206]. Low vitamin D levels have also been associated with markers of allergy and asthma severity^[207]. Corticosteroids that are used to treat asthma patients function better in the presence of vitamin D, suggesting that low vitamin D concentrations might also impair therapy^[208]. Serum 25-D level of less than 30 ng/mL has been associated with impaired lung function, airway hyper responsiveness and increased asthma exacerbations^[209]. Vitamin D deficiency in children with asthma being treated with inhaled corticosteroids is associated with reduced lung function^[209]. However, one study did not find any correlation between the level of vitamin D and airway inflammation in a group of asthmatic children^[210].

However, vitamin D supplementation has not been suggested as a therapeutic in patients with symptomatic asthma^[211]. A case control study performed in asthmatic children observed a possible association of vitamin D sufficiency status and *VDR* gene polymorphism^[212]. Impaired maternal 1, 25D3 level during pregnancy impairs the production of Treg cells^[213]. Even though most cohort studies have suggested that there is inverse correlation between maternal vitamin D level and early childhood risk of allergic sensitization, this is not consistent in all studies^[214-216]. A recent study argued that neither serum 25-D level nor dietary intake of calcitriol is associated with allergy^[217].

Retinoic acid-treated rabbit tracheal epithelial cells lead to enhanced mucin gene expression suggesting the potential role of retinoic acid in asthma^[218]. Normal bone marrow cultures treated with *atRA* suppress eosinophil and basophil differentiation^[219]. Furthermore, human bronchial epithelial cell line BEAS-2B treated with RA, abrogates IL-4-induced production of eotaxin, an eosinophil chemoattractant^[220]. The transrepression of eotaxin is not due to altered STAT6 binding or AP-1 activity^[220]. *atRA* reduces total cell counts and inflammatory cells in BAL fluid in asthmatic rats which could be due to reduced expression of NF- κ B and ICAM1^[221]. RA also inhibits human bronchial smooth muscle cell migration by modulating PI3K/Akt pathway and is associated with inhibition of platelet-derived growth factor-induced actin migration^[222]. In order to prevent *atRA* catabolism and clearance, liposome encapsulated *atRA* has been developed. When liposome-encapsulated *atRA* is administered intraperitoneally, OVA-induced IgE production and accumulation of IL-5 is increased^[223]. However, when administered intranasally those parameters were not affected^[223]. When inhaled *atRA* is given to a patient suffering from severe emphysema, inflammation and lung destruction are reduced^[224]. In mouse models of allergic inflammation, *atRA* attenuates the asthmatic phenotype by inhibiting both Th2 and Th17 responses^[225,226]. In contrast, the expression of Foxp3 is not altered after *atRA* treatment in the spleen of the asthmatic mice^[226]. In an allergic asthma model when mice are treated with RAR inhibitor to block RA signaling the number of Foxp3+ cells were reduced *in vivo*^[227]. A study has shown that *atRA* upregulates the expression of *ORMDL3*, a childhood asthma onset candidate gene *via* PKC/CREB pathway^[227].

Autoimmune diseases: The function of vitamin D and retinoic acid have been studied in EAE (mouse model of multiple sclerosis) and T1D^[228].

EAE: When naïve T cells with a TCR specific for myelin basic protein are transferred to recipient mice that are immunized later with MBP, recipients do not have EAE symptoms when they are treated with 1, 25D3^[229]. This effect is mediated *via* *VDR* in T cells^[230,231]. Peripheral CD4⁺ T cell priming and myelin-specific T cell trafficking

remain unchanged after vitamin D treatment owing to an anergic T cell phenotype in vitamin D-treated groups^[229,230]. A reduced number of Th1 cells in the periphery could also contribute to the therapeutic effect of vitamin D in treating EAE^[232]. Vitamin D administered to rodents correlated with fewer CD4⁺ T cells in CNS, attenuated CNS pathology and reduced IFN- γ -production^[81]. In healthy volunteers, vitamin D decreases the percentage of Th1 cells in circulation^[233]. In contrast, it has been reported that vitamin D deficiency actually delays the onset of EAE^[234]. Oral vitamin D treatment in healthy volunteers and in MS patients does not have a significant effect on the level of circulating IFN- γ ^[235,236].

Apart from Th1 cells, development of Th17 cells is affected by vitamin D in EAE. In EAE, there are reduced numbers of Th17 cells in the CNS after vitamin D treatment^[237]. There are fewer Th17 cells in spleen in vitamin D-treated mice compared to placebo controls^[125,238]. Vitamin D can either inhibit Th17 cytokine production or can suppress *Il17* gene transcription in EAE^[125,126]. When human CD4⁺ T cells are treated with vitamin D, the frequency of Th17 cells are attenuated, whereas increased doses of vitamin D reduce the proportion of Th17 cells in peripheral blood^[137,233]. However, vitamin D supplementation may not affect Th17 cells in MS patients^[239]. Th2 cells might also play a role in EAE^[240]. Disruption of IL-4 slightly reduces the protective function of vitamin D in EAE, and there are increased IL-4 transcripts in the lymph nodes when vitamin D is administered before EAE induction^[241,242].

In autoimmune diseases including MS there are defects in CD4⁺Foxp3⁺ Treg cells in peripheral blood cells and thymus^[243-245]. There is correlation between percentage of Tregs and 25-D level as well as correlation of suppressive capacity of CD4⁺CD25⁺ Treg cells with serum 25-D level^[239,246]. Seasonal increases in 25-D and 1, 25D3 in healthy volunteers correlate with enhanced Foxp3⁺ Treg expression^[247].

Retinoic acid suppresses EAE in rodents^[176,248]. Production of IL-4 is correlated with better disease outcome when retinoic acid is used to treat EAE, while the level of IFN- γ , TNF- α , and IL-2 mRNA is attenuated^[249]. Intraperitoneal injection of high dose of *atRA* protects animals from developing EAE and is associated with reduced IFN- γ and IL-17 production^[184]. When synthetic retinoid AM80 is administered orally, early development of EAE is ameliorated concomitant with reduced Th17 cell differentiation^[250]. The amelioration of EAE is mediated by down-regulation of MHC II molecules, CD80 and CD86 on the surface of DCs that results in altered polarization of both Th1 and Th17 cells^[172]. As an additional potential mechanism, one report has suggested AM80 induces MDSCs thereby increasing the severity of EAE^[251]. When *atRA* is combined with atorvastatin, a synergistic effect is observed in ameliorating EAE^[252]. Tributyltin, which activates RXR delays the onset of EAE^[253]. Ligands for PPAR γ and RXR generate additive anti-inflammatory

effects on EAE^[254]. The decrease of pro-inflammatory cytokine production following helminth infection in multiple sclerosis patients is dependent on RA^[255].

T1D: T1D is an autoimmune disorder where the immune system attacks the insulin-producing beta cells in the pancreas. The relationship between T1D and vitamin D is still not completely understood as most of the studies have been observational. Studies have observed that vitamin D supplementation may improve insulin sensitivity whereas, vitamin D deficiency has been suggested to affect the risk of T1D^[256]. People diagnosed with T1D have a reduced level of vitamin D^[257]. Approximately 15%-90% prevalence of vitamin D deficiency in patients with T1D has been reported^[258-260]. A steady dose of vitamin D could reduce the risk of T1D^[261,262]. Lower serum vitamin D level and insufficient supplementation during infancy may enhance the risk of T1D^[263]. In addition, genetic association of VDR and age of onset of T1D has been assessed^[264-266]. One progressive study observed no association between diabetes progression and vitamin D level^[267]. Vitamin D deficiency during pregnancy may increase the incidence of T1D^[268]. However, whether vitamin D intake during pregnancy would prevent children from developing T1D is not clear due to contradictory results^[269]. A reduced level of vitamin D has been observed in children with T1D and multiple islet autoantibodies^[270]. Both 1, 25D3 and its analog TX527 suppress effector cytokine production and induce the production of IL-10-secreting CD4⁺CD25^{hi}CD127^{lo} T cells with regulatory function^[271]. In NOD mice 1, 25D3 establishes tolerogenic DCs which are responsible for Treg expansion and IL-10 production^[272]. In the presence of vitamin D there is increased frequency of Tregs in the pancreatic lymph node in NOD mice^[273]. Overall these results suggest that vitamin D deficiency correlates with increased risk of T1D. Thus vitamin D could help in managing some spectrum of the disease.

RA has been shown to attenuate inflammation in diabetic rat kidney and reduced proteinuria in diabetic rats^[274]. In NOD mice with insulinitis, *atRA* treatment suppresses diabetes associated with reduced IFN- γ -producing T cells^[170]. However, the percentages of IL-17- or IL-4-secreting cells are not affected. When CD8⁺ T cells from 8.3-NOD splenocytes are cultured in the presence of *atRA* and TGF- β , an induction of Foxp3⁺ CD8⁺ T cells is observed that suppress diabetogenic T cells both *in vitro* and *in vivo*^[275]. *atRA* and exendin-4 (which increases beta-cell mass and function) treatment before the onset of T1D prevents and reverses the disease; however, after the onset of the disease reversal of hyperglycemia or improved survival is not observed^[276]. The suppression of T1D by retinoids is dependent on the presence of Tregs^[277].

CLINICAL APPLICATIONS

As vitamin D deficiency is endemic and associated

with various diseases, it is no surprise that vitamin D has its uses in the clinical practice. As previously mentioned, vitamin D aids in the suppression of allergic inflammation and autoimmune diseases including T1D and multiple sclerosis. A meta-analysis of 18 randomized controlled trials have suggested that even at a low dose vitamin D significantly reduces mortality^[278]. Subsequent studies have indicated that the statin class of drug could attenuate mortality rate by enhancing the level of 25-D^[279]. A 30% increase in the baseline dose of 25-D reduces internal cancer by 60%, while the level of serum 25-D is an independent predictor of cancer risk^[280]. Vitamin D attenuates the incidence of respiratory infections in children^[281]. Vitamin D supplementation in food has been suggested to decrease the risk of autism^[282]. Deficiency of vitamin D has been associated with increased risk of multiple chronic disorders^[283,284]. Vitamin D is recommended to treat osteoporosis^[285]. Seasonal deficiency of antimicrobial peptide (AMP) is secondary to vitamin D seasonal deficiency and could be linked, to flu epidemics^[281]. Interestingly though vitamin D-sensitive AMPs inhibit HIV virus but vitamin D has been suggested play a role in the pathogenesis of HIV^[286]. Vitamin D could provide respite against invasive bacterial diseases including meningococci and pneumococci. A therapeutic effect of vitamin D is also seen in non-small-cell lung cancer patients^[287]. Consistent with its anti-inflammatory property, vitamin D enhances the life span in mouse model of SLE and inhibits contact hypersensitivity^[288,289]. Vitamin D has also been successfully used to treat psoriasis^[290]. It has also been suggested that vitamin D can potentially be used during transplantation. In rat model of renal transplantation vitamin D and its analogue prevents chronic allograft rejection while vitamin D delayed chronic allograft rejection in mouse model of aortic transplantation^[291,292]. Intriguingly, VDR polymorphisms are associated with increased GVHD in bone-marrow transplantation patients^[293]. It has been noted that the vitamin D analogue may be safer alternative to vitamin D-mediated immune modulation owing to its adverse effect in bone resorption and hypercalcemia^[91].

Retinoic acid also has multiple clinical applications. A correlation between vitamin A supplementation and reduced diarrhea and mortality in HIV-infected children has been reported^[294,295]. Retinoic acid has been suggested to induce terminal cellular differentiation in tumor cells^[296]. Vitamin A has been demonstrated to maintain natural killer cell numbers that play important role in anti-tumor immunity^[297]. *atRA* treatment doubles the survival rate of acute promyelocytic leukemia (APL) patients^[298]. In the presence of HDAC inhibitor sodium phenylbutyrate, *atRA* induces remission in APL patient^[299]. In addition, *atRA* along with trichostatin A, another HDAC inhibitor demonstrated significant inhibition of tumor growth associated with increased level of RAR β 2 level^[300]. RAR α proteins play crucial role in the attenuating APL^[54]. RA also initiates growth arrest of leukemia-initiating cells in APL mouse model^[301].

Retinoids have been suggested to induce irreversible differentiation of cancer stem cells^[54]. Apart from cancer treatment, retinoic acid has also been suggested to treat autoimmune inflammation including EAE, adjuvant arthritis and experimental nephritis^[180,249,302]. Retinoic acid has also been used for treating atopic dermatitis and psoriasis^[303,304].

CONCLUSION

There is a paucity of data on the role of vitamin C, E, and K on adaptive immune system including the function in Th cell development. We have highlighted the role of vitamin D and retinoic acid on CD4⁺ T cells and their role in inflammatory disorders, but other vitamins likely also impact many of the responses we have described. Vitamin D-mediated paracrine signaling to CD4⁺ T cells affects its immunoregulatory mechanisms. The sources of vitamin D and the signals required to induce and inhibit vitamin D *in vivo* is crucial to the understanding of its role in health issues. Because of the use of common receptor RXR, vitamin D and retinoic acid could potentially oppose each other's contribution in the development of T helper cells and modulation of inflammatory responses.

A better understanding of the role of vitamin D in controlling effector CD4⁺ T cells could be achieved using animals that have T-cell-specific deletion of VDR. A study has demonstrated that vitamin D in combination with IL-2 induce the production of CTLA-4-expressing Treg cells^[136]. CTLA-4 regulates the spectrum of T cell responses and CTLA-4 deficiency increases follicular T helper cells (Tfh cell) numbers^[305]. Vitamin D also up-regulates the expression of Helios and CD200, other markers of Tfh cells^[81,306]. Whether vitamin D plays a role in the differentiation of Tfh cells has not been ascertained. Consumption of foods enriched with specific vitamins could alter the microbiota in our body leading to better immune responses.

There is ample indication that both vitamin D and retinoic acid could be used as future therapeutics to treat inflammatory diseases. Vitamin D and oral mycophenolate mofetil lead to tolerance and acceptance of fully mismatched mouse islet isograft associated with increased frequency of CD4⁺CD25⁺CD152⁺ Tregs in a transplant model^[307]. Mucosal vaccination might be aided by retinoic acid while vitamin D could be applied in transplant patients^[308]. The role of vitamin D in cardiovascular disease remains unclear. The function of retinoic acid is also not explored in other inflammatory disease pathologies. However, the challenge remains in translating promising animal model results into humans. Once established, purchasing vitamins over the counter could be a safe and efficient approach to modifying immune responses.

KEY POINTS

Dietary supplementation of vitamin D and retinoic

acid can be used to treat certain diseases; Skin, bone, dental health, cognitive function, and other physiological processes are maintained by vitamin D and retinoic acid; both vitamins A and D regulate immune system by affecting CD4⁺ T helper cell differentiation and lymphocyte activation; allergy and autoimmune diseases such as type I diabetes and EAE can be modulated indirectly by vitamin D and retinoic acid; Both vitamin D and retinoic acid have clinical implications for treating various inflammatory disorders.

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Review of immunological responses to porcine coronaviruses and implications on population based control strategies in epidemic and endemic infections

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Abstract

Five major porcine coronaviruses (COVs) have been identified which cause severe gastrointestinal (GI) and respiratory disease in pigs. They include transmissible

gastroenteritis (TGEV), porcine epidemic diarrhea virus (PEDV), porcine deltacoronavirus, porcine respiratory coronavirus, and porcine hemagglutinating encephalomyelitis. These diseases, especially TGEV and PEDV, have caused epidemics in Europe, Asia, and the Americas over the past 50 years, causing significant economic losses to swine producers. As pigs are a major protein source worldwide there is great interest in understanding, controlling, and preventing these diseases. These diseases have no cure, and current vaccines are not fully protective. On-farm prevention and biosecurity are difficult to enforce and have not stopped the spread of these diseases between herds. Recent advances in the immunology of porcine COVs has revealed that the immune response to porcine COVs shares many similarities with the response to human COVs, leading to increased interest in pigs as models for human disease. Highlights of these advances include the key role of local antigen presenting cells in the gastrointestinal tract in stimulating a protective immune response. This understanding has led to new proposed vaccines. Advances in the understanding of the ways the viruses evade and degrade the host immune system have also led to novel proposed therapies. Many of these therapies are in the early development stages, as researchers attempt to create efficacious, cost-effective, and practical therapies for these diseases.

Key words: Immunology; Porcine; Corona viruses; Population; Control; Zoonotic; Epidemic

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Core tip: Coronaviruses (COVs) cause severe disease in both pigs and humans. New immunological research in pigs has revealed many similarities between porcine and human responses to COVs. Understanding the immunological responses of pigs to COVs may prove

that they are a viable human model to study these diseases, as well as providing new and more efficacious control mechanisms for veterinarians and swine producers worldwide.

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INTRODUCTION

Coronaviruses (COVs) are pathogens that naturally infect both humans and pigs, as well as other mammals and birds^[1]. These viruses mutate rapidly and spread easily, and present a complex prevention and control challenge^[2]. Known and emerging COVs continue to cause dangerous and costly diseases in humans and pigs, and some have proven zoonotic potential. For example, the global outbreak of severe acute respiratory syndrome in 2003, which killed 774 people, was caused by a COV of animal origin^[3]. Emerging COVs have also caused outbreaks with high mortality in pig populations. A recent example was the 2010 outbreak of porcine epidemic diarrhea virus (PEDV) in China, which killed over 1000000 piglets^[4]. New research has also shown that pigs may be a viable human model to study COVs in both species^[5]. Recent research has made many advances in the field of coronavirus immunology, which will be crucial in improving future prevention and control techniques of COVs in both humans and pigs.

BACKGROUND

Review of COVs

COVs are enveloped, single strand positive-sense RNA viruses that primarily target the upper respiratory and gastrointestinal (GI) tract of humans, pigs, and a variety of other mammals and birds^[2,6,7]. They belong to the *Coronaviridae* family, subfamily *Coronavirinae*^[8].

Once in the body, COVs bind to surface receptors on susceptible cells^[2]. This allows the virus to enter the cell, and replicate in the cytoplasm^[8]. New copies of the virus are then assembled in the rough endoplasmic reticulum^[9]. From there, move through the Golgi apparatus, fuse with the cell membrane of the host cell, and are released^[10].

Porcine COVs

Five COVs have been identified in pigs. They include: Transmissible gastroenteritis (TGEV), PEDV, porcine deltacoronavirus (PDCoV), porcine respiratory coronavirus (PRCV), and porcine hemagglutinating encephalomyelitis (PHEV). Of these, the most damaging to the worldwide pork production industry and therefore the

most studied are TGEV and PEDV^[2]. These two diseases will be the focus of this review.

TGEV was the original COV shown to cause clinical disease in pigs. It was first documented in the United States in 1946, and today has high seroprevalence in pig operations throughout the world^[2]. Because of its longevity and negative impact worldwide, it has received the most attention and research. Epidemics are less common in the United States and Europe today since PRCV has become more ubiquitous which seems to provide immunological protection against TGE, but outbreaks in naïve herds still occur^[11]. However, TGEV continues to cause major outbreaks in Asia to date, and a new strain has been documented in 2012 that shares a common ancestor with the United States strain^[12]. This highlights the ability of COVs to mutate rapidly, and the challenges and risks of our increasingly global economy, which may bring new virulent strains back to the United States and Europe.

PEDV was the next virus to emerge as a global problem in pig production. PEDV initially caused outbreaks in Europe. It was identified as the cause of outbreaks of diarrhea in 1971. In 1984 PEDV was identified in Asia. The most recent outbreak in Asia was 2010-2012, when the appearance of new strains caused massive vaccine failures and high mortality among affected pigs^[13]. From 2013-2014, two new strains of PEDV were identified in the United States. Despite control efforts, these strains spread rapidly through the United States, Canada, and Mexico, causing the death of several million piglets^[14]. Additionally, an outbreak in Germany in 2014 revealed strains nearly identical to those found in the United States^[15]. The fact that multiple strains are present worldwide that may be related again highlights the need for new, more effective control measures^[2].

PDCoV is the most recent porcine COV to be identified, it was detected for the first time in the United States in 2014. Although the consequences of infection tend to be less severe than TGEV and PEDV, it is an emerging disease that requires further study^[16].

PRCV is a variant of TGEV caused by small deletions in the TGEV genome, and generally causes an asymptomatic infection. Its emergence in Europe in the early 1980's corresponded with the disappearance of TGEV in Europe, as it acted as a natural modified-live vaccine to produce active immunity in infected sows, who then passively transferred immunity to their suckling piglets. Infections are usually subclinical, or a mild respiratory disease can be detected. It is endemic in most swine herds in affected countries, and pigs are not routinely tested for the disease. However, it still remains of interest because of potential protection against TGEV afforded by previous exposure to PRCV^[2].

PHEV is a ubiquitous virus, and causes mostly subclinical infections. It can cause clinical disease upon entering a naïve herd that can affect both the GI and central nervous system^[17]. However, morbidity and mortality are low for both forms, making this disease

less of a concern for pig producers and researchers at this time^[2].

TGEV and PEDV pathogenesis and clinical signs

TGEV and PEDV enter *via* the oral cavity, survive the low pH of the stomach, and primarily infect villous epithelial cells of the intestinal brush border of the small intestine. Both viruses can also infect the alveolar macrophages of the respiratory tract resulting in pneumonic lesions^[2].

TGEV uses its spike (S) glycoprotein to bind to the receptor aminopeptidase N expressed by epithelial cells of the small intestine. The virus then destroys the mature villous cells, leading to reduction and blunting of the villi, and reducing normal enzymatic activity. This leads to alterations in digestion, cellular transport, and hydrolysis of lactose from milk. Tight junctions between the intestinal epithelial cells are also weakened by both TGEV and PEDV infections^[18]. The result is a malabsorptive syndrome caused by increased osmotic force in the unhealthy intestine as lactose and sodium accumulate, leading to diarrhea, metabolic acidosis, dehydration, and death in a naïve animal^[2].

The impact of TGEV on a herd depends on the immune status of herd that is infected. The most devastating is the epizootic infection, which occurs when a herd with no prior immunity herd is exposed. It is common in these herds for neonatal mortality to reach 100% for as long as 4 wk secondary to profuse diarrhea and the resulting dehydration. In young growing pigs and adults inappetence, vomiting, and diarrhea are commonly seen with morbidity approaching 100% but very low to no mortality. At the herd level the infection is usually self-limiting, and ends within several weeks. However, recovered adults often become carriers^[19]. Other herds which have an endemic or enzootic infection, or where adult females have been previously exposed and can provide passive immunity to the piglets, may experience outbreaks, but those outbreaks have much lower mortality rates, usually not exceeding 20%. PEDV is closely related to TGEV in both pathogenesis, behavior in a herd, and clinical signs^[2]. It is necessary to use diagnostic tests to differentiate the diseases from each other on an infected farm^[20].

Epidemiology of TGEV and PEDV

TGEV is shed in both the feces and the nasal secretions of infected animals during an epidemic infection. The virus has been found in the feces of young pigs for up to 2 wk, and in the nasal secretions for 10 to 11 d. The virus is then spread to other animals *via* fecal-oral contact or aerogenously over short distances, and can travel between pens and farms by means of mechanical vectors which include farm workers and equipment, dogs, cats, foxes, starlings, and flies^[2].

The infection may become endemic on the farm after an epidemic. Those pigs that survived the initial TGEV infection may continue to shed the virus, and further outbreaks have been documented up to 9 mo later. Also, in the absence of thorough cleaning and

disinfection, the virus may persist in the environment, especially during colder months^[21].

PEDV offers similar epidemiological challenges, and has been documented to be shed in the feces of recovered pigs for up to 42 d^[13]. However, the numbers of studies currently available for PEDV epidemiology are far fewer than those available for TGEV, and offers another avenue for continued study that may yield important information for the control of both diseases.

THE PORCINE IMMUNE RESPONSE

When a pig is infected with TGEV or PEDV, both single stranded RNA (ssRNA) viruses, the virus primarily targets the epithelial cells of the small intestines^[2]. As the virus invades the cells and begins its process of replication, the immune system attempts to mount a response. However, given the high mortality rate among pigs less than four weeks of age (neonates), it is known that the response is unable to protect very young animals.

Innate immune response

The major difference in the behavior of the villous epithelial cells in neonates compared to older animals is the rate of replacement of the mature cells. Neonatal pigs require up to 10 d to replace these cells, while older pigs only require 2 to 4 d^[2]. Therefore, clinical signs caused by the destruction of the villous epithelium rapidly overwhelm younger pigs, while older pigs can repair the epithelium quickly enough to mount an appropriate immune response and recover.

The first immune cells to respond to TGEV, PEDV, or other viral infections are the professional antigen presenting cells (APCs) of the innate immune system^[19]. These are a family of white blood cells including monocytes and macrophages, B lymphocytes, and, of most recent interest in TGEV and PEDV, dendritic cells (DCs)^[22]. After antigen recognition, APCs migrate to lymphoid tissues and interact with T lymphocytes, which are responsible for cell-mediated immunity^[5]. This interaction leads to the activation of B cells, which are responsible for humoral immunity (antibody production). Memory cells of both types are also produced in response to an antigen that are present and prepared to respond upon antigen reexposure^[23].

The DCs are of great interest in viral infections such as TGEV because these cells are the only APCs capable of presenting antigen to naïve T lymphocytes. The other APCs are only able to stimulate existing memory T lymphocytes^[22]. In pigs, these APCs, including DCs, are present in various organs and locations throughout the body, but of particular interest to TGEV, are found in the gut-associated lymphoid tissue (GALT) of the small intestines^[24]. The GALT is the largest immune organ in the body, and is composed of peyer's patches, lymphoid follicles, and mesenteric lymph nodes^[14].

The DCs and other APCs in the GALT recognize TGEV using pathogen associated molecular patterns

(PAMPs) on the virus particles that correspond to pattern recognition receptors (PRRs) on the APCs^[25]. One of the primary families of PRRs involved in ssRNA virus detection are called toll-like receptors (TLRs)^[25].

DCs in pigs, as in humans, are divided into two families, and have slightly different roles in mediating the initial immune response to a virus like TGE. These two families are: Plasmacytoid and myeloid. Myeloid dendritic cells (mDCs) are also called conventional myeloid dendritic cells (cDCs) and are most similar to monocytes. Plasmacytoid dendritic cells (pDCs) are most similar in appearance to plasma cells, but share characteristics of the mDCs. pDCs have a further subset of special interest called natural interferon producing cells (NIPCs). pDCs, including NIPCs, differ from mDCs in several ways, including that they can be triggered by viral glycoprotein structures without a live virus present or without a concurrent viral infection^[26].

Once the TGEV PAMP matches to the DCs TLRs, a process called dimerization occurs. Initial signaling pathways are then initiated which lead to the MyD88 signaling cascade, ultimately resulting in the production of interferon-alpha (IFN- α) and other proinflammatory cytokines^[25]. IFN- α is a type 1 interferon that can help to induce viral resistance as well as initiating its own signaling cascade to activate the adaptive immune response^[27]. Specifically, it can mediate inhibition of viral replication, and activate local cell-mediated immunity^[28].

Adaptive immune response

In TGEV, the adaptive response involves both cell-mediated and humoral immunity. The production of the antibody IgA has been identified as particularly important in protecting pigs against future infection^[27]. The importance of stimulating this local IgA and cell-mediated response is a finding that has led to an increased interest in oral vaccines, which may be a more effective route of administration than traditional injectable vaccines^[28].

The stimulation of a protective adaptive response in pregnant pigs is crucial in allowing for the transfer of effective lactogenic immunity to newborn piglets^[29]. Lactogenic immunity refers to the passive transfer of antibodies to newborn pigs through the mother's milk. This is necessary for disease protection in piglets because in pigs, unlike in humans, maternal antibodies are not passed to the neonate *in utero*. They must be consumed in the first milk produced by the mother that is very rich in antibodies called colostrum, and continue to be consumed in subsequent nursing for the first several weeks of life as their immune systems develop^[14].

IgA has been identified as the key antibody produced that must be present in milk to protect piglets from severe disease. Once it is stimulated in the intestines, IgA travels to both the lamina propria of the intestines and the mammary glands^[30]. The mammary gland then produce specific IgA that is transferred to the milk. Protective levels for piglets in milk are only

produced by mothers exposed to a virulent form of the virus *via* the oral route. Attenuated oral vaccines given to pregnant mothers offer only partial protection to neonates^[14].

To achieve the best level of protection in the neonate, piglets must be continually nursing or being fed milk with high levels of anti-TGEV or PEDV IgA. However, even with continuous consumption of IgA the lactogenic immunity is incomplete. Limiting the amount of virus in the environment is also critical for the protection of neonates^[30].

CURRENT CONTROL STRATEGIES

Given what is known about the pathogenesis, immune responses, and transmission of TGEV and PEDV, current population based control strategies focus on prevention of disease, as there is no curative treatment available. These strategies include herd management, strict biosecurity on the farm, and vaccination protocols^[30].

In epidemic infections, affected piglets are treated with fluids in an effort to maintain hydration and improve survival rates. In those facilities with adequate space, mothers who are due to give birth are isolated in order to protect the newborn piglets. In those where space is not available, the only effective option to stimulate lactogenic IgA production or offer future protection from disease in naïve animals is to feed the feces of infected piglets to expectant mothers or other herd members^[30].

However, this is both time and labor intensive, and offers its own challenges and risks. Because only general guidelines have been agreed upon in terms of amount of feces that should be used to inoculate mothers, in some cases the doses have been more virulent than expected and caused disease. In other cases they have been less virulent, and were not protective. Also, there is no guarantee that the animals are only being infected with TGEV or PEDV - other diseases can be transmitted throughout the herd in the feces. Furthermore, intentionally infecting animals leads to more challenges with biosecurity to avoid transmission to neighboring farms^[30].

Those farms not currently experiencing an outbreak or where the diseases are endemic are not able to use this method to stimulate immunity in their herds. They must turn to available injectable vaccines, which do not offer full protection against the disease, and do not stimulate sufficient lactogenic immunity to protect newborn piglets. Available oral attenuated vaccines also lack effectiveness in preventing disease in naïve animals^[30]. However, the vaccines have been shown to be effective as boosters in previously exposed pigs, and are able to stimulate appropriate levels of IgA to offer lactogenic immunity to piglets^[14].

FUTURE CONTROL STRATEGIES

Therefore, future control strategies for TGEV and PEDV

are largely focused on the creation of more effective vaccines to control these diseases and prevent further spread. Proposals for improved modified live and attenuated vaccines are currently under investigation.

Other areas of research include improved treatment options for those animals that have already been infected to lessen clinical signs and decrease mortality. Research in these areas include methods to directly stimulate the innate immune response to overcome the dysregulatory effects of these viruses, as well as preventing the extensive cell death that also leads to the debilitating and often fatal clinical signs.

Proposed vaccines

Given the recent increase in understanding of the gut-mammary gland-IgA axis as described above, creating a safe, effective, and cost-effective live oral vaccine to induce lactogenic immunity as well as providing immunity to naïve herds is one of the main goals of TGEV and PEDV vaccine research. Several modified live (attenuated) vaccines have been proposed. Some strategies for creating these vaccines include deleting proteins and genes in order to decrease virulence. However, modified live vaccines still carry the risk of reverting to virulence and causing disease^[31].

One protein deletion investigation involves non-structural protein 1 (nsp1), which has been shown in mammalian studies to be the first mature viral protein to be expressed in the cytoplasm of the host cell. It may be responsible for the degradation of the host cell mRNA leading to apoptosis, as well as interfering with the IFN- α release that is one of the key components to effective innate immune response in early infection. A study in mice infected with a modified coronavirus where nsp1 had been deleted showed that the virus replicated as efficiently as the unmodified virus, but with greatly reduce virulence. It was able to stimulate efficient memory T cell responses that were protective^[32].

Another study using swine testicular (ST) cells as a model targeted a gene called *ORF 7*, and showed that when it is targeted and inactivated, viral replication and expression of genes in ST cells are inhibited^[33]. These types of investigations may lead to the development of more effective and safer modified live vaccines for swine producers. Because of the inherent risks involved in administering modified live vaccines, subunit vaccines are another area of interest in preventing TGEV and PEDV.

One study demonstrated that recombinant plasmids containing the "S" proteins of TGEV and PEDV could be created, and stimulated a robust proliferation of T lymphocytes and specific antibodies for the viruses^[20]. Producing inexpensive vaccines made from transgenic plants expressing the S glycoprotein from TGEV have also been proposed^[34].

Proposed treatments for infected animals

Some interest has also been generated in stimulating

the innate immune response after an infection has occurred. How COVs like TGEV downregulate or dysregulate the immune system is not completely understood, but new evidence strongly suggests this viral ability^[33].

One proposed treatment to counteract this dysregulation is to directly stimulate NIPCs. Research has shown that porcine NIPCs can recognize a PAMP called an unmethylated CPG motif, which is a short RNA sequence. Exposure to this PAMP leads to production of IFN- α and other inflammatory cytokines. This NIPC response has also been documented in humans. These unmethylated CPG motifs may be useful as adjuvants in new vaccines for both human and porcine COVs^[35].

It is now understood that TGEV causes cell death (apoptosis) by irreversibly activating proteases found in the cytoplasm called caspases. These caspases lead to fragmentation of the host cell nucleus, resulting in the death of the cell. Studies have shown that apoptosis caused by TGEV can be significantly reduced using a caspase inhibitor called Z-VAD.fmk. This does not impede virus production, but does have a protective effect on infected cells^[36]. Treatment with lithium chloride on cells infected with TGEV and PEDV also showed that early and late cell apoptosis was inhibited^[37].

Protecting host cells during disease to reduce clinical signs and improve survival rates is another area of research that may offer another an alternative to supportive care or herd inoculation during an outbreak.

CONCLUSION

Members of the *Coronaviridae* family are important viruses today for both humans and animals. Emerging coronavirus diseases threaten health and productivity. More in-depth understanding of these viruses is the key to future control and safety.

In pigs, the study of TGEV and PEDV provide a window into the complexities of viral disease pathogenesis, control, and prevention. Study of these viruses in pigs may also have direct benefits on human coronavirus control, as porcine responses to these viruses have been increasingly found to be very similar immunologically to human responses.

Important advances have been made in the understanding of the immunological response of pigs to TGEV and PEDV over the last decade. Highlights include the role of NIPCs in early IFN- α production, the immunogenicity and function of the structural and non-structural proteins of the virus, the caspase-dependent pathways resulting in apoptosis, and the role and creation of lactogenic immunity in pig populations.

These advances have led to detailed and promising research for population based control strategies in epidemic and endemic infections. Increased understanding of the best methods to induce lactogenic immunity may play a role in slowing future epidemics on individual farms. The use of caspase inhibitors to

reduce the incidence of cell apoptosis may also increase survival rates for infected pigs during an outbreak.

New vaccines may be able to induce more complete immunity with fewer side effects for naïve herds, making vaccination a more reliable and cost-effective method of disease prevention, and enabling farmers to eradicate endemic infections with TGEV and PEDV over time. These efforts will need funding to facilitate new vaccine development, testing, and deployment.

Because these diseases pose such a threat to food security and both animal and human health, a worldwide effort to eradicate these diseases is likely an ultimate goal. Cooperation between human doctors and veterinarians will be essential in this endeavor. Increased understanding of the immunological responses to these diseases and future treatments will be at the forefront of new control strategies going forward.

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Noncanonical intercellular communication in immune response

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Abstract

The classical view of signaling between cells of immune system includes two major routes of intercellular communication: Through the release of extracellular molecules or a direct interaction between membrane bound receptor and its membrane bound ligand, which initiate a cascade of signaling in target cell. However, recent studies indicate that besides these canonical modes of signaling there are also noncanonical routes of intercellular communications through membrane stripping/membrane exchange/trogocytosis, extracellular traps, exosomes and ectosomes/microparticles. In this review we discuss what are the components of noncanonical pathways of signaling and what role they play in immune cells interactions.

Key words: Trogocytosis; Membrane stripping; Extracellular traps; Exosomes; Ectosomes; Microparticles

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Core tip: Noncanonical routes of intercellular communications through membrane stripping, trogocytosis, extracellular traps, microparticles and exosomes and their function in immune response are highlighted.

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INTRODUCTION

For many decades general belief has been that during an immune response the immune cells communicate either by a direct contact between molecules anchored at the plasma membrane of adjacent cells (juxtacrine signaling) or *via* short (autocrine and paracrine) or long (endocrine) distance signaling using various cytokines or hormones and their cognate receptors systems (Figure 1). However, recent years studies, amounting to hundreds of publications, indicate that besides these well-known (canonical) signaling pathways there is a cornucopia of nonclassical (noncanonical) signaling mechanisms, which modify behavior of immune cells and shape the immune response. Below we give a brief overview of features and functions of these noncanonical signaling pathways.

MEMBRANE STRIPPING/MEMBRANE EXCHANGE/TROGOCYTOSIS

The trogocytosis - the nibbling (gnawing) off the fragments of live cells is probably an ancient mechanism applied by feeding amoebas^[1,2]. However, in contrast to amoebic trogocytosis, which ultimate goal is devouring, and death of cellular prey, the immune cells developed mechanisms of vital trogocytosis or membrane stripping or exchange (Figure 2). During such a process, upon close contact between cells, the recipient cells acquire (borrow) foreign molecules, which they normally do not produce, and the donor cells either completely lose given molecules or the level of these molecules become, at least temporarily, reduced^[3,4]. Recent studies indicate that the membrane exchange between various cell types, including immune cells, is a much more common and frequent phenomenon than previously thought. The *in vitro* and *in vivo* studies in different model systems showed that upon disengagement from immunological synapse with antigen presenting cells (APCs) or from a direct contact with other target cells, the activated CD4⁺, CD8⁺ T cells, regulatory T cells, helper T cells, macrophages, B cells, monocytes, granulocytes and natural killer (NK) cells, are able to internalize fragments of APCs/target cell membrane together with monoclonal antibodies, antigens, ligands, major histocompatibility complex (MHC), adhesion or co-stimulatory molecules^[5-18]. For example, studies of Baba *et al.*^[19] showed that OX40 ligand (OX40L) expressed by COS-1 cells is transferred to CD4⁺ T (OX40L⁻, OX40⁺) cells, and that the acquired OX40L is functionally active. Other studies showed that the trogocytic acquisition of m157 (the murine cytomegalovirus-encoded ligand for the Ly49H-activating receptor) from target cells regulates NK cells function making them hypo-responsive both

in vivo and *in vitro*^[8]. In contrast, acquisition of anti-CD19 chimeric antigen receptors by NK cells enhances their cytotoxicity against the B-cell acute lymphoblastic leukemia cells^[7]. Trogocytosis can also lead to acquisition of the MHC complexes by the non-APCs, which in turn may reinforce and/or propagate immune response, and activate or regulate T cells^[4]. There are indications that trogocytosis/membrane internalization depends on GTPase TC21 and RhoG-dependent phagocytosis pathway (Figure 2)^[16,20]. Membrane internalization not only leads to the acquisition of novel qualities by recipient cells but may also down-regulates the MHC/antigen/co-stimulatory molecules level in bestower APCs^[11,16]. There are also instances of multicellular exchange and serial trogocytosis when immune cells acquire novel molecules from multiple sources and then transfer them to other recipient cells. For example the membrane bound molecules from multiple cancer cells can be acquired by CD4⁺ and CD8⁺ T cells and monocytes through multiple trogocytosis^[21]. It has been shown that monocytes are able to transfer these molecules to other T cells^[21]. Thus, trogocytosis/membrane exchange/stripping leads to acquisition/depletion of molecules and their cognate functions in recipient/donor cell, and ultimately modify or modulate an immune response (Figure 2)^[3,4,6,18,22,23]. Trogocytosis and its outcomes can be either beneficial or harmful for the organism. Depending on circumstances and cell partners involved the trogocytosis may either promote or prevent development of various pathological conditions or diseases. For example trogocytosis is involved in the ablation of red blood cells in autoimmune hemolytic anemia^[24] but when it removes antibodies binding to self-antigens it can prevent autoimmune diseases^[25]. Another example of harmful trogocytosis is "oncologic trogocytosis" occurring between ovarian epithelial cancer cells and stromal cells allows cancer cells to acquire multiple drug resistance protein and thus chemoresistance^[26].

EXTRACELLULAR TRAPS

Extracellular traps (ETs) were discovered in 2004 in neutrophils and thus have been named Neutrophil Extracellular Traps (NETs)^[27]. The process of ETs and NETs formation is called ETosis and NETosis, respectively. ETs consist of filamentous network of chromosomal and/or mitochondrial DNA, which is released from the cell after the break of nuclear/mitochondrial membrane (Figure 3). Because the process of ETosis involves nuclear/mitochondrial/plasma membrane breakage it usually leads to a suicidal, distinct from apoptosis or necrosis, death of ETs' producing cells^[28]. However, there are instances of non-suicidal (vital) NETosis, when nucleus-deprived neutrophils retain motility and chemotactic and phagocytic functions^[29,30]. Another example is the vital mitochondrial NETosis when neutrophils primed, for example, with granulocyte/

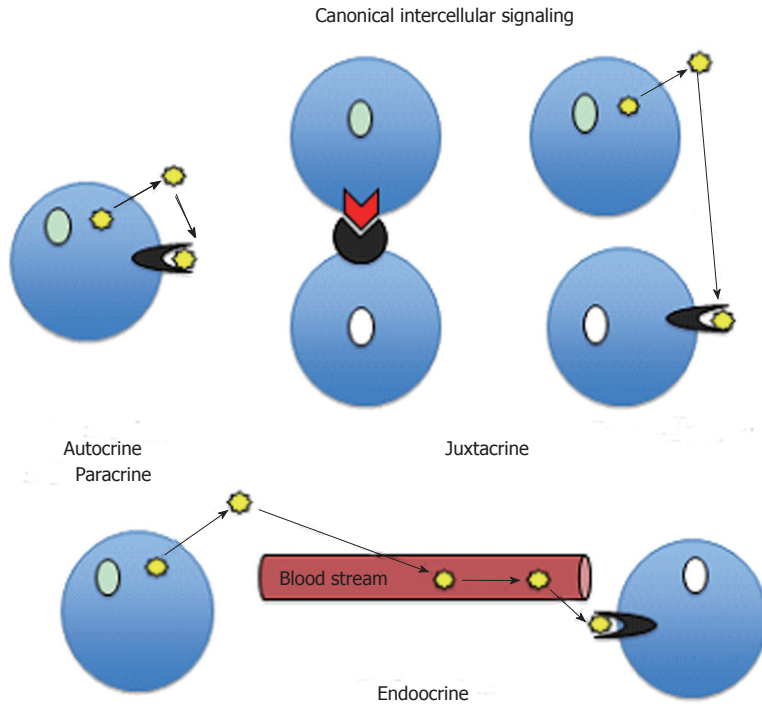


Figure 1 Types of canonical signaling. In autocrine signaling, the cell regulates itself (autoregulates) through internally produced signaling molecules, which after release from the cell bind to cell's own receptors. The examples are: Interleukin-1 produced by monocyte in response to external stimuli binds to its own receptor on the same monocyte; IL-2 released from activated T cell binds to its own receptor leading to self-stimulation. The juxtacrine signaling occurs between closely apposing cells when signaling molecule attached to one cell interacts with its receptor on adjacent cells or when signaling molecule excreted to the intercellular matrix of one cell binds to the receptor on neighboring cell. In juxtacrine signaling the signaling molecules do not diffuse freely between cells. The examples include cytokine signaling in immune system and Notch pathway signaling. In paracrine signaling, released signaling molecules such as, for example, cytokines or retinoic acid diffuse at short distances and act on the cells located in vicinity. In endocrine signaling, signaling molecules such as hormones or cytokines are transported through the circulation to the target cells.

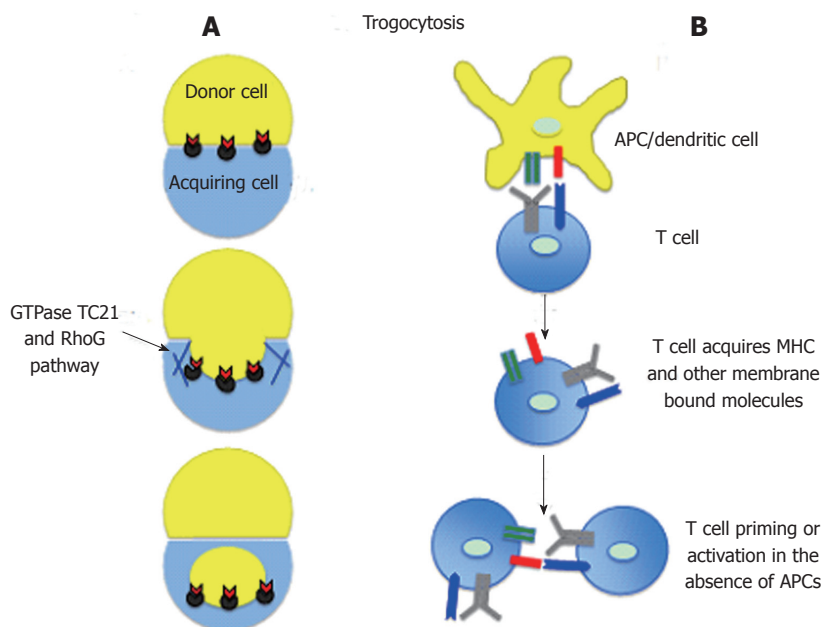


Figure 2 Trogocytosis/ membrane exchange/stripping. A: During trogocytosis the recipient cell acquires membrane bound molecules from donor cell. The process of internalization of donor cell membrane is similar to phagocytosis and depends on actin ring contraction and small GTPases; B: During APC/T cell interaction the T cell may acquire MHC/peptide complexes and co-stimulatory molecules. Subsequently, such T cells can prime/activate naïve T cells in the absence of APCs, and/or by interacting with activated T cells lead to propagation of immune response^[16,20]. APC: Antigen presenting cell; MHC: Major histocompatibility complex.

macrophage colony-stimulating factor and stimulated with short-term toll-like receptor 4 or complement

factor 5a receptor retain intact nucleus and produce NETs containing exclusively mitochondrial DNA^[31].

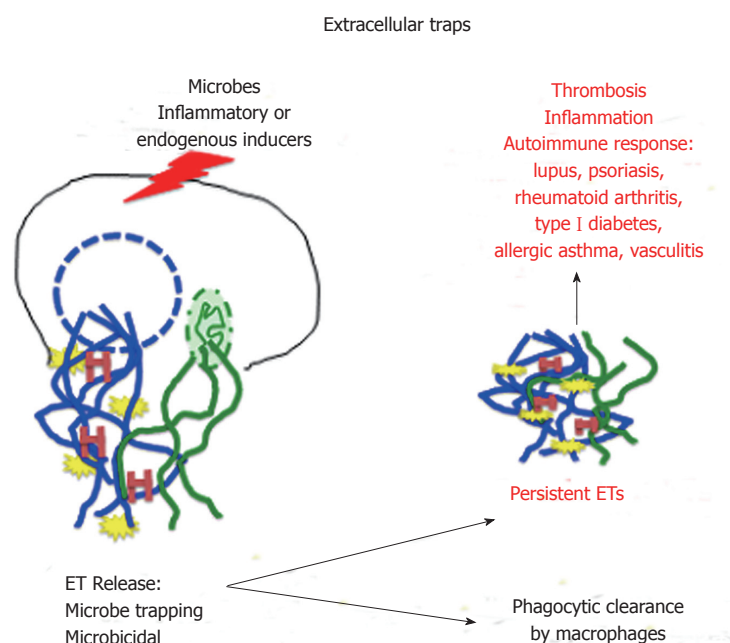


Figure 3 Extracellular traps formation and side effects. Various external or internal inducers may lead to the breakage of nuclear or mitochondrial (or both) membranes and release of extracellular traps (ETs). The ETs contain network of nuclear/mitochondrial DNA (blue/green), antimicrobial compounds (yellow) such as LL37, myeloperoxidase (lysosomal protein) and elastase (chymotrypsin-like protease), and deiminated (citrullinated) histones (red H). Under normal circumstances the ETs are promptly removed by macrophages, however if the ETs persist they can lead to inflammatory and autoimmune response^[31,32].

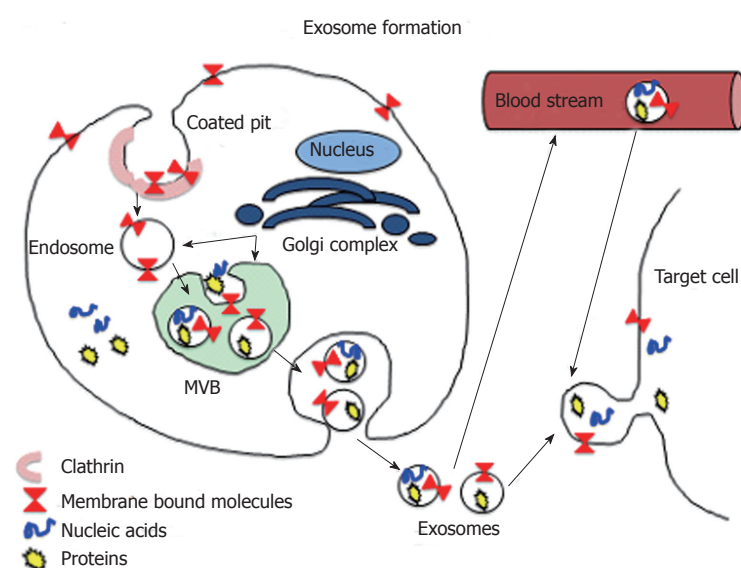


Figure 4 Exosome pathway. Exosome forms through endocytosis, which starts from the invagination of clathrin coated domain of plasma membrane (coated pit) bearing the receptors and other membrane-bound molecules. After entering cell interior, the coated vesicle loses clathrin coat and becomes the endosome. Subsequently, after acquiring variety of other molecules from Golgi apparatus and cytoplasm, the endosome membrane undergoes inward budding resulting in the formation of multivesicular multivesicular body (MVB) containing exosomes. Ultimately, the MVB fuses with plasma membrane and in the process of exocytosis releases exosomes outside the cell. The exosomes either fuse with the membrane of neighboring target cell or enter blood stream to be transported to distant targets. In the alternative outcome (not shown here), which serves as the pathway degradation pathway, the MVB fuses with lysosomes, which degrade its content^[67,69].

Besides DNA, the ETs contain various histones (which by themselves have antimicrobial properties) and a plethora of antimicrobial enzymes (Figure 3)^[32-35]. The main role of ETs is the immobilization of microbes, which prevents dissemination, and exposing them to a high concentration of antimicrobial agents. Interestingly, many microbes developed defense mechanisms allowing them to escape from or neutralize ETs or

ETs producing cells. For example *Staphylococcus* and *Vibrio cholera* produce endonucleases, which digest NETs, or/and convert NETs' DNA into toxic metabolite (deoxyadenosine), which induces apoptosis and promotes death of immune cells^[35-37]. The fact that ETosis occurs in many different cell types, and not only in vertebrates but also in invertebrates and plants, suggests that ETs are one of the primordial

and evolutionary ancient mechanism of host defense. Studies of molecular pathways involved in initiation and execution of NETosis indicate that stimulation with microbes, inflammatory molecules or endogenous inducers leads, *via* protein kinase C and NADPH oxidase, to the production of reactive oxygen species and nitric oxide^[35]. These, in turn, induce nuclear/mitochondrial/granule membrane rupture, followed by proteolytic cleavage, deamination (citrullination) of histones, chromatin decondensation and eventual release of NETs^[38]. It has been shown that besides consistent presence of DNA the other components of ETs vary, as they are stimulatory signal-specific and cell type-specific^[32]. Although ETs play beneficial role in host defense, the presence of DNA and various enzymes makes ETs harmful, especially if they persist for long period of time; they become a very potent inducer of autoimmune response and various pathological conditions, such as lupus, psoriasis, vasculitis, rheumatoid arthritis, type I diabetes, allergic asthma and deep-vein thrombosis (Figure 3)^[33,35,39]. It has been also shown that, at least *in vitro*, ETs influence the behavior of immune cells. NETs are able to down regulate lipopolysaccharide-induced activation of monocyte-derived dendritic cells, inhibit their capacity to activate proliferation of CD4⁺ T lymphocytes and to polarize naïve CD4⁺ T cells toward Th1/Th17 phenotypes, promoting Th2 response instead^[40]. In addition, prolonged exposure to NETs can induce macrophage and dendritic cells death, which may limit ongoing inflammation^[41]. However, it is still unknown, which components of NETs are responsible for these effects. The fact that persisting ETs can modify molecular and cellular components of immune system indicates that fast clearing of ETs is extremely important for proper functioning of immune response^[42]. Recent studies indicate that macrophages serve as such clearing agents. Thus, macrophages seem to have a dual role; they can produce ETs and also remove them through phagocytosis^[35]. Macrophage ETs, named METs, were discovered in 2010 in murine RAW 264. 7 (Abelson murine leukemia virus transformed macrophages) cell line and since then have been described in many different macrophage types^[35,43]. In contrast to the neutrophils where the NETs formation is their main strategy (neutrophils are short-lived “by design”) the formations of METs in macrophages, which are long-lived cells, is an auxiliary strategy and is (regardless of stimulation) self-limited to less than 25% of total macrophage population^[35]. Recently, the eosinophil extracellular traps (EETs) and their role in allergic diseases such as human eosinophilic chronic rhinosinusitis and eosinophilic otitis and eosinophilic esophagitis have been described^[44,45]. Eosinophil traps released during local cytotoxicity contain DNA/histone H1 complex, which form globular fibers thicker than those present in neutrophil-derived traps. The EETs can trap fungi and bacteria and at least in eosinophilic esophagitis (characterized by esophageal epithelial barrier defects) can guard against pathogens infiltration through the

Table 1 Molecular content of exosomes^[49,55-61,74,75]

Nucleic acids	Exosome lumen proteins	Exosome membrane proteins	Lipids
mRNA	Actin	Annexins	Cholesterol
miRNA	Cofilin	Channels	Diglycerides
tRNA	GAPDH	EGFR	Eicosanoids fatty acids
rRNA	Hsp70	FasL	Gangliosides
mitochondrial	Rab	I-CAM1	Lyso-phosphatidylcholine
DNA	Tubulin	Integrins	Lyso-bis phosphatidic acid
retrotransposons		LBPA/CD63	Phosphatidylcholine phosph
		LAMP1/2	hatidylethanolamine
		MHC	Phosphatidylserine
		PD-1L	phosphatidylinositol
		Tetraspanins	Sphingomyelin
		Tsg 101	

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; I-CAM1: Interleukin adhesion molecule 1; EGFR: Epidermal growth factor receptor; LBPA: Lactoferrin-binding protein; LAMP1/2: Lysosomal-associated membrane protein 1; MHC: Major histocompatibility complex; PD-1: Programmed death 1.

impaired esophageal wall^[45].

EXOSOMAL AND ECTOSOMAL/MICROPARTICLE SIGNALING

Exosomes are small (30-150 nm) endocytic membranous vesicles, which are produced by various cell types including immune cells^[46]. They were discovered over 30 years ago and for many years they were believed to be the non-functional cell debris or debris disposal vehicles. However, over the last several years numerous studies have proven that exosomes are an important component of intercellular communication circuit and as such play a crucial role in initiation and/or modulation of immune response^[46-54]. Exosomes form inside the cell through endosome/multivesicular body (MVB) pathway in which they acquire various cytoplasmic, membrane bound and/or nuclear components (Figure 4). Fully matured exosomes released (*via* exocytosis) from a founder cells deliver, sometimes over long distances, various molecules to their targets. Exosomes may contain a variety of molecules such as: (1) genetic material: retrotransposomal DNA, mitochondrial DNA, mRNAs, miRNAs, rRNA, tRNA; (2) lipids; and (3) proteins: Cytoskeletal proteins, heat shock proteins, channels and transporters, adhesion proteins, tetraspanins and various receptors (Table 1), listed in Exocarta database <http://www.exocarta.org>^[49,55-61]. There are many studies showing how exosomal signaling can influence and modify immune cells and immune response. For example the exosomes released by B cells and dendritic cells contain functional MHC - antigenic peptide complexes, which induce adaptive immune responses *in vitro* and *in vivo*^[62,63]. Andreola *et al.*^[64], showed that death-inducing Fas ligand - bearing exosomes secreted by tumor cells induce lymphocyte apoptosis, which

in turn suppress anti-tumor response. In addition, other studies showed that exosomes derived from B lymphocytes expressing FasL can kill T helper (TH) lymphocytes^[65], and that antigen-specific suppression of immune response is exerted by microRNA-150 (miRNA-150)-containing exosomes derived from T CD8⁺ suppressor (Ts) cells^[66]. There are also studies showing that exosomes participate in the signaling between pathogens and immune cells. For example exosomes derived from *Schistosoma japonicum* worm induce macrophage differentiation into M1 subtype^[67] and *Leishmania*-derived exosomes deliver *Leishmania* specific molecules into the host macrophages and induce secretion of IL-8^[68]. Bhatnagar *et al.*^[69] showed that pathogen-associated molecular patterns (PAMPs)-rich exosomes secreted from macrophages infected with various mycobacteria are able to stimulate proinflammatory response in naïve macrophages, and when transferred into mice they stimulate synthesis of IL-12 and TNF- α and promote infiltration of lungs with neutrophils and macrophages. Authors suggest that PAMPs-containing exosomes play a major role in immune surveillance^[69]. Because exosomes' content is cell/pathogen specific and they are able to carry and deliver biologically active molecules to the target cells there is also tremendous interest in application of exosomes as biomarkers and the custom engineered exosomes as an immune and anti-cancer therapeutics^[70-75].

Besides exosomes, various cells are able to release another type of membranous vesicles called the ectosomes/microparticles (MPs). The MPs are 0.2-2 μ m in diameter and unlike exosomes they bud off the plasma membrane without the involvement of endosome/MVB pathway. The MPs contain a variety of bioactive molecules such as procoagulation compounds (for example P-selectin glycoprotein ligand-1 and tissue factor TF) and/or oncogenic proteins, mRNAs and micro RNAs^[76-78]. Similar to exosomes, the circulating MPs may promote/inhibit inflammation, immune response, resistance to chemotherapeutics or activate oncogenic pathways^[76-78].

CONCLUSION

One of the most fascinating aspects of noncanonical signaling is the fact that its cellular processes such as trogocytosis/membrane exchange, ETs, exosomes and microparticles are evolutionary ancient (amoebic trogocytosis, ETs in acoelomate) and conserved in plants, invertebrates and vertebrates^[79]. This indicates that noncanonical processes served as the primordial defense mechanisms and canonical signaling had developed later in evolution adding a new and more sophisticated quality to the ancient safeguards. Ironically, the existence of these ancient noncanonical pathways has been discovered much latter than canonical pathways, and only in recent decade they have been recognized as an extremely important regulators

of innate and adaptive immunity and inflammatory responses.

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Probiotics and allergic diseases

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Abstract

The prevalence of allergic diseases including atopic dermatitis, asthma, allergic rhinitis (AR) and food allergy is increasing worldwide and they cause a big economic and social burden. Understanding of reasons that

contribute to the etiology of allergic diseases as well as new treatment approaches are very important for the follow-up and prevention of these diseases. In recent years, probiotics seem to be promising for allergic diseases. The effect of probiotics in the prevention and treatment of eczema is more extensively studied, but little is known about the association of the microbial flora of the host and allergic airway diseases and the efficacy of probiotics in decreasing the symptoms of patients with asthma and rhinitis. Hitherto, there is no strong evidence for use of probiotics in the treatment of eczema; however, administration of probiotics in breastfeeding mothers in the prenatal period and infants in the postnatal period can be accepted as a safe and helpful option in the prevention of eczema. In contrast, there is not yet reliable evidence or recommendations on use of probiotics for the prevention or treatment of asthma, AR, food allergy, and anaphylaxis currently. More standardized studies should be performed with different strains of probiotics to evaluate the protective and therapeutic effects of probiotics on other allergic diseases as well as eczema. In this review, the relationship between allergy and probiotics is handled in the light of current literature.

Key words: Probiotic; Allergy; Atopic dermatitis; Asthma; Allergic rhinitis

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Core tip: The data in recent years concerning probiotics seem to be promising for allergic diseases. In this review, the relationship between allergy and probiotics is handled in the light of current literature.

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INTRODUCTION

Probiotics are beneficial bacteria that have valuable effects on health if taken in sufficient amounts. They are live single cell microorganisms which suppress the growth of harmful bacteria by competition at the adherence sites and by the direct effects of the antimicrobials they secrete. Probiotics secrete several vitamins such as thiamine, vitamin K₂ and folic acid, aid digestion by producing digestive enzymes and help the body absorb the nutrients^[1]. Probiotics increase the production of mucin, decrease the permeability of intestinal lining and have effects on both innate and adaptive immunity. Members of *Lactobacillus*, *Bifidobacteria*, *Bacteroides*, *Enterococci* genera, *Streptococcus thermophilus* and *Saccharomyces boulardii* are used as probiotics^[2-4]. In order to have the better utility, probiotics can be used with prebiotics. Prebiotics are non-digestible food ingredients that promote the growth and activity of probiotics. Fermentation of prebiotics by the gut microbiota reveals short chain fatty acids such as acetate propionate and butyrate which serve as the energy sources for the regeneration of the colon epithelium. Some of the prebiotics are inulin, oligofructose, polydextrose, fructooligosaccharides, galactooligosaccharides, xylooligosaccharides, and lactulose^[5-7]. Probiotics are used in a variety of diseases such as gastroenteritis, inflammatory bowel diseases, irritable bowel syndrome, celiac disease, peptic ulcers, colon cancer, lactose intolerance, bacterial vaginosis, childhood obesity, hyperlipidemia, anxiety, depression, stress, autism and allergic diseases^[8-10].

The prevalence of allergic diseases is increasing worldwide. Asthma, atopic dermatitis (AD), allergic rhinitis (AR) and food allergy are common and important diseases which cause a big economic and social burden. Understanding of reasons that contribute to the etiology of allergic diseases as well as new treatment approaches are very important for the follow-up and prevention of these diseases. The data in recent years concerning probiotics seem to be promising for allergic diseases. The effect of probiotics in prevention and treatment of eczema is more extensively studied, but little is known about the association of the microbial flora of the host and allergic airway diseases and the efficacy of probiotics in alleviating the symptoms of patients with asthma and rhinitis. Although methodologies and results of the studies investigating the protective effects of probiotics in the development and treatment of allergic airway diseases are variable and controversial, they give a promising perspective and offer new insights for further studies^[11,12]. In this review, the relationship between allergy and probiotics is handled in the light of current literature.

Prenatal factors such as maternal diet, stressful life during pregnancy and gestational diabetes are suggested to be responsible for the development of atopic diseases. In the postnatal period, diet content in the early life, microorganisms and allergen exposure

are potential risk factors and are target of treatment^[13]. Hygiene hypothesis has been suggested during a few decades in order to explain the prominent increase in the incidence of atopic diseases. Incidence of allergic diseases increases in the children growing in smaller family, while infections experienced in the children living together with their older siblings have been proposed to prevent allergic diseases^[11]. Correlations between allergic diseases and microbial agents have begun to be discussed over time. The idea that the existence and proper maintenance of natural flora have positive effects on the immune system and could prevent the development of allergic or autoimmune inflammation in these people has been exciting. Numerous studies have been conducted about the potential role of probiotics in the prevention and intervention of asthma, food allergy and eczema. The promising outcomes obtained from probiotics lead to the interest in this subject intensified in allergic diseases^[11,14].

PROBIOTICS AND IMMUNE RESPONSE

Probiotics contribute education of the immune system and appropriate response to the antigen. The beneficial effect of probiotics on allergy is often associated with enhancement of Treg cells. Specific probiotics have been shown to induce an increase in Treg cells as well as to suppress Th17 responses. Th17 cells secrete IL-17 that induces tissue inflammation^[15,16]. The pathogenesis of allergic diseases was first described as an imbalance of Th1/Th2 cells. Probiotics inhibit allergic diseases by suppressing the Th2 response^[17,18]. The timing of the bacterial colonization in the gut is crucial. The timing of bacterial colonization early in life is important for appropriate immune education and the transmission from mother to the fetus during pregnancy. The early colonization with *Bacteroides* and *Bifidobacterium* species might play a crucial role in the development of immune regulation^[15]. Clinical studies evaluating the effects of probiotics in allergic diseases are seen in Table 1.

PROBIOTICS AND AD

AD is a common important disease which causes an economic and social burden. Its incidence differs among the societies with an incidence reported to reach up to 20% in developed countries^[19]. AD is the first allergic disease seen in the childhood. Infantile eczema usually occurs in the first year of life and the majority of cases resolve before 6 years of age. Most of AD cases are mild, although up to 30% are seen as moderate to severe cases. AD is recognized as a precursor of the development of atopic diseases such as asthma, AR and food allergy in the forthcoming years of children^[12,20,21]. Increased incidence of eczema in recent years has made new research a current issue in epidemiology, prevention and treatment of this disease^[22]. Given that it is a frequently seen disease, causes a substantial proportion

Table 1 Clinical studies evaluating the effects of probiotics in allergic diseases

Disease	Ref.	Number of participants	Strains	Age	Results
Atopic dermatitis	Kalliomäki <i>et al</i> ^[54] , 2007	159	<i>Lactobacillus</i> GG	Prenatal and postnatal	Decreased incidence of eczema
	Taylor <i>et al</i> ^[55] , 2007	178	<i>L. acidophilus</i> LAVRI- A1	Postnatal	No change in eczema rates
	Kopp <i>et al</i> ^[56] , 2008	94	<i>Lactobacillus</i> GG	Prenatal and postnatal	No difference
	Griskevicius <i>et al</i> ^[57] , 2008	446	Group 1: <i>L. Rhamnosus</i> HN001 Group 2: <i>B. animalis</i> ssp. <i>lactis</i> HN019	Prenatal and postnatal	Decreased incidence of eczema by the intake of <i>L. rhamnosus</i>
	Enomoto <i>et al</i> ^[27] , 2014	130	<i>Bifidobacterium breve</i> M-16V, <i>Bifidobacterium longum</i> BB536	Prenatal and postnatal	Decreased incidence of eczema
	Rautava <i>et al</i> ^[28] , 2012	241	<i>Lactobacillus rhamnosus</i> LPR and <i>Bifidobacterium longum</i> BL999, <i>Lactobacillus paracasei</i> ST11 and <i>Bifidobacterium longum</i> BL999	Prenatal and postnatal	Decreased incidence of eczema
Atopic sensitization	Huurre <i>et al</i> ^[58] , 2008	171	<i>Lactobacillus</i> GG, <i>B. lactis</i>	Prenatal and postnatal	Decreased incidence of sensitization
Allergic airway diseases	Abrahamsson <i>et al</i> ^[59] , 2007	232	<i>L. reuteri</i>	Prenatal and postnatal	No difference in wheeze
	Wickens <i>et al</i> ^[60] , 2012	425	<i>B. lactis</i> , <i>Lactobacillus</i> HN001	Prenatal and postnatal	Decreased incidence of rhinoconjunctivitis
	Chen <i>et al</i> ^[40] , 2010	105	<i>L. gasseri</i> A5	School children	Decrease in asthmatic symptoms
Food allergy	Wassenberg <i>et al</i> ^[41] , 2011	31	<i>Lactobacillus paracasei</i> ST11	Adults	Decreased cytokine release
	Dupont <i>et al</i> ^[47] , 2015	119	Probiotic supplemented formula	Infants with cows' milk protein allergy	Decreased incidence in food related symptoms
	Tang <i>et al</i> ^[48] , 2015	62	<i>Lactobacillus rhamnosus</i> CGMCC 1.3724	Children with peanut allergy	Increased incidence of desensitization
Urticaria	Arslanoğlu <i>et al</i> ^[5] , 2008	134	Prebiotic-supplemented hypoallergenic formula	Postnatal	Decreased cumulative incidence of allergic urticaria
Systemic nickel allergy syndrome	Randazzo <i>et al</i> ^[43] , 2015	22	<i>Lactobacillus reuteri</i> DSM 17938	Adults	Decrease in symptoms

of severe cases and accompanies other allergic diseases, meticulous treatment and follow-up of pediatric eczema have become compulsory.

There is a correlation between the development of AD and gut microbiota though it is not exactly clear^[23]. A randomized double blind study conducted with administered bacterial lysate shown a correlation between gut microbiota and AD^[24]. A greater number of elder siblings were found to be correlated with an increase in lactobacilli and bacteroides colonization and a decrease in clostridia in the gut. Risk for development of AD increased in the infant aged 5-13 mo with a higher gut concentration of clostridia^[24]. This study demonstrated that gut microbiota is one of the reasons underlying the development of AD. A more diverse intestinal microbiota in the first week of life is associated with a reduced risk of subsequent eczema in infants at an increased risk of allergic disease^[25]. This study suggested that interventions that enhance microbial diversity in early life may provide an effective means for the prevention of eczema in high-risk infants.

The knowledge about whether probiotics prevent the development of AD is increasing day by day. In a cohort from Norway with a wide population, the potential association was investigated between the intake of probiotics during pregnancy and infancy periods and

the development of questionnaire-reported atopic eczema, rhinoconjunctivitis and asthma. The intake of probiotic milk products was found to be correlated with a decreased incidence of atopic eczema and rhinoconjunctivitis; however, this was not correlated with the incidence of asthma in the 36-mo follow-up^[26]. Probiotics used by pregnant women or breast-feeding mothers and/or given to infants reduced the risk of eczema in infants; however, the certainty in the evidence is low. No effect was observed for the prevention of other allergic conditions^[14]. Enomoto *et al*^[27] examined the effect of the intake of *Bifidobacterium breve* (*B. breve*) M-16V and *Bifidobacterium longum* (*B. longum*) BB536 over prenatal 1 mo and postnatal 6 mo on the development of allergic disease. They found that the incidence of eczema was lower in the probiotic group than in control in a short follow-up period of 18 mo. Rautava *et al*^[28] investigated the role of probiotic intervention regimens in reducing the risk of eczema in infants. Mothers with a history of allergic disease and atopic sensitization were randomly assigned to the *Lactobacillus rhamnosus* LPR and *B. longum* BL999 (LPR1BL999) group, the *Lactobacillus paracasei* (LP), ST11 and *B. longum* BL999 (ST111BL999) group, or the placebo group during 2 mo before and after the expected date of delivery. They found that infants whose

mothers received any of the probiotic supplements developed significantly less episodes of eczema when compared with the placebo group. However, skin prick test results were similar between the groups. It is noteworthy in the mentioned study that, probiotics did not prevent atopic sensitization and prevented only non-atopic eczema^[29]. Based on this result, long-term effects of the use of probiotics may be limited. It has been noted in a recent meta-analysis that, additionally administered probiotics in the early life prevented AD in long term compared to placebo^[30]. There are several studies suggesting *Lactobacillus rhamnosus* GG (LGG), a mix of specific probiotic strains and prebiotic + prebiotic mixtures have a positive effect in the prevention of the incidence or decrease of the severity of AD. Nevertheless, these studies are not enough for use of probiotic with this purpose^[31].

There are also studies demonstrating that probiotic support is ineffective in the prevention of the development of AD. In a study by Allen *et al.*^[32] with women from 36 wk gestation and their infants to the age of 6 mo, the incidence of eczema was investigated in the 2nd year of age in infants who received daily either the probiotic (*Lactobacillus salivarius* CUL61, LP CUL08, *B. animalis* subspecies lactis CUL34 and *Bifidobacterium bifidum* CUL20) or placebo. Use of probiotics was found not to prevent the development of eczema during the study. In that study, probiotics were found to decrease the incidence of food allergy in early childhood as an indirect effect. There are interesting results in the evaluations carried out according to the type of probiotics. For example, according to a meta-analysis use of lactobacilli during pregnancy was stated to prevent atopic eczema in children between 2 and 7 years old, but a mixture of various bacterial strains did not affect the development of eczema^[33].

The role of probiotics in the prevention of atopic eczema is yet to be clarified. Evidence presented by the studies suggesting that probiotics may prevent pediatric eczema is not enough strong. A positive effect, if any, may be related with onset time, dose, duration and use of specific probiotics. Support of this issue with further and longer research would be helpful in elimination of the limited uncertainties in the use of probiotics. Another question is that can probiotics be used for the treatment of pediatric eczema? Evidence about the use of probiotics in the treatment of eczema is not enough good as their use in the prevention. It is suggested in a limited number of studies that probiotics decrease the severity of eczema. In a randomized double-blind placebo-controlled study examining the effect of the use of *Lactobacillus plantarum* (*L. plantarum*) CJLP133 for 12 wk in children with AD aged 12 mo and 12 years, an improvement in SCORing of Atopic Dermatitis (SCORAD) scores was noted, while levels of eosinophil counts, interferon-gamma (IFN- γ) and IL-4 were decreased. However, no significant reduction was observed in total use of topical corticosteroids^[34]. Wang *et al.*^[35] compared children who received LP, *Lactobacillus fermentum*

(LF) and LP + LF with those who received placebo in a double-blind, prospective, randomized study. SCORAD index was lower in the probiotic groups compared to placebo until 4 mo after probiotics were discontinued. In brief, the use of LP and LF mix probiotics was found to be correlated with clinical improvements in the children having AD. In another study, Niccoli *et al.*^[11] showed that the use of *L. salivarius* LS01 led to improvements in quality of life in the children having AD.

Consequently, numerous studies investigating the role of probiotics in the prevention or intervention of AD present clinical suggestions for the use of probiotics. Even if the evidence is not strong enough, use of probiotics for the prevention of eczema is recognized by a considerable number of clinicians. Another difficult-to-answer question about the use of probiotics is that "when should probiotics be initiated?" Factors that can influence early-life colonization include antibiotic treatment, method of delivery, maternal and infant diet, and biodiversity in the home, surrounding environment and in family members. Initiation of probiotics in the early life is a better option in order to prevent eczema, a result that we want to obtain. Early initiation of probiotics is of key importance, if a benefit would be obtained. Even if possible, administration of probiotics to mothers in the prenatal period can be helpful in the infant under risk of eczema. Considering all critical outcomes, because there is a net benefit resulting primarily from prevention of eczema, World Allergy Organization (WAO)-McMaster University Guidelines for Allergic Disease Prevention suggest using probiotics in pregnant women and women who breastfeed infant at a high risk for allergy in their children. Similarly, the WAO guideline panel suggests using probiotics in infants at a high risk of developing allergies^[36]. However, there are still questions to be answered in the future about probiotic administration for eczema and other allergic diseases: Which probiotic, at what dose, when, how long, and to whom? Clinical use of probiotics will gradually become more widespread as these questions find their answers^[23].

PROBIOTICS AND ALLERGIC AIRWAY DISEASES

The prevalence of asthma as well as other allergic diseases is increasing worldwide. Maturation of the immune system in the first years of life may play a role in this increase and this may be related to hygiene hypothesis and imbalances in lymphocyte T-helper 1 (Th1)/lymphocyte T-helper 2 (Th2) balance and activation of Th2 cytokines such as IL-4, IL-5, and IL-13^[11].

Asthma is a chronic airway disease characterized by airway inflammation and hyperresponsiveness. Although asthma is a heterogeneous disease with different phenotypes and endotypes, Th2 mediated inflammation is central to the pathogenesis in most of the patients^[4]. Therapeutic effects of probiotics in the prevention and

treatment of eczema are more extensively studied, but little is known about the association of the microbial flora of the host and allergic airway diseases and the efficacy of probiotics in alleviating the symptoms of patients with asthma and rhinitis. Methodologies and results of the studies investigating the protective effects of probiotics in the development and treatment of allergic airway diseases are variable and controversial, but they give a promising perspective and offer new insights for further studies^[11].

The heterogeneity of gut microbiota is required for the development of mucosal tolerance. The composition of the gut microbiota differs in children with asthma. Some species of *Clostridium difficile* were found in higher amounts whereas *Bifidobacterium* amounts were lower in the intestinal microbiota of asthmatic children^[4]. Probiotics modulate immune responses through toll-like receptors, activation of dendritic cells, maintaining a Th1 response and thereby suppressing Th2 response^[4,11,12].

Evidence from ovalbumin (OVA)-sensitized murine asthma models showed significant reductions in the number of inflammatory cells in bronchoalveolar lavage fluid and airway hyperreactivity and an increase in the levels of regulatory T cells after administration of *Lactobacillus reuteri* ATCC 23272, LGG or *Bifidobacterium lactis* in single challenges. However, *Lactobacillus salivarius* has not been shown to slow down allergic airway responses or reduce the number of inflammatory cytokines and cells^[4,37]. In another study, OVA-sensitized mice were given *B. breve* M-16V, *Bifidobacterium infantis* NumRes251, *Bifidobacterium animalis* (*B. animalis*) NumRes252 and NumRes253, *L. plantarum* NumRes 8 and *Lactobacillus rhamnosus* NumRes6. After challenge with OVA, airway reactivity response to metacholine and numbers of inflammatory cells in bronchoalveolar fluid were measured. Of the 6 strains, only *B. Breve* M16V and *L. plantarum* NumRes8 were found to inhibit the metacholine response, reduce the number of inflammatory cells in bronchoalveolar lavage and reduce OVA-specific IgE and IgG1. These outcomes indicate that the immunomodulatory effects of probiotics can be strain specific^[38].

A meta-analysis of randomized, placebo-controlled trials that evaluated the effect of probiotics given prenatally or postnatally within the first year and were published between 2001 and 2012 revealed statistically significant reductions of total IgE levels and the risk of atopic sensitization, but a significant decrease in asthma/wheeze development was not seen. Atopic sensitization risk was decreased significantly when probiotics were given prenatally and postnatally, but the same effect was not seen when given only postnatally^[11]. In another meta-analysis of randomized controlled trials of 17 studies enrolling 4755 children, asthma, rhinitis and wheezing development was not changed significantly after prenatal and postnatal probiotic supplementation^[39]. In a double-blind, randomized, placebo-controlled study, 105 asthmatic children aged 6-12 years with a history of mild to moderate persistent asthma and persistent

AR were enrolled. *Lactobacillus gasseri* A5 was given to the study group ($n = 49$). Study outcomes revealed decreases in the severity of asthmatic symptoms, improvements in the CACT score ($P < 0.05$) and decreases in bronchial hyperreactivity ($P < 0.001$) and significant improvements in the FEV1, FVC, FEV1/FVC and MEF25-75 levels in the study group^[40].

Rhinitis plays an important role in the life quality of the host because of the frequent co-occurrence of AR and asthma and the united airway disease hypothesis^[2]. Sinus bacterial diversity is important in the healthy functioning of upper airways and decrease in sinus inflammation^[6]. In a study, 31 adults with AR received LP ST11 for 8 wk. IL-5, IL-8 and IL-10 levels from peripheral blood mononuclear cells decreased after the probiotic supplementation^[41]. Peripheral blood eosinophils and IFN- γ levels as well as the need for medication were reported to be decreased in patients with Japanese cedar pollinosis after supplementation with *B. longum*^[42].

The probiotics used must be resistant to acid and bile for enough efficacy and they should be taken regularly. At least 9 d of probiotic application was reported to be essential for significant inhibition of airway eosinophilia and airway hyperreactivity in mice^[11,37]. But experimental animals such as mice have a gut microbial flora which has less than 50% DNA identity to the human microbiota. Therefore, interpretation of the results should be cautiously made^[4].

More studies done with different strains of probiotics and standardization of methodologies will offer advances in the understanding of microbial flora of patients with asthma and rhinitis and the therapeutic effects of probiotics for these patients. Also the duration of therapy, selection of probiotics in different situations and patients and the dosage of the probiotics need to be considered in the future studies.

PROBIOTICS AND OTHER ALLERGIC DISEASES

There are few studies about the effects of probiotics on urticaria. In a prospective, randomized, double-blind, placebo-controlled study, 134 infants (68 in placebo, 66 in study group) received either a placebo-supplemented hypoallergenic formula or a prebiotic-supplemented (8 g/L scGOS/lcFOS) hypoallergenic formula during the first 6 mo of life. Cumulative incidence of allergic urticaria at 2 years of age was 1.5% in the study and 10.3% in the placebo group ($P < 0.05$)^[5]. In patients with systemic nickel allergy syndrome, after 2 wk of *Lactobacillus reuteri* DSM 17938 strain supplementation, a significant improvement was seen in cutaneous symptoms such as urticaria, presence of itch and eczema in both the study and control groups. Gastrointestinal symptoms improved only in the patients supplemented with *Lactobacillus reuteri*^[43]. In a case study, probiotic supplementation and antihistamines

were given to a 17-mo-old boy with urticaria pigmentosa and a decrease in pruritus, and improvement of appetite and sleeping was reported^[44].

Food allergy is seen early in the course of atopic march and is a known aggravating factor of AD. Food induced anaphylaxis is the most common cause of anaphylaxis in outpatients especially in children and can be fatal. In a study, LP L9 was given to β -lactoglobulin (BLG)-sensitized mice. Levels of CD4⁺CD25⁺Foxp3⁺ Treg cells in mesenteric lymph nodes increased by 51.85% after the supplementation^[45]. Among the probiotic strains, LGG was reported to be the most studied probiotic in the prevention of food allergy, especially cow milk allergy^[46]. In a study, 119 infants with cow milk protein allergy were given a probiotic supplemented extensively hydrolysed casein-based formula. Food related symptoms and SORAD index were improved^[47]. A double-blind, placebo-controlled randomized trial of 62 children with peanut allergy on oral immunotherapy *Lactobacillus rhamnosus* CGMCC 1.3724 supplementation was given. In the study group 89.7% and in the placebo group 7.1% of children were desensitized ($P < 0.001$) at the trial end^[48]. Probiotic-supplemented peanut sensitized mice have been shown to have increased splenic naturally occurring T regulatory cell populations and reduced gene expression IL-13 compared to controls^[49]. In spite of these encouraging results, a meta-analysis of 10 trials did not show preventive effects of prenatal and postnatal usage of probiotics on food allergy^[50].

In a murine model of food allergy induced systemic anaphylaxis, VSL3 probiotic was reported to suppress TH2 mediated reactions and anaphylactic reactions^[51]. Probiotics can contribute to decreases in the allergic symptoms as well as anaphylactic reactions through induction of Treg cells, suppression of Th2 responses, inhibition of mast cell membrane potassium (IKCa) current and degranulation^[52,53]. Clinical studies evaluating the effects of probiotics in allergic diseases are seen in Table 1.

The formation and maintenance of gut flora in a proper way in the early life are crucial especially for AD and other allergic diseases. Although there is partial debate over the prevention of the development of AD, here it is worth noting to mention some recommendations. Although it alone does not prevent eczema yet, breast-feeding of infants should be promoted. Normal vaginal delivery which is quite important for gut microbiota of the infant should be promoted except medical contraindications. Unnecessary use of antibiotics which impair gut flora should be avoided in early life. To the date of this study, there is no strong evidence for use of probiotics in the treatment of eczema; however, administration of probiotics in breastfeeding mothers in the prenatal period and infants in the postnatal period can be accepted as a safe and helpful option in the prevention of eczema. In contrast, there is not yet reliable evidence or recommendations on use of probiotics for the prevention or treatment of asthma,

AR, food allergy, and anaphylaxis currently. More standardized studies should be performed with different strains of probiotics to evaluate the protective and therapeutic effects of probiotics on other allergic diseases as well as eczema.

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Basic Study

Intracellular localisation of *Mycobacterium marinum* in mast cells

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Author contributions: Siad S designed this study as part of her PhD thesis; Byrne S assisted in critical technical aspects; Mukamolova G and Stover C supervised the work and are joint last authors; Stover C assembled the data; all authors read and approved the contents.

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Abstract

AIM: To study the bacteriocidal or bacteriostatic role of mast cells during infection with *Mycobacterium*.

METHODS: *Mycobacterium marinum* (*M. marinum*) (BAA-535/M strain) was investigated for its ability to grow at a temperature relevant to the mammalian host. Primary mast cells were differentiated from bone marrows of mice, a human mast cell line (HMC-1) and a human monocytic cell line (MonoMac6) were maintained in culture. Mice were stimulated by intra-peritoneal injection of heat-killed *M. marinum* to study cytochemically the degranulation of peritoneal mast cells. HMC-1 cells were stimulated with *M. marinum* to analyse mRNA expression for inflammatory reactant genes, while HMC-1 and primary mouse mast cells were infected with *M. marinum* to establish in parallel cell viability (lactate dehydrogenase release and cell counts) and viable mycobacterial counts. Flow cytometry was used to assess intracellular presence of fluorescein isothiocyanate labelled *M. marinum* after trypan blue quenching and to measure the extent of infection-induced apoptosis or necrosis in HMC-1. A GFP expressing recombinant *M. marinum* strain was used to assess intracellular location by fluorescence microscopy. Light microscopy of osmium tetroxide and Gram Twort

stained sections of 0.5 µm and transmission electron microscopy were undertaken as sensitive methods.

RESULTS: Since its isolation, *M. marinum* has adapted to grow at 37 °C. This study found that *M. marinum* infects HMC-1 cells and primary murine mast cells, where they survive, replicate, and cause dose dependent cell damage over the analysis period of up to 120 h. Amikacin was an effective aminoglycoside antibiotic to eliminate extracellular or membrane attached *M. marinum* in order to adequately quantify the intracellular bacterial loads. *In vivo*, intraperitoneal injection of heat-killed *M. marinum* led to the release of mast cell granules in mice. HMC-1 cells stimulated with *M. marinum* showed a biphasic pattern of increased mRNA expression for LL-37 and COX-2/TNF-α during 24 h of stimulation. In HMC-1, *M. marinum* localised to the cytoplasm whereas in primary mast cells, *M. marinum* were found in vacuoles.

CONCLUSION: The effector role of mast cells in infection with *M. marinum* can be studied *in vitro* and *in vivo*.

Key words: Mast cells; *Mycobacterium marinum*; Microscopy; Infection; Degranulation

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Core tip: *Mycobacterium marinum* (*M. marinum*) is easily culturable and shows promise as a model to understand in mammalian cells the pathogenicity of *M. tuberculosis*. We used *M. marinum* to study uptake and elimination of *M. marinum* by mast cells, being abundant immune effector cells. A range of imaging techniques was used to unequivocally show the intracellular presence of *M. marinum*. Mast cells did not control the replication of *M. marinum* but reacted in a pro-inflammatory way. This is consistent with mast cells being orchestrators of inflammation. In summary, we clearly show that *M. marinum* can infect mast cells, survive and replicate within.

Siad S, Byrne S, Mukamolova G, Stover C. Intracellular localisation of *Mycobacterium marinum* in mast cells. *World J Immunol* 2016; 6(1): 83-95 Available from: URL: <http://www.wjgnet.com/2219-2824/full/v6/i1/83.htm> DOI: <http://dx.doi.org/10.5411/wji.v6.i1.83>

INTRODUCTION

Mycobacterium marinum (*M. marinum*) is the causative agent of mycobacterial disease in poikilotherms and of the so-called "swimming pool" granuloma contracted by the human host^[1]. Because it is a relatively fast-growing (generation time approximately 4 h) and a containment level 2 pathogen, *M. marinum* is used

as a model organism to understand pathogenicity and host response to *M. tuberculosis*, which is slower growing (generation time approximately 20 h), and requires higher levels of containment^[2]. The natural host, zebrafish, is used as a model organism to develop understanding of mycobacterial pathogenesis in general^[3], but *M. marinum* has also been studied in mouse models of topical or systemic infections^[4,5].

The primary host cell for *M. tuberculosis* infection is the macrophage where pathogenic mycobacteria initially infect and spread into resting macrophages. These in turn serve as a habitat for survival and replication of the bacteria^[6]. Dissemination of mycobacteria by macrophages has been observed in real-time using *M. marinum* infected zebrafish embryos, showing the transfer of mycobacteria from one macrophage to another *via* membrane tethers^[7]. Normally, activated macrophages possess potent microbicidal activity or at least control the growth of pathogenic mycobacteria^[8]. However, the mycobacteria cell envelope confers to the bacilli the ability to survive and replicate within phagosomes of macrophages where they arrest phagosome maturation^[9]. Macrophages respond to *M. tuberculosis* infection with apoptosis and secretion of inflammatory cytokines and are tightly involved in the formation of granulomas.

Mast cells have been found associated with tuberculous granuloma^[10]. Abundant in mucosal tissues, intratracheal injection of guinea pigs with *M. tuberculosis* led to degranulation of mast cells^[11]. Pre-treating mice with C48/80, a potent mast cell activator, by repeated intranasal administrations before intratracheal infection with *M. tuberculosis* impeded neutrophil and mononuclear cell recruitment in the bronchoalveolar space and clearance of bacteria^[12]. This experiment illustrated the orchestrating role mast cells have in mounting a pro-inflammatory response. Reconstitution of mice with interleukin-3 (IL-3) differentiated bone marrow derived mast cells (BMDMC) from TLR2^{+/+} or TLR2^{-/-} mice showed that TLR2 expression of mast cells was important for the normal inflammatory response to *M. tuberculosis* (myeloid cell recruitment, pro-inflammatory cytokine production, granuloma formation and bacterial clearance)^[13].

Infection of the rat basophilic cell line RBL-2H3 (used as a model of mast cells) and peritoneal mast cells with *M. tuberculosis* triggered the release of pre-stored reactants such as histamine and β-hexosaminidase, and the *de novo* synthesis of pro-inflammatory cytokines TNF-α and IL-6 at 30 min and 6 h^[14,15]. Mast cells may form cholesterol dependent pseudopod like structures (lipid rafts) to take up *M. tuberculosis*, which may then replicate within the mast cells^[15].

However, it is unclear whether mycobacteria are susceptible to the inhibitory and/or killing actions posed by mast cells directly, whether and to what extent mast cells internalise mycobacteria. It is also not known to what extent mycobacteria survive inside the activated

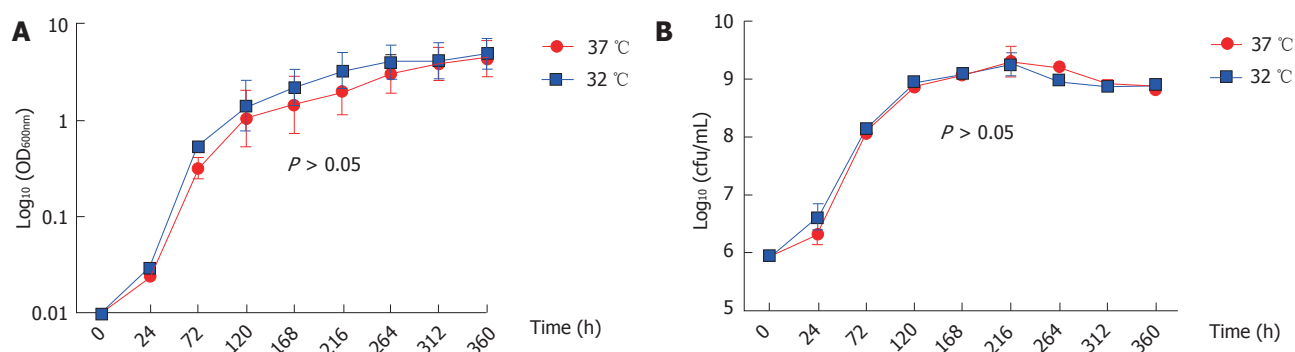


Figure 1 Growth of *Mycobacterium marinum* at 32 °C and 37 °C. Optical density (A) and colony-forming units count (B) of *Mycobacterium marinum* in growth medium cultured at 32 °C and 37 °C. There is no difference between bacterial growths and viabilities at the two growth temperatures tested. The values are mean $\pm$ SEM of five independent experiments (A); two independent experiments (B), each in triplicate.

mast cell.

MATERIALS AND METHODS

Bacterial strains

M. marinum BAA-535/M strain (isolated from human lesions) was originally obtained from the clinical laboratories of Moffitt Hospital at the University of California, United States, and provided by Dr. Hagedorn M (University of Geneva). *M. marinum* strain expressing GFP was generated by introduction of pMind plasmid^[16] expressing GFP from a Tet-promoter and was maintained in 50 μ g/mL kanamycin. The bacilli were grown to mid log phase in Middlebrook 7H9 broth (Difco, Detroit, MI) supplemented with 0.5% (v/v) glycerol, 0.05% (v/v) Tween 80 and 10% (v/v) Albumin/Dextrose complex (ADC) at 32 °C without shaking. The strain was identified as *M. marinum* prior to experiments by acid fast staining, photochromogenic test, and sequencing of *16SrDNA*. Single cell suspensions were obtained by passing through a 25G gauge needle to disperse cell clumps prior to infection. Whilst being a fish pathogen, the clinical isolate of *M. marinum* that was used in this work was able to grow at 37 °C (Figure 1).

Cell cultures

Primary murine mast cells were differentiated from bone marrows of mice (BMDMC) with 100 ng/ μ L of recombinant mouse IL-3 (Peprotech) over 5 wk^[17]. Cells were characterised as mast cells by flow cytometry for the expression of high affinity mast cell receptor Fc ϵ RI (BioLegend) (Figure 2). A human mast cell line (HMC-1) was cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% (v/v) heat-inactivated iron supplemented foetal calf serum (Fisher, Loughborough, United Kingdom), 1.2 mmol/L 1-thioglycerol (Sigma-Aldrich) and 100 U/mL penicillin, 100 μ g/mL streptomycin. The cells were split 1:3 every 3-4 d and re-suspended in fresh medium. The human monocyte cell line MonoMac6^[18] was maintained in RPMI1640 medium supplemented with 2 mmol/L L-glutamine, 1% (v/v) non-essential amino acids, 1 mmol/L pyruvate, 10% (v/v) heat-inactivated FCS and

100 U/mL penicillin, 100 μ g/mL streptomycin.

In vivo stimulation

C57Bl/6J mice were used at 4 mo. A volume of 100 μ L of heat-killed *M. marinum* (OD_{600nm} 0.7), at a dose comparable to that of Complete Freund's Adjuvant, was injected intraperitoneally in two mice, while two control mice received 100 μ L PBS. All appropriate measures were taken to minimise pain or discomfort. The mice were monitored and culled at 4 h by cervical dislocation in the absence of any clinical signs of illness in any of the mice. Peritoneal lavage was performed with cold phosphate-buffered saline (PBS) with 4% (v/v) foetal calf serum (FCS) to collect peritoneal cells. Cytological staining of the peritoneal mast cells was then performed using Toluidine blue, Wright's stain, Bismarck Brown and Kinyoun stain. Slides were air dried and mounted with cover slips using Xylene and DPX mounting medium.

In vitro infection

Mast cells (2×10^5 cells/mL) were infected with *M. marinum* at a multiplicity of infection (MOI) of 0.5:1 (bacteria per cell) in 24 well-plates and incubated for 4 h (37 °C, 5% CO₂ humidified atmosphere). Cells were washed three times with PBS and treated with 200 μ g/mL amikacin for 2 h (optimised). Thereafter, the cells were washed and re-suspended with complete IMDM without antibiotics and incubated further for up to 120 h. At each time point, viability count (using trypan blue dye-exclusion method) and colony-forming units (CFU) count [after lysis of the infected cells with 0.1% (v/v) Triton X-100 for 5-10 min] were carried out. Ten microliters drops containing serially diluted bacteria were spotted on ADC supplemented 7H10 agar plates. The plates were stored in a plastic sleeve to maintain humidity and incubated at 32 °C for 7-14 d to obtain *M. marinum* colonies. For some experiments, cells (2×10^6 cells/mL) were infected with *M. marinum* at MOI 0.5, 10, 25, 50. At each time point cells were washed once with PBS to remove the extracellular bacteria and were processed for flow cytometry or stored at -80 °C for total RNA extraction. The supernatants were kept at -80 °C for further investigation.

Lactate dehydrogenase assay

The release of lactate dehydrogenase (LDH) from the cytoplasm in the supernatants was measured for BMDMCs infected with *M. marinum* at MOI 0.5, 10 and 25 for 8 h in comparison with uninfected control cells. The assay was performed according to the manufacturer's instructions (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega United Kingdom Ltd, Southampton, United Kingdom).

Flow cytometric analysis

To assess purity of BMDMCs after differentiation with IL-3, the cell population was harvested ($300 \times g$, 5 min); next, 1×10^6 cells were re-suspended with 100 μ L Fc Block [CD16/32, clone 2.4G2, diluted 1:200 in FACS buffer, 2% (w/v) BSA in sterile PBS]. The samples were then incubated for 15 min on ice, centrifuged at $500 \times g$ for 5 min at 4 °C and stained with 100 μ L FACS buffer containing PE conjugated anti-mouse Fc ϵ RI alpha (clone MAR-1, BioLegend, 1:200) or its isotype control (Armenian Hamster IgG). The samples were incubated on ice for 15-30 min in the dark. After two-three washes in FACS buffer, the samples were re-suspended with 500 μ L FACS buffer. The data were acquired using a FACSCalibur instrument (Becton Dickinson) and were analysed using the CellQuest Pro Software (Becton Dickinson) and Flow-Jo Software.

Quantification of mycobacterial uptake by mast cells was determined using an established fluorescence-quenching technique. A single cell suspension of *M. marinum* (1×10^9 CFU/mL) was labelled by incubation with 0.5 mg/mL of fluorescein isothiocyanate (FITC; Sigma) in 0.1 mol/L carbonate buffer (pH 9.0) at 37 °C for 2 h. FITC labelled *M. marinum* were washed twice with PBS to remove unbound FITC and re-suspended with fresh IMDM (complete without antibiotics). Thereafter, 1×10^6 cells/mL mast cells were infected at MOI 10:1 (bacteria per cell) for 24 h (37 °C in humidified 5% CO₂ incubator). After each time point, the infected cells were washed three times with washing buffer [2% (w/v) BSA-PBS] to remove the extracellular bacteria and incubated with sodium acetate buffer (0.05 mol/L; pH 4.5) containing 0.06% trypan blue for 5 min at 4 °C. Cells were harvested ($300 \times g$, 5 min) and re-suspended with 500 μ L of fixation buffer [2% (w/v) paraformaldehyde in PBS] to carry out the acquisition step of the flow cytometer.

Discrimination of apoptotic and necrotic cellular subpopulations was achieved by simultaneously staining infected and uninfected HMC-1 cells, with Annexin V (FITC) and propidium iodide (PI) in the presence of 2.5 mmol/L of CaCl₂. Samples were incubated on ice in the dark for 30 min and analysed by flow cytometry.

Microscopic analyses

To visualise intracellular mycobacteria using GFP expressing *M. marinum* (pMIND), epifluorescence microscopy and confocal laser scanning microscope

(Olympus FV1000) were applied. The cells were fixed with 4% (w/v) paraformaldehyde for 10 min at room temperature, followed by 30 min to dry in the dark. Thereafter, two drops of 3 μ L mounting media [75% (v/v) glycerol] were added onto the cover slip and then pressed onto a microscope slide and allowed to adhere overnight at room temperature in the dark. For epifluorescence microscopy, the preparations were observed using a Nikon Diaphot 300 inverted microscope with a 100 W mercury light source. Images were recorded using a 12/10 bit, high speed peltier-cooled CCD camera (FDI, Photonic Science) using Image-Pro Plus (Media Cybernetics) software. Preparations for confocal laser scanning microscopy were observed using Olympus FV1000 inverted IX81 motorised confocal laser scanning microscope.

Zero point five micron-cross-sections of Mono Mac 6 and HMC-1 cells (infected with *M. marinum* at MOI 10 for 24 h) were produced using an ultramicrotome (Reichert, glass knife). After fixing the cells with 4% (v/v) glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4), cells were post fixed with 1% (w/v) osmium tetroxide (OsO₄) and dehydrated in an ethanol series. Cells were then exposed to the intermediate solvent epoxypropane (propylene oxide) before embedding in Agar Low Viscosity Resin (epoxy resin) and baking at 60 °C for 16 h. Sections were baked onto glass slides, then stained using Gram-Twort's. All the steps were carried out on a 80 °C hotplate. The images were then viewed by light microscopy (Olympus CH2) using $\times 100$ oil immersion and images were captured using iPhone 4S.

For transmission electron microscopy (TEM) analysis, cells (1×10^6 cells/mL) were infected with *M. marinum* at MOI 10 for 24, 72 and 120 h. After each timespan of infection, the cells were harvested ($300 \times g$, 5 min) and washed once with PBS. The pellets were re-suspended with 1 mL 1% (v/v) glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4) at RT for 30 min. After three washes of 0.1 mol/L sodium cacodylate at $250 \times g$ for 20 min, the cells were fixed with 1% (w/v) osmium tetroxide in 0.1 mol/L sodium cacodylate for 90 min at 4 °C, and thoroughly washed with distilled de-ionised water three times for 20 min each wash^[19]. The samples were embedded in 3% (w/v) agar and were dehydrated through an ethanol series. 100% analytical grade ethanol was used to wash the samples twice for 15 min, followed by two changes of propylene oxide (PO) for 15 min and then transferred through changes of PO: Agar low viscosity resin mix 1:1 ratio for 60 min in two changes and lastly 3:1 ratio for 60 min. The samples were transferred into 100% low viscosity agar overnight and then loaded into capsules with fresh agar low viscosity resin for 3 h, followed by polymerisation at 60 °C for 16 h to produce a solid block for ultrathin sectioning (80-100 nm, diamond knife).

Quantitative RT-PCR

Total RNA was prepared using TRIzol Reagent (Invitrogen, Paisley, United Kingdom) according

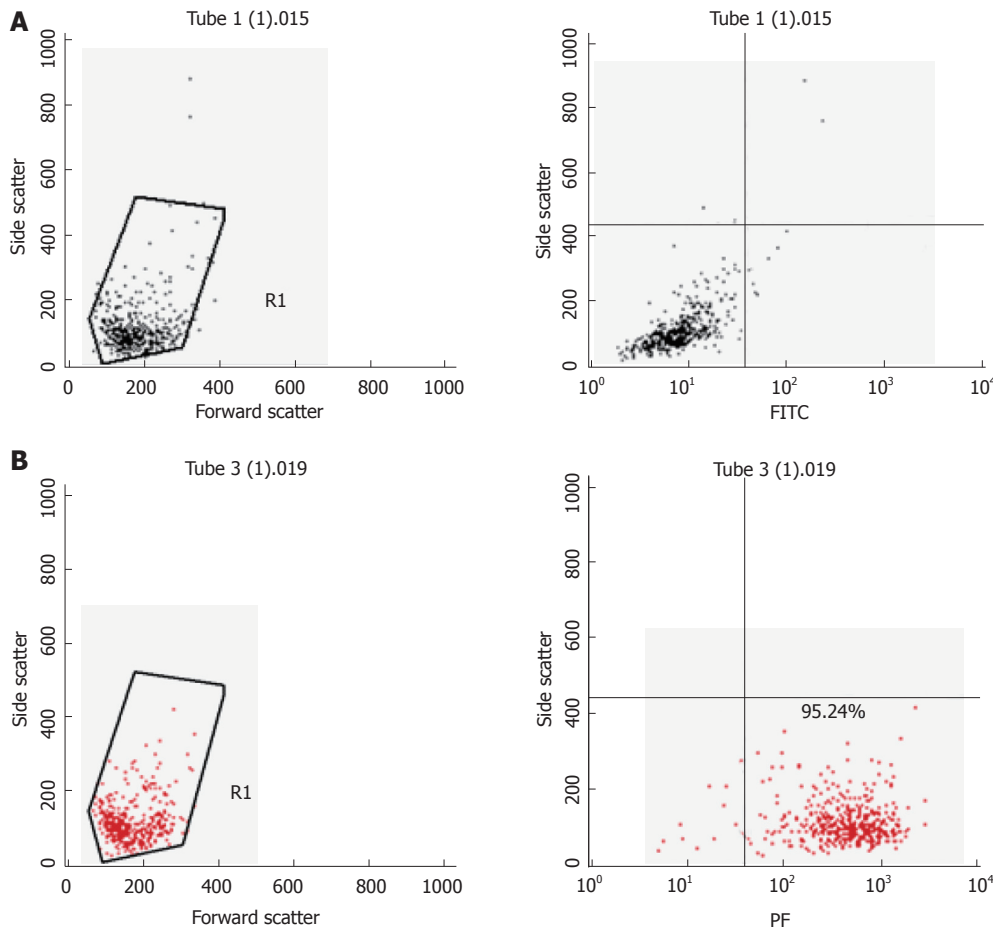


Figure 2 Characterisation of primary mast cells. Bone marrow derived mast cells (BMDMCs) were isolated from C57Bl/6J mice and were differentiated with 100 ng/ μ L interleukin-3 over 5 wk. A: Isotype PE [Hamster immunoglobulin G (IgG) with isotype fluorescein isothiocyanate (FITC) (Rat IgG2b, κ) stained BMDMCs; B: Isotype FITC (Rat IgG2b, κ) with Fc ϵ RI (PE) antibody stained BMDMCs. Ninety-five percent of cells stained positively with Fc ϵ RI (PE) antibody.

to the manufacturer's instructions. After DNaseI digest and extraction, first strand cDNA synthesis was carried out according to the manufacturer's instructions (Thermo Scientific United Kingdom). Primer sequences for subsequent PCR were for TNF- α 5'-CCCGACTATCTCGACTTTGC-3' and 5'-GTTGGATGTTTCGTCCTCCTC-3', for NOD2 5'-GGCAGCCTCTTCAAAATGAG-3' and 5'-GGGAAGAAGTCAATGGCAAA-3', for COX2 5'-CGCCCTCATAATCATTTTCC-3' and 5'-GAGGGCGATGAGGACTAGG-3', for LL-37 5'-GAAGACCCAAAGGAATGGCC-3' and 5'-CAGAGCCAGAAGCCTGAGC-3', for β 2microglobulin 5'-GGCTATCCAGCGTACTCCAAAG-3' and 5'-CAACTTCAATGTCGGATGGATG-3'. The Livak or $2^{-\Delta\Delta CT}$ method was used to calculate the normalised expression ratio of the target gene with reference to β 2M gene^[20].

Ethical consideration

Animal care and use statement: The animal protocol was designed to minimise pain or discomfort to the animals by using heat-killed *M. marinum* to cause immune stimulation, not disease. Mice were taken from a breeding colony within the unit, were acclimatised

to 21 °C, 50% humidity, with 12/12 h light/dark cycle, and had *ad libitum* access to food (EURodent Diet 14%, LabDiet, International Product Supplies, London, United Kingdom) and water. Controls and experimental animals were each jointly housed. Intraperitoneal injection and cervical dislocation at the end of the experiment were performed by trained staff competent in these techniques in accordance with Home Office regulations.

Statistical analysis

Data are presented as means + standard deviations. Both Microsoft Excel and GraphPad Prism (v.4.02 for Windows; GraphPad Software, San Diego, CA) were used to analysis data in this study, and where appropriate statistical analyses was done using One-way and Two-way ANOVA (Analysis of Variance) with Bonferroni post-test using GraphPad Prism program, unless stated otherwise. A *P* value of < 0.05 was considered statistically significant.

RESULTS

Mast cells are stimulated by *M. marinum*

To demonstrate mast cell involvement in an *in vivo*

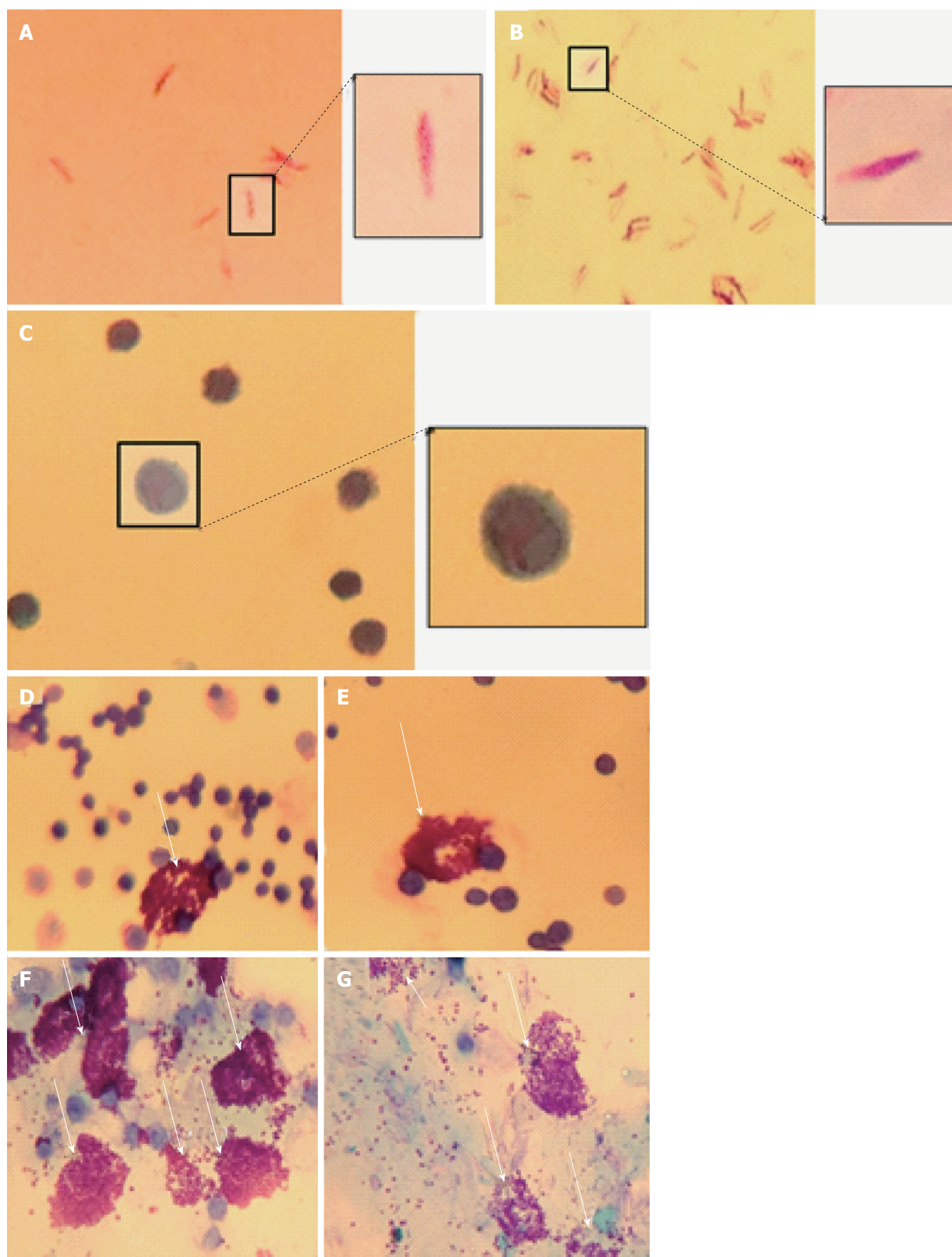


Figure 3 Peritoneal mast cells stimulated with *Mycobacterium marinum* degranulate. Heat-killed (A) and untreated (B) *Mycobacterium marinum* (*M. marinum*) positive for Kinyoun's acid fast stain, while peritoneal mast cells from mice injected with heat-killed *M. marinum* were negative (C). Metachromatic granules of peritoneal mast cells are stained with toluidine blue from unstimulated mice (D, E) and mice injected with heat-killed *M. marinum* (F, G). An exemplary image of each experimental mouse is shown (Olympus CH2, $\times 100$ oil immersion, captured using iPhone 4S).

model, C57/Bl6J mice were stimulated intraperitoneally with heat-killed *M. marinum* and peritoneal lavages

were performed. Cytologically, there was no evidence of bacteria associated to or visibly internalised by mast

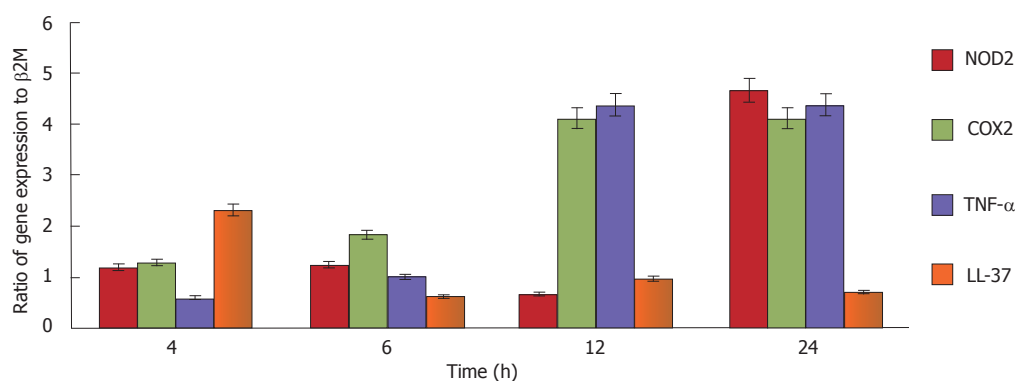


Figure 4 quantitative reverse transcriptase- polymerase chain reaction analysis of inflammatory genes from human mast cell line incubated with *Mycobacterium marinum* (MOI 10). The target inflammatory genes were normalised to individual housekeeping gene ($\beta 2M$), thereafter the target genes were calculated against control human mast cell line cells and expressed as fold change. The error bars show standard deviation of two-four independent experiments, each in either duplicate or triplicate. NOD2: Nucleotide-binding oligomerization domain-containing protein 2; COX2: Cyclooxygenase 2; TNF- α : Tumour necrosis factor- α .

Table 1 Lactate dehydrogenase release was measured photometrically

Mast cells infected with <i>M. marinum</i> at different MOIs for 8 h	LDH release, % increase over uninfected control
0.5:1 (bacteria:cell)	4.20%
10:1 (bacteria:cell)	31.10%
25:1 (bacteria:cell)	43.70%

Mean of triplicate determinations was related to the control and expressed as % increase. *M. marinum*: *Mycobacterium marinum*; LDH: Lactate dehydrogenase; MOI: Multiplicity of infection.

cells but significant degranulation (Figure 3). To assess the inflammatory response in a greater number of mast cells (replacement of animal models), a cell line was used. After incubation of HMC-1 with *M. marinum* for 4, 6, 12, 24 h, qRT-PCR analysis showed early expression of LL-37 mRNA, significant expression of COX-2 and TNF- α mRNA at 12 h and delayed expression of NOD2 mRNA at 24 h (Figure 4).

***M. marinum* infects mast cells, where it replicates**

Assessment of LDH release, a measure of cytoplasmic leakage, from HMC-1 incubated with *M. marinum* revealed significant damage incrementally at MOI 10 and 25 compared to the uninfected control (Table 1). Flow cytometric analysis using Annexin V and PI showed that early apoptosis was detected in about 1/3 of the infected cell population. It is possible that internalised bacteria account for some PI staining in late apoptotic and necrotic cells (Table 2).

Next, HMC-1 and primary murine mast cells were infected with *M. marinum* (MOI 0.5) for up to 120 h. Among the aminoglycoside antibiotics tested, only amikacin proved efficacious against *M. marinum*, whilst leaving the viability of mast cells unimpaired (data not shown). It was used at 0.2 mg/mL for 2 h to kill all extracellular bacteria after infection of mast cells - this was verified by the lack of CFU grown from the supernatants - so that subsequently, the intracellular viable bacterial load could be determined. At 4 h after

Table 2 A representative flow cytometric analysis of apoptotic (Annexin V⁺/PI⁻, early; Annexin V⁺/PI⁺; late) and necrotic (Annexin V/PI⁺) human mast cell line cells after 8 h infection with *Mycobacterium marinum*

Uninfected/infected HMC-1	Annexin V ⁺ /PI ⁻	Annexin V ⁺ /PI ⁺	Annexin V ⁺ /PI ⁺	Annexin V ⁺ /PI ⁺
Uninfected (control)	99.80%	0.20%	0%	0%
Infected with <i>M. marinum</i> at MOI 10	58.52%	31.76%	2.98%	6.70%
Infected with <i>M. marinum</i> at MOI 25	58.24%	29.37%	4.25%	8.14%

Percentage gated population. HMC-1: Human mast cell line; MOI: Multiplicity of infection; *M. marinum*: *Mycobacterium marinum*.

infection, there was significant presence of viable *M. marinum* in the experimentally lysed eukaryotic cells, especially in HMC-1. Cultures of HMC-1 and primary murine mast cells infected in parallel and analysed over longer times maintain a considerable intracellular bacterial load of *M. marinum*. Though intracellular growth of *M. marinum* appeared to be somewhat controlled in BMDMC at 24 h, both types of mast cells harboured significantly elevated CFU at 120 h with concomitant increase in cell damage (12%-14%), as assessed by the trypan blue exclusion method (Figure 5).

Intracellular presence of *M. marinum* in mast cells

Flow cytometric analysis was performed using fluorescently labelled mycobacteria and quenching any extracellular signal with trypan blue. With this method, the intracellular content of the labelled material can be reliably measured. Because our GFP-expressing *M. marinum* strain showed growth restriction at 37 °C, it could not be used for this purpose. Alternatively, *M. marinum* were instead labelled with FITC and used to infect HMC1 and primary murine mast cells at MOI 10 for 24 h. After washing, trypan blue was added and cells were analysed by flow cytometry. Intracellular presence detected in this manner varied from 5%-8% of gated intact cells for BMDMC and HMC-1 cells, respectively

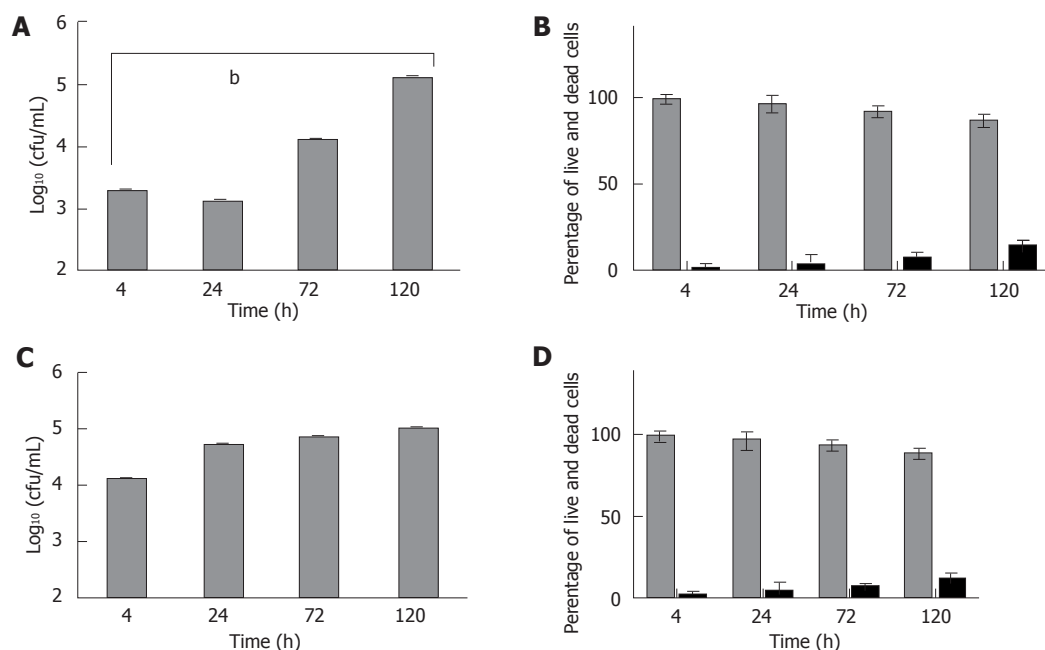


Figure 5 Mast cells infected *in vitro* with *Mycobacterium marinum*: Viable counts and cell viability. Bone marrow derived mast cells (A, B) and human mast cell line (C, D) were infected with *Mycobacterium marinum* at MOI 0.5 for up to 120 h. After 4 h of infection, cells were rinsed and treated with 200 µg/mL amikacin for 2 h. At each time point intracellular bacteria were determined by enumerating colony forming units (CFU) (A, C). The values are mean $\pm$ SD of three to four independent experiments, each in triplicate. A: $P < 0.0008$. Viability was assessed for mast cells at these timepoints (B, D). After 4 h of infection, the cells were washed and treated with 200 µg/mL amikacin for 2 h. The values are mean $\pm$ SD of three independent experiments, each in triplicate. MOI: Multiplicity of infection.

(Figure 6).

Various techniques were employed to visualise the intracellular presence of *M. marinum* in mast cells. Whilst fluorescence microscopy did not convincingly differentiate between adsorption and intracellular presence of *M. marinum* (Figure 7), clear evidence was for the first time obtained from the analysis of 0.5 µm cross-sections prepared of HMC-1 cells, in comparison with infected MonoMac6 cells (Figure 8). Though the monocytic cell line seems more avid in the bacterial uptake, a comparable picture is obtained when producing and analysing thin sections from mast cells. Lastly, to confirm this observation beyond doubt, TEM was used for both, HMC-1 cells and BMDMCs, which were infected with *M. marinum* at MOI 10 for 24 h (Figure 9). *M. marinum* sub-localised to the cytoplasm of HMC-1 cells whereas the bacilli sub-localised in vacuoles of BMDMCs.

DISCUSSION

This study shows that the involvement of mast cells in infection with *M. marinum* was manifold; mast cells degranulated, expressed mRNA of pro-inflammatory genes, lost cytoplasmic integrity, underwent apoptosis and to a lesser extent necrosis, and also internalised the bacilli. At the MOI chosen, the study could not evaluate a bactericidal or bacteriostatic effect of mast cell activity against mycobacteria, although the bacteria did not grow in culture medium alone.

We employed a human mast cell line (HMC-1 cells) which was established from a leukaemia patient by

Butterfield *et al.*^[21]. As this cell line has a phenotype typical of immature mast cells, we isolated primary mast cells from the bone marrows of C57/BL6J mice. The cells were differentiated with IL-3, and were 95% pure according to their surface staining for FcεRI. Other sources to study mast cell interaction with *M. tuberculosis* include a rat basophilic leukaemia cell line (RBL-2H3)^[14,15] although there is contention about the relationship of these cells to mast cells^[22].

Firstly, we established that *M. marinum* does lend itself to studies of eukaryotic infection because its growth at 37 °C compared well to that at the more typical temperature for this bacterium of 32 °C. This observation had precedence in the description by others of growth at 37 °C for the *M. marinum* strain^[23-25]. In accordance with other studies, we found that GFP or ds-Red expressing *M. marinum* strains could not grow at 37 °C^[26].

Next, we showed that *M. marinum* can infect, survive and replicate inside mast cells (HMC-1 and BMDMCs), where the bacilli sub-localise in the cytoplasm of HMC-1 cells, yet show a vacuolar presence in the more mature BMDMCs. In HMC-1 cells, no bacteria were seen surrounded by a vacuolar membrane at 24, 72 and 120 h (data not shown). Given that the overall uptake was low, it seems unlikely that *M. marinum* would have escaped from a vacuole in the interval of sampling time points. By contrast, *M. marinum* were regularly found in phagocytic vacuoles in the mature BMDMCs, with occasionally larger numbers of *M. marinum* occupying a single vacuole. These subcellular locations may reflect

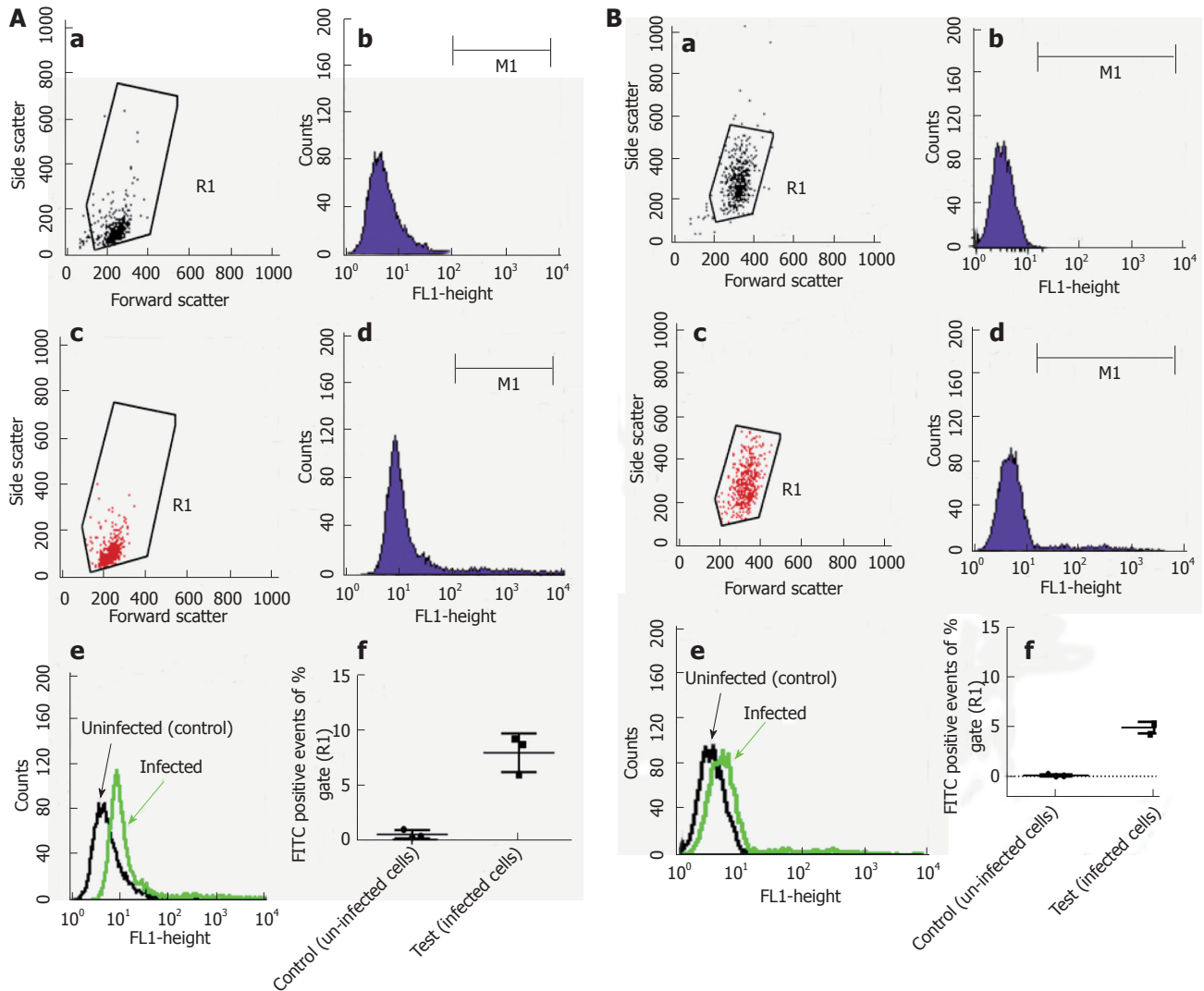


Figure 6 Flow cytometric detection of intracellular fluorescein isothiocyanate labelled *Mycobacterium marinum* (trypan blue quenching method). A: bone marrow derived mast cells; B: Human mast cell line. Density plot and histogram to measure R1 gate of uninfected control cells (a, b) and of cells infected with *Mycobacterium marinum* at MOI 10 for 24 h (c, d); e: Overlay of b and d; f: Portion of fluorescent signal of R1 gate (fluorescein isothiocyanate labelled bacteria), expressed in percentage. This data is a collection of three independent experiments, each performed in duplicate; error bars represent standard deviations. MOI: Multiplicity of infection.

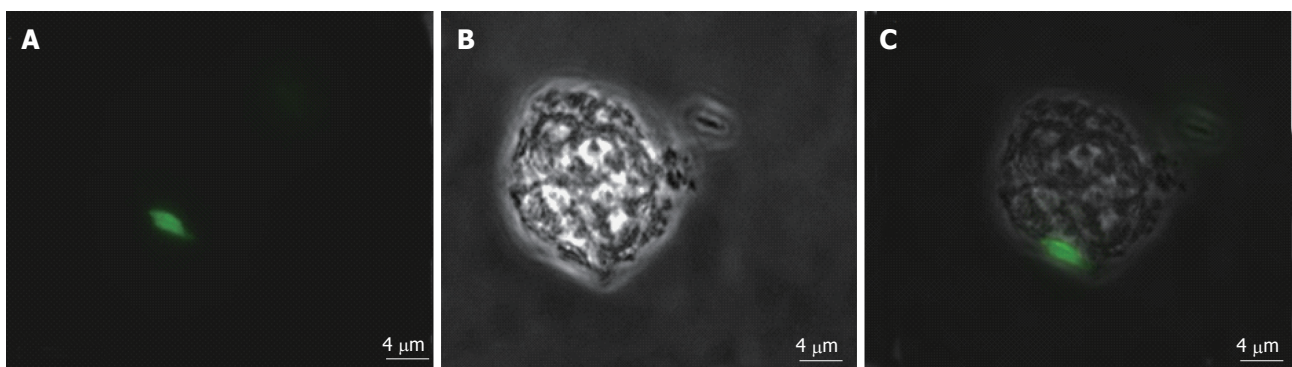


Figure 7 Representative image of human mast cell line cell infected with green fluorescent protein expressing *Mycobacterium marinum*. A: Green fluorescent protein expressing *Mycobacterium marinum*; B: Human mast cell line in phase contrast; C: Overlay image of A and B.

uptake mechanisms which differ between immature and mature, non-professional phagocytic cells.

During the analyses of the cytoplasmic presence of *M. marinum* in TEM images, the presence of damaged

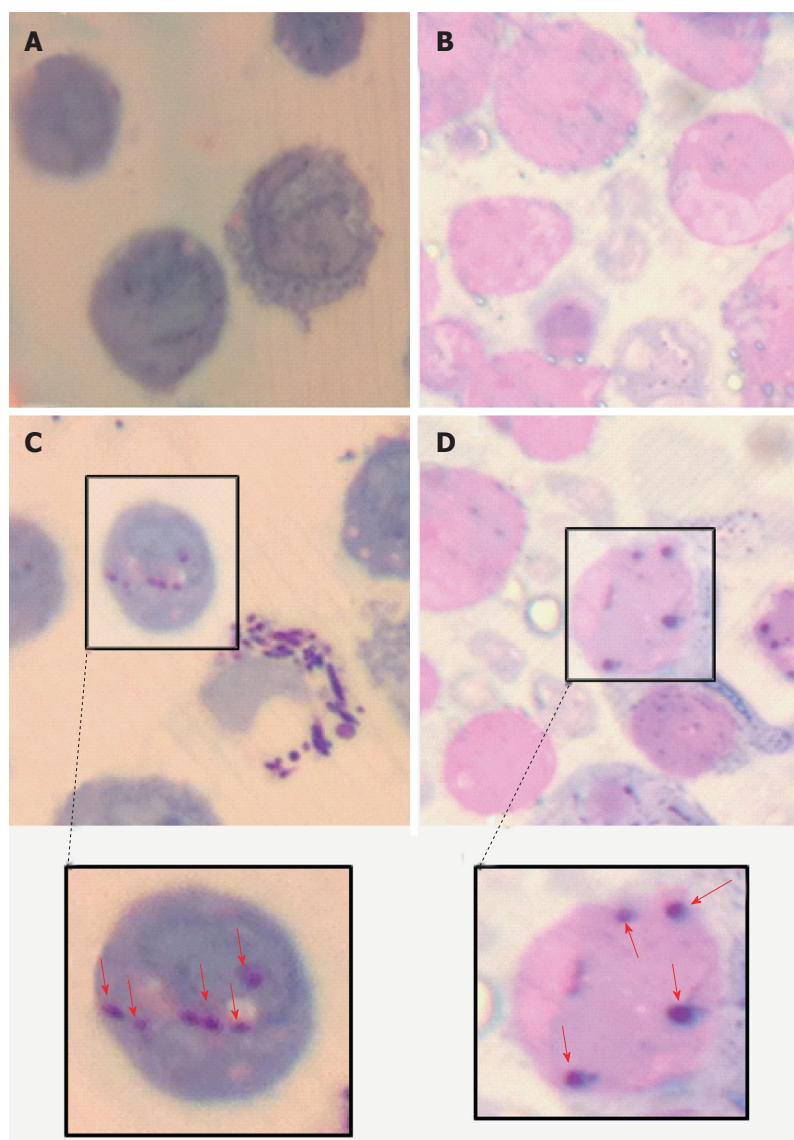


Figure 8 Light microscopic evidence of the intracellular presence of *Mycobacterium marinum* in human mast cell line. Gram-Twort stained, 0.5 µm thin cross sections of Mono Mac 6 cells [A: uninfected; C: infected with *Mycobacterium marinum* (*M. marinum*) at MOI 10 for 24 h] and human mast cell line (HMC-1) cells [B: Uninfected; D: Infected HMC-1 cells with *M. marinum* at MOI 10 for 24 h]. The red arrows in insets C and D indicate internalised *M. marinum* (Olympus CH2, × 100 oil immersion, captured using iPhone 4).

cells was noted. Viability of mast cells after low dose infection (MOI 0.5) for 120 h had shown only mild impairment. Measurement of cytoplasmic LDH release due to infection-induced injury revealed significant damage at MOI 10 and 25 compared to the uninfected control, contrary to low MOI (0.5). Up to this time point, it was known that *M. marinum* caused the release of LDH from infected macrophages^[27]. In flow cytometric analysis using Annexin V and PI, we demonstrated that this damage was likely caused by an increase in necrotic cells.

Cruse *et al.*^[28] (2010) showed that mast cells (including HMC-1 cells) were able to kill *Streptococcus pneumoniae* and in response to pneumolysin, they rapidly released the antimicrobial peptide cathelicidin LL-37 (which is capable of inducing pneumococcal cell death), and the pro-inflammatory mediator LTC₄. In

relation to the pneumococcus, Cruse *et al.*^[28] (2010) also showed that although cell contact may increase the cytotoxicity of mast cells to pneumococci, HMC-1 cells can elicit antimicrobial activity to pneumococci in the absence of cell contact. In contrast, von Köckritz-Blickwede *et al.*^[29] (2008) demonstrated that HMC-1 cells can kill directly *S. pyogenes* without phagocytosis but cellular contact was required. They showed that LL-37 is caught in extracellular traps and H₂O₂ was required for extracellular trap formation (a process which is required for antimicrobial activity of HMC-1 against *S. pyogenes*). This process is at the cost of HMC-1 cell viability as the cells seemed to have undergone nuclear degradation to form these traps. The investigators referred to this so-called cell death, which is between apoptosis and necrosis, as "NETosis". Whilst Arock *et al.*^[30] (1998) showed that mast cells phagocytose *Staphylococcus*

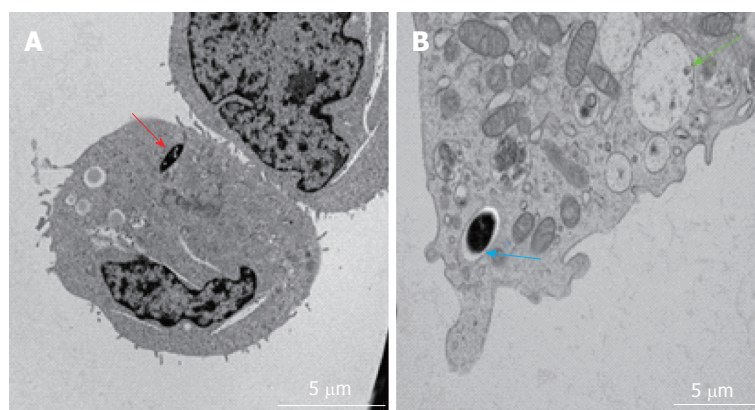


Figure 9 Transmission electron microscopy images of intracellular localisation of mycobacteria in human mast cell line (A) and bone marrow derived mast cells (B). Cells infected with *Mycobacterium marinum* at MOI 10 for 24 h. A, red arrow: Cytoplasmic localisation; B, blue arrow: Vacuolar localisation; green arrow: Empty vacuole comparable to those described for degranulating mast cells^[11]. MOI: Multiplicity of infection.

aureus, Cruse *et al.*^[28] (2010) and von Köckritz-Blickwede *et al.*^[29] (2008) reported that mast cells do not phagocytose *S. pneumoniae* or *S. pyogenes*, we showed for the first time that mast cells can internalise *M. marinum* where the bacilli survive and replicate.

In spite of the fact that the viability of infected mast cells at MOI 0.5 decreases with prolonged infection (up to 120 h), a large proportion of the infected cells (HMC-1 and BMDMCs) were trypan blue negative and therewith deemed viable (88%-86%). Based on our analysis of intracellular viable count, it is these viable cells which harbour a significant number of viable *M. marinum*. These in turn are likely to have adapted to this intracellular environment and to the action of synthesised inflammatory products. mRNA for the antimicrobial peptide LL-37 was an early indicator of the antimycobacterial response. COX2 TNF- α and NOD2 mRNA were increased later. The release of TNF- α from the supernatants of infected HMC-1 cells at MOI 0.5, 1 and 10 for 24, 72 and 120 h, using TNF- α bioassay (L929 cells) was undetectable (data not shown). This is consistent with previous observations that the TNF- α produced by HMC-1 is not bioactive^[31]. Further studies are needed to evaluate the transcriptomic changes undergone by *M. marinum* in response to its intracellular localisation.

ACKNOWLEDGMENTS

The Schlumberger Foundation Faculty for the Future is gratefully acknowledged for its continuous support throughout the fellowship (Sadiyo Siad). We thank Natalie Allcock and the late Stefan Hyman from the University of Leicester Electron Microscopy Unit. University of Leicester Biomedical Services staff is acknowledged for excellent maintenance and care of mouse colonies. We appreciate support from Professor Peter Andrew (University of Leicester) and helpful discussions with Dr Anthony Tsolaki (University of

Brunel).

COMMENTS

Background

Host pathogen interactions in *Mycobacterium tuberculosis* (*M. tuberculosis*) infection have been extensively studied for macrophages, dendritic cells, and have recently been described for epithelial cells and mast cells. The study of *M. tuberculosis*, however, requires sophisticated containment facilities. Isolation of *M. marinum* from zebrafish, its characterisation in granulomatous waterborne disease in man and comparative analysis of its genome against other mycobacterial species eventually led to its wider introduction in experimental work. Mast cells are important immune effector cells, which tailor the release of their granular content to different modes of activation (crosslinking of Fc ϵ R bound antibodies, activation of complement, recognition of pathogen associated molecular patterns, integrin signalling). Whilst tissue resident macrophages originate from the yolk sac, mast cells differentiate from myeloid bone marrow precursors, are much longer lived than macrophages and proliferate. Mast cells reside close to mucosa, nerves, lymphatics and vessels.

Research frontiers

M. smegmatis does not replicate intracellularly and incompletely models characteristics of *M. tuberculosis*. Use of *M. marinum* to study mammalian immune responses was hampered by its being primarily a pathogen of poikilotherms and causative of skin disease in humans ("fish tank granuloma"). However, its adaptation to 37 °C, its sensitivity to amikacin, and its ability to replicate intracellularly render *M. marinum* to be of interest to those studying host-pathogen interactions of *M. tuberculosis*.

Innovations and breakthrough

This is the first study to use 0.5 μ m thin cell sections to demonstrate the intracellular location of *M. marinum* after infection. This technique, for this question, rivals the use of transmission electron microscopy.

Applications

Because the natural host of *M. marinum* is different (zebra fish), the study does not address mycobacterial pathogenesis in the natural host. However, *M. marinum* does infect humans and causes fish tank granuloma which sometimes can resolve without treatment and implies an efficient, still not fully characterised role of the immune system. The authors do not know in which other ways the strain that has adapted to 37 °C differs from the isolate with an optimal temperature of 32 °C. The major focus of most recently published studies is on zebra fish, even so, fish tank granuloma is often misdiagnosed (probably, because of the authors' lack of knowledge). Non-*M. tuberculosis* mycobacterial

infections are an emerging clinical problem and they often require special drug regimes (different from *M. tuberculosis*). For these reasons, there is a need for an easy-to-handle, relevant, *in vitro* infection system. In addition, further studies could now evaluate the ability of *M. marinum* to adapt their metabolism and expression of lipid rich bodies in order to persist in cells, and thereby give an indication whether this is indeed a feature in those infected with *M. marinum*.

Terminology

Primary mast cells describe those cells differentiated from mouse bone marrow precursors using IL-3 expressing Fc ϵ R.

Peer-review

The role of mast cells in infection secondary to *M. marinum* is explored in this study. The results demonstrate that a human mast cell line stimulated with *M. marinum* showed increased mRNA expression of pro-inflammatory genes including *LL-37* and *COX2/TNF- α* , internalized the bacilli and underwent apoptosis. Furthermore the *M. marinum* can infect, survive and replicate inside mast cells.

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New insights on chitinases immunologic activities

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Abstract

Mammalian chitinases and the related chilectins (ChiLs) belong to the GH18 family, which hydrolyse the glycosidic bond of chitin by a substrate-assisted mechanism. Chitin the fundamental component in the

coating of numerous living species is the most abundant natural biopolymer. Mounting evidence suggest that the function of the majority of the mammalian chitinases is not exclusive to catalyze the hydrolysis of chitin producing pathogens, but include crucial role specific in the immunologic activities. The chitinases and chitinase-like proteins are expressed in response to different proinflammatory cues in various tissues by activated macrophages, neutrophils and in different monocyte-derived cell lines. The mechanism and molecular interaction of chitinases in relation to immune regulation embrace bacterial infection, inflammation, dismetabolic and degenerative disease. The aim of this review is to update the reader with regard to the role of chitinases proposed in the recent innate and adaptive immunity literature. The deep scrutiny of this family of enzymes could be a useful base for further studies addressed to the development of potential procedure directing these molecules as diagnostic and prognostic markers for numerous immune and inflammatory diseases.

Key words: Chitinases; Chitinase like proteins; Chronic inflammation; Immune regulation; Autoimmunity

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Core tip: The chitinases and chitinase-like proteins are expressed in response to different pro-inflammatory signals by activated macrophages and in different monocyte-derived cell lines. The mechanism and molecular interaction of chitinases in the immune regulation embrace bacterial infection, inflammation, dismetabolic and degenerative disease. The concept of the chitinases involvement in human diseases discussed herein may stimulate the development of new studies leading to a deeper understanding on the biochemical mechanisms inducing chitinases regulation and on the consequences that the increases in chitinases levels impact with immunity and autoimmunity in different conditions. The future understanding on chitinase functions will lead to the opportunity to develop selective and specific chitinase inhibitors.

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INTRODUCTION

Mammalian chitinases and the related chilectins (ChiL) belong to the GH18 family^[1]. Chitinases embraces members both with and without glycohydrolase enzymatic activity against chitin. Chitotriosidase (CHIT1) and acidic mammalian chitinase (CHIA) are recognized as true chitinase because are the only two chitinases demonstrating chitinolytic (glycohydrolase) activity^[2]. In contrast none of the other mammalian chitinases, encompassing chitinase 3-like-1 (CHI3L1), chitinase 3-like-2 (CHI3L2), chitinase domain-containing 1 (CHID1), display enzymatic activity in the face of the retention and conservation of the substrate-binding cleft of the chitinases^[3] and for this reason they are called chitinase-like-lectins (Chi-lectins) or chitinase-like proteins (C/CLPs). Mammalian chitinases with enzymatic activity have a chitin binding domain containing six cysteine residues predisposed for the binding of chitin^[4]. Instead, CLPs do not contain such typical chitin-binding domains, but still can bind to chitin with high affinity^[5]. A number of evidence reports that the expression of the majority of the mammalian chitinases is differentially regulated during specific immunologic activities and has important biological roles in chronic inflammatory diseases^[6-8]. Additionally chitinases have been widely shown to have an antipathogen function, through their capability to degrade both colloidal chitin and chitin in the cell wall of the fungal pathogen. Similarly, mammalian ChiLs may play a role in immunomodulation. The majority of chitinase families are produced by monocyte/macrophages lineage. In addition, macrophages induce inflammatory responses by producing cytokines, chemokines, and lipid mediators. Interestingly, chitinase play a role in modulating the local and/or circulating concentration of chitins in the body and, therefore, in regulating the immune response to this polysaccharide. Hypothetically, when exogenous chitin from sources such as fungi or dust mites are present in the tissues, chitinases act by cleaving chitin which consequently prevent chitin from stimulating immune responses. Hence, it is possible that without active chitinases, chitin accumulate in tissues triggering an excessive inflammatory response. Therefore is clear that induction of chitinase and CLPs is associated with inflammatory disease, including allergy, asthma, dismetabolic and degenerative diseases and several types of cancer^[9].

In the last decade various investigations have brought new insights on the immune properties of chitinases and their functions in inflammatory pathologies. Both chitinases and CLPs can activate specific receptors and

signaling pathways stimulating immune mediators' generation and amplification of inflammation. New studies are helping to understand the beneficial as well the detrimental properties of chitinases. Characterizing the role of induced chitinases activity promises interesting perspectives. As well, understanding the molecular signalling pathways involved in the immune function influenced by chitinases might be a valuable approach to investigate new therapeutic alternatives for pathological conditions in which the increased immune response and inflammation are involved.

CHITOTRIOSIDASE AND IMMUNITY

CHIT1 was the first mammalian chitinase measured in disease states^[4]. CHIT1 has been encompassed as one of the secreted biomarkers for Gaucher's disease^[10]. The elevation of CHIT1 in these patients may reflect a particular state of activation of macrophages^[11]. CHIT1 is a very critical enzyme to regulate the susceptibility to infection of organisms containing chitin as structural components^[2].

The *CHIT1* gene is localized in chromosome 1q31-q32^[12] and consists of 12 exons and spans approximately 20 kb of genomic DNA^[12]. Recombinant CHIT1 inhibits hyphal growth of fungi, suggesting a physiological role in the host defense mechanism against the invasion/attack of chitin-containing pathogens^[13] which to act as adjuvants by stimulating the production of cytokines and chemokines^[6]. Further evidence indicates that the enzymatic role of CHIT1 extends to bacteria^[4,13]. Usually, CHIT1 activity is very low and originates in the circulating polymorphonuclear cells^[12]. CHIT1 rises significantly in response to various pro-inflammatory signals in a complementary fashion in neutrophils and macrophages^[4]. The evidence that TLR signaling is a potent inducer in neutrophils, while NOD-2 signaling induces *CHIT1* in macrophages^[14], strongly confirms the importance of this enzyme in the immune response. A defect in *CHIT1* gene consisting of 24-bp duplication in exon 10 that activates a cryptic 39 splice site in the same exon generates an abnormally spliced mRNA with an in-frame deletion of 87 nucleotides. This spliced mRNA encodes an enzymatically inactive protein that lacks an internal stretch of 29 amino acids^[12]. CHIT1 deficiency appears as an autosomal incompletely dominant disorder, with no activity in homozygous subjects for the defective allele and approximately half-normal activities in heterozygous subjects. *CHIT1* gene mutation has been encountered with high incidence in different Caucasian populations^[12], instead, in African peoples living in malaria parasite endemic areas CHIT1 mutation shows a low prevalence. The absence of homozygosis for CHIT1 deficiency in malaria endemics area suggests the hypothesis that susceptibility to parasitic disease influences the CHIT1 allele composition. In sub-Saharan regions the maintenance of the wild-type *CHIT1* gene confirms that CHIT1 provides innate protection from malaria infection^[15]. As well the studies reporting that

individuals bearing the mutant allele exhibit an increased susceptibility to chitin-containing pathogens including *Wuchereria bancrofti* filarial, *Plasmodium falciparum* malaria, *Cryptococcus neoformans* and *Candida albicans*^[16] confirmed the CHIT1 allele arrangement hypothesis. Nevertheless, in others studies have been reported that a functional polymorphism produces protective effect in human longevity^[17] and protects from nonalcoholic fatty liver disease progression^[18]. CHIT1 may have organ- as well as cell-specific effects in the setting of infectious diseases and inflammatory disorders. In fact, CHIT1 overexpression in Kupffer cells is involved in the modulation of the tissue remodeling processes in fibroblastic hepatic tissue^[18]. Furthermore, the CHIT1 produced by macrophages enhances atherosclerotic plaques formation and subsequent thrombosis^[19]. Therefore this enzyme produced by differentiated macrophages can also be damaging to host tissues and are implicated in the progression of a number of chronic inflammatory diseases^[20]. In this context, it is important to note that CHIT1 displays different role in the specialized macrophages. CHIT1 modulation changed during the diverse stages of macrophages maturation and in polarized M1 and M2 macrophages^[6]. This data could explain why the expression of CHIT-1 is particularly elevated in the later inflamed stages of infection-induced diseases such as tuberculosis and leprosy^[21,22]. Remarkably, was also reported that in monocytes interleukin-4 (IL-4) treatment induced a significant increase on CHIT-1 expression^[6]. Since IL-4 promotes immune responses to parasites, this finding set straight why CHIT-1 increased secretion is closely associated with pathophysiological conditions dominated by T-helper type 2 (Th2) cells including infections with fungal pathogens and malaria parasites, fibrosis, allergy, and asthma^[23-25]. Macrophages are involved in both generation of fibrosis and its resolution. Conversely M2 polarization generates a positive feedback loop during resolution of inflammation, therefore it is unclear what are the events influencing M2 differentiation and interrupting tissue repair/remodeling as well fibrotic outcomes. The finding reporting that CHIT1 increases in M2 subset suggest that CHIT1 could be involved in the modulation of the extracellular matrix affecting cell adhesion and migration during the tissue remodeling processes that take place in fibrogenesis^[26,27]. CHIT1 is also involved in human airway hyper-responsiveness and asthma^[28], as well as being active to IL-13-driven alveolar fibrosis by augmenting transforming growth factor beta (TGFβ) and mitogen-activated protein kinase signaling in mice^[29]. Therefore, it is conceivable that chitinase inhibition might have beneficial effects on the expression of genes associated with tissues remodeling. Additionally, the recent findings demonstrating that CHIT1 is not exclusively produced by macrophages but is expressed in other cells involved in the immune response such as osteoclasts^[30,31] and monocyte-derived DCs^[32] confirm the active role of CHIT-1 in the immune

response and in disease states where inflammatory responses prevail^[21,22,28,33-35].

ACIDIC MAMMALIAN CHITINASE AND IMMUNITY

The second true chitinase called AMCase or CHIA has a 30-kDa N-terminal catalytic domain that hydrolyze chitin, and it expressed mainly in the gastrointestinal tract and lung of both mouse and human^[36]. Similarly to CHIT1, is located on chromosome 1q13.1e 21.3, and in addition to the N-terminal catalytic domain acidic mammalian chitinase (AMCase) contain a C-terminal chitinase binding domain^[5]. The presence of AMCase in the gastrointestinal tract and lung indicates that it plays a crucial role as a food processor in stomach and its involvement in lung inflammation^[5,37]. As well, the expression of AMCase in the lung suggests that the enzyme may have a dual function in digestion of chitinous substrates and host defense^[38]. This enzyme plays protective role against parasites. AMCase acts as chemotactic agents and synergistically with other chemokines attracting eosinophils and T cells to sites of parasitic infection, appears to modulate tissue inflammation, immunity, and therefore plays active roles in anti-infective defense and repair responses^[8]. Recently it has been demonstrated that AMCase and CHIT1 play different rule in the immune response^[8]. Comparing the modulation of both AMCase and CHIT1 expression during monocyte/macrophages differentiation and polarization was found that AMCase was not selectively expressed and highly regulated in activated macrophages. The slight increases of AMCase in M1 stage following treatment with pro-inflammatory stimuli indicated AMCase is ineffective against infections and therefore may be involved only in innate immunity^[8]. It has been reported that AMCase is specifically upregulated in response to Th2 inflammation in the lung, and is strictly related to pathophysiological conditions dominated by Th2 type cells such as allergy and asthma^[39-41]. The early up regulation of AMCase expression in undifferentiated monocytes treated with IL-4 suggested that an inhibition of AMCase prevents this immune response^[8]. In addition, genetic studies of the AMCase gene have indicated that certain polymorphisms and haplotypes of AMCase are associated with bronchial asthma in humans^[40]. In contrast, other studies revealed that a haplotype encoding an AMCase isoform displaying a significant enzymatic activity was associated with protection from asthma in several United States ethnic populations^[41]. These data indicated that an increased AMCase enzymatic activity could be protective against the development of human asthma, possibly through cleavage of inflammatory chitin polymers^[41]. This protective isoform of AMCase may reproduce an improved activity in the stomach, where the degradation of ingested polymeric environmental chitin or chitin-containing microorganisms could induce changes of the bowel commensal flora or to alterations in immune

responses to ingested allergens^[42,43]. Ingested polymeric chitin has been observed to disrupt interactions with host proteins involved in regulating bacterial adherence to the gastrointestinal epithelium, such as RegIII, and to be used as the preferred energy source by certain gut bacteria^[44,45]. Alterations in intestinal microflora alter the subsequent immune response to allergens in the lung in experimental models^[46]. It has been reported that inhibition with the transition-state analog allosamidin, an inhibitor of chitinase, enhanced the Th2 driven, IL-13-dependent inflammation, endorsing that its chitinase activity play a role in asthma, even in the absence of chitin^[47]. The enzymatic activity of AMCase was found critical in the regulation of pulmonary Th2 inflammation in both murine models exposed and unexposed to polymeric chitin. Since AMCase expression is regulated by active Th2 inflammation it is possible that the active isoform predominates in severe asthmatics and/or during asthma exacerbations. Furthermore, expression of the active isoform could be up-regulated by environmental chitin exposures. Chitin microparticles induce alternative macrophage activation through CCL2 signaling in response to binding of chitin by airway epithelial cells^[45]. Moreover, chitin induces the release of IL-25, IL-33 and thymic stromal lymphopoietin that are able to activate the production of the type 2 cytokines such as IL-5 and IL-13 in innate lymphoid type 2 cells. This induction also led to both eosinophilia and alternative activation of macrophages^[48]. It has been reported that chitin itself is a pattern recognition molecule stimulating the tissue accumulation of innate immune cells associated with asthma, such as eosinophils and basophils^[43]. In addition, AMCase preserves airway epithelial cells from undergoing apoptosis by stimulating phosphoinositide 3-kinase (PI3K) and AKT signaling, through a mechanism associated to its chitin-binding site^[45].

CHITINASE-3-LIKE-1 AND IMMUNITY

Chitinase-3-like-1 (CHI3L1) protein or YLK-40 binds chitin polymers in the absence of the active site residues necessary for cleavage. CHI3L1 is produced by neutrophils, monocytes/macrophages, monocyte derived dendritic cells and osteoclasts^[32,49,50]. CHI3L1 is a pro-inflammatory biomarker^[51] and is capable of inducing inflammatory mediators including chemokines (CCL2, CXCL2) and metalloproteases (MMP-9)^[51]. Local inflamed tissues including intestinal mucosa in inflammatory bowel disease (IBD)^[52] and adipose tissues in type 2 diabetes produce CHI3L1^[53]. Induction of CHI3L1 has been reported in autoimmune disorders, in pulmonary sarcoidosis, systemic sclerosis, liver fibrosis, rheumatoid arthritis, bronchial asthma, coronary artery disease, Alzheimer's disease and inflammatory-related illnesses in humans^[54-62]. CHI3L1 secretion is induced by interferon (IFN)- γ ^[5] and IL-6^[61] and is an acute phase reactant associated with disease severity and mortality in numerous infectious diseases.

The expression of CHI3L1 has been reported to be significantly associated with migration of human macrophages^[52] bronchial smooth muscle cells^[62] and glioma cells^[63]. In inflammation activated macrophages are the major CHI3L1 producers^[50]. Substantial evidence supports a role of CHI3L1 in endothelial dysfunction and atherosclerosis^[52,60]. CHI3L1 expression was found variably modulated during macrophages activation and polarization supporting that CHI3L1 plays a crucial role during the initial innate immune responses at the site of pathogen invasion^[64]. The modulation of CHI3L1 following treatment with pro-inflammatory stimuli in monocytes and its strong increases in M1 polarized macrophages indicates that the antimicrobial pathway in human macrophages involves also a vigorous activation of CHI3L1. Additionally the higher expression in M2 polarized macrophages highlight that CHI3L1 is a mediator of innate and acquired immunity^[7]. Remarkably, some evidence indicated that CHI3L1 may play a role in type 2 helper cell-mediated inflammation^[65]. Additionally, CHI3L1 is involved in intestinal inflammation and diverse pathologies concerning the mucosal barriers of the stomach and gastrointestinal tract integrity such as inflammatory bowel disorders. Specifically, *CHI3L1* is upregulated in inflammatory conditions of the gut. Moreover, infection studies have suggested a function in both development and resolution of intestinal inflammation as well as bacterial removal^[66]. The infection stimulating effects have been found to arise from enhanced adhesion of bacteria to intestinal epithelial cells (IECs)^[66], precisely through bacterial interaction with N-glycosylation patterns on *CHI3L1* expressed by IECs^[66]. *CHI3L1* also stimulates clearance and resolution of bacterial infections and inflammation in colitis *via* Stat3 signaling^[66]. Moreover, elevated serum levels of *CHI3L1* promote a marked protection against *Streptococcus pneumoniae* infection, improving the aptitude of macrophages to kill bacteria and simultaneously protecting the immune cells from pyroptosis by inhibiting IL-1 β -driven inflammasome activation^[66]. Serum levels of *CHI3L1* are elevated in patients with pathogen-induced inflammation, including purulent meningitis, and endotoxaemia caused by endotoxin of *Escherichia coli*^[66]. In both meningitis and pneumonia, CHI3L1 is secreted by locally activated macrophages^[66] and neutrophils^[67], and thus, has been proposed as a specific supplementary serological marker for the activation of granulocytes and macrophages in inflamed tissues^[68]. These evidences confirm that CHI3L1 may have a particular affinity with some pathogenic bacteria. Though chitin is not expressed in bacteria, the majority of chitinase-producing pathogenic microorganisms encode a gene encoding for the chitin binding protein, which possibly interacts with the binding ability between chitinase producing bacteria and chitin^[69]. In a knock-out model of the murine CHI3L1 analogue, CHI3L1 is important in establishing Th2 polarized immune responses and enhance the

recruitment of macrophages, dendritic cells and T-cells by inhibiting apoptosis^[70].

Genetic variants of CHI3L1 are associated with reduced lung function in asthmatics^[71]. The increase of this protein in the lung has been found also in patients with COPD and pulmonary sarcoidosis^[72]. Both macrophages and giant cells in pulmonary sarcoid granuloma express CHI3L1, and serum levels of CHI3L1 are indicative for sarcoid disease activity and ongoing fibrosis^[73]. In addition, CHI3L1 promotes the proliferation and antagonizes catabolic or degradative processes during the inflammatory response of connective tissues^[74]. Increased concentrations of CHI3L1 have been detected also in serum of patients with rheumatoid arthritis (RA). The ability of CHI3L1 to regulate cell proliferation, adhesion, migration, and activation, as well as to regulate extracellular matrix assembly, correlates well with elevated level of CHI3L1 in the sites of chronic inflammation and active connective tissue turnover. Local release of CHI3L1 in the arthritic joint is followed by a secondary increase of CHI3L1 concentration in serum. Neutrophil-released CHI3L1 acts as an autoantigen in RA. In contrast to healthy individuals, who show strong bias to regulatory response to CHI3L1, patients with RA exhibit polarization towards Th1 phenotype^[73]. At the same time CHI3L1 is able to suppress the TNF α and IL-1-induced secretion of matrix metalloproteases and IL-8 in both human skin fibroblasts and articular chondrocytes^[74]. In contrast, in RA the serum levels of CHI3L1 positively correlated with serum levels of IL-6 and CRP^[75]. Increased levels of CHI3L1 in serum reflect the degree of the synovial inflammation and joint destruction in patients with RA and OA^[76]. Moreover, elevated level of CHI3L1 is a marker for joint involvement in IBD^[77] and for the activity of the disease^[59]. Rheumatic symptoms are also common for extra-intestinal manifestations of IBD, which is an autoimmune inflammatory disorder of the colon and small intestine. CHI3L1 also colocalises with lactoferrin, but not with gelatinase in both stimulated and non-stimulated neutrophils. Moreover, release of CHI3L1 from specific neutrophil granules was suggested to lead to the post-transfusional complications, which were avoided depleting leukocytes by filtration of whole blood in order to inhibit extracellular CHI3L1 accumulation during storage of erythrocyte components^[78]. CHI3L1 promotes proliferation of human synovial cells, skin and fetal lung fibroblasts, an effect that occurs in synergy with the insulin-like growth factor^[79]. CHI3L1 is upregulated in distinct subsets of macrophages, particularly, in early atherosclerotic lesions and in macrophages which infiltrated deep in the lesion^[80]. Later proteomics study identified elevated levels of CHI3L1 in supernatants of macrophage cell line THP-1 treated with oxidized LDL^[81], proving that CHI3L1 expression is indicative for the differentiation of macrophages during formation of atherosclerotic plaque^[79].

CHI3L2 AND IMMUNITY

CHI3L2 was originally isolated from the cultured medium of primary human articular cartilage chondrocytes^[82]. CHI3L2 is homologous to the family 18 chitinases in the human genome, it lacks of chitinase activity but possesses a chitinase-like fold and putative lectin properties^[83]. CHI3L2 is recognized as a biochemical marker for the activation of chondrocytes and the progress of the osteoarthritis in human. CHI3L2 mRNA is significantly up-regulated in cartilage of patients with osteoarthritis (OA) vs normal subjects, while no significant up-regulation was detected for CHI3L2 mRNA in OA cartilage^[82]. Particularly CHI3L2 expression is upregulated both in early degenerative and late stage of osteoarthritis. Proteomic analysis established that CHI3L2 is secreted by human osteoarthritic cartilage in culture^[5]. The contribution of CHI3L2 to the OA progression is suggested by the induction of autoimmune response^[84] and by its involvement in tissue remodeling. However these finding suggested that synovial fibroblasts do not represent the exclusive producers of CHI3L2 in OA. Recently, CHI3L2 has been found slightly expressed in macrophages differentiated in the presence of IFN- γ or IL-4^[85]. Only classically activated or M1 macrophages are able to produce CHI3L2, whereas in response to IFN- γ and LPS stimulation undifferentiated monocytes were unable to produce CHI3L2^[85]. Thus, IFN- γ which is one of the main cytokines in OA tissues that is able to induce the production of CHI3L2 by monocyte-derived macrophages. In patients with OA, the amount of autoantibodies to CHI3L2 and other autoantigens on early phases of disease indicates that the autoimmune response occurs during the initial phase of cartilage degeneration^[86]. It has been demonstrated that Th1 cells prevail in the synovium of patients with OA^[87]. In addition the co-treatment of IL-4 and TGF- β promotes stimulatory effect on the expression of CHI3L2 in macrophage cultures^[88]. So far, the studies on biological activity of CHI3L2 are limited, therefore further studies are necessary to elucidate the role of CHI3L2 in immunopathology and inflammatory diseases.

CONCLUSION

Chitinases synthesis occurs in most innate immune responses against fungi, bacteria and other non-viral pathogens. In the context of infectious diseases, it is likely that chitinases activity can be both detrimental and beneficial for the host organism. In addition, it cannot be excluded that chitinases augmentations have negative consequences in those conditions in which they are regarded as biochemical markers of macrophage activation. Although we do not yet fully understand the implications of chitinases production in response to chitinous pathogens, the concept of their function as "more than just antipathogens and antifungicidals" seems reasonable. In support to this opinion, the

aforsaid investigations confirming that CHIT-1, CHI3L1 and CHI3L2 can be regarded as mediators of the immune and inflammatory responses and are involved in the progression of degenerative and dismetabolic disorders. The general concept of the chitinases involvement in human diseases discussed in this review may stimulate the development of new planning and experiments leading to a deeper understanding, not only on the biochemical mechanisms inducing chitinases regulation, but also on the consequences that the increases in chitinases levels impact with immunity and autoimmunity in different conditions. The future understanding will lead to the opportunity to develop selective and specific chitinase inhibitors.

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Basic Study

Regulatory T cells suppress autoreactive CD4⁺ T cell response to bladder epithelial antigen

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Abstract

AIM: To investigate the role of regulatory T (Treg) cells in CD4⁺ T cell-mediated bladder autoimmune inflammation.

METHODS: Urothelium-ovalbumin (URO-OVA)/OT-II mice, a double transgenic line that expresses the membrane form of the model antigen (Ag) OVA as a self-Ag on the urothelium and the OVA-specific CD4⁺ T cell receptor specific for the I-A^b/OVA₃₂₃₋₃₃₉ epitope in the periphery, were developed to provide an autoimmune environment for investigation of the role of Treg cells in bladder autoimmune inflammation. To facilitate Treg cell analysis, we further developed URO-OVA^{GFP-Foxp3}/OT-II mice, a derived line of URO-OVA/OT-II mice that express the green fluorescent protein (GFP)-forkhead box protein P3 (Foxp3) fusion protein.

RESULTS: URO-OVA/OT-II mice failed to develop bladder inflammation despite the presence of autoreactive CD4⁺ T cells. By monitoring GFP-positive cells, bladder infiltration of CD4⁺ Treg cells was observed in URO-OVA^{GFP-Foxp3}/OT-II mice. The infiltrating Treg cells were functionally active and expressed Treg cell effector molecule as well as marker mRNAs including transforming growth factor- β , interleukin (IL)-10, fibrinogen-like protein 2, and glucocorticoid-induced tumor necrosis factor receptor (GITR). Studies further revealed that Treg cells from URO-OVA^{GFP-Foxp3}/OT-II mice were suppressive and inhibited autoreactive CD4⁺ T cell proliferation

and interferon (IFN)- γ production in response to OVA Ag stimulation. Depletion of GITR-positive cells led to spontaneous development of bladder inflammation and expression of inflammatory factor mRNAs for IFN- γ , IL-6, tumor necrosis factor- α and nerve growth factor in URO-OVA^{GFP-Foxp3}/OT-II mice.

CONCLUSION: Treg cells specific for bladder epithelial Ag play an important role in immunological homeostasis and the control of CD4⁺ T cell-mediated bladder autoimmune inflammation.

Key words: Bladder; Autoimmunity; Regulatory T cell; CD4⁺ T cells; Antigen

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Core tip: Evidence suggests that autoimmune inflammation may cause interstitial cystitis/bladder pain syndrome (IC/BPS) in subgroups of patients. However, the role of regulatory T (Treg) cells in the control of bladder autoimmunity has not yet been identified. In this study we developed novel transgenic autoimmune cystitis models and demonstrated that Treg cells specific for bladder epithelial Ag play an important role in immunological homeostasis and the control of CD4⁺ T cell-mediated bladder autoimmune inflammation. Our results suggest that loss of functional Treg cells may contribute to IC/BPS pathology in subgroups of patients.

Liu WJ, Luo Y. Regulatory T cells suppress autoreactive CD4⁺ T cell response to bladder epithelial antigen. *World J Immunol* 2016; 6(2): 105-118 Available from: URL: <http://www.wjgnet.com/2219-2824/full/v6/i2/105.htm> DOI: <http://dx.doi.org/10.5411/wji.v6.i2.105>

INTRODUCTION

The mechanisms of autoimmune responses in the urinary bladder have not been well studied. Regulatory T (Treg) cells, a special subset of CD4⁺ T cells, are crucial for immunological homeostasis and play an important role in preventing autoimmune pathogenesis. Predisposition to immunopathology due to loss of functional Treg cells has been observed in numerous autoimmune diseases and animal models^[1]. Studies have shown the involvement of Treg cells in the pathogenesis of bladder carcinoma^[2-4], suggesting the importance of Treg cells in bladder immunosurveillance. Interstitial cystitis/bladder pain syndrome (IC/BPS) is a chronic inflammatory condition of the bladder characterized by pelvic pain, irritative voiding symptoms, and sterile and cytologically normal urine. The etiology of IC/BPS is currently unknown and may involve multiple causes. Although autoimmunity is debated as a potential cause of IC/BPS, clinical evidence

suggests that it may play an important role in the pathophysiology of the disease. It has been reported that IC/BPS patients develop antinuclear and anti-urothelium autoantibodies^[5-11], overexpress urothelial HLA-DR molecules^[12-14], and co-present with other autoimmune diseases such as bronchial asthma, systemic lupus erythematosus, Sjögren's syndrome, rheumatoid arthritis and ulcerative colitis^[15-21]. Considerable data have been published on the histopathology of bladder specimens, demonstrating a role of cell-mediated immune mechanisms in IC/BPS^[14,22]. Hence, autoimmune inflammation may be a component in the pathophysiology of IC/BPS in subgroups of patients. However, despite these observations, the role of Treg cells in bladder autoimmunity has not been identified.

Prior studies on bladder autoimmunity have been based on the use of rodent models of experimental autoimmune cystitis (EAC) in which animals developed bladder inflammation after immunization with urothelial components^[23-28]. These EAC models demonstrated many clinical correlates seen in IC/BPS, offering a unique property for controlled examination of specific aspects of the disease. Using genetic engineering technology, we previously developed a novel transgenic model of EAC (URO-OVA mice) that expresses the membrane form of the model antigen (Ag) ovalbumin (OVA) as a self-Ag on the urothelium and develops bladder inflammation upon introduction of OVA-specific T cells^[29-32]. In addition to the many features of conventional EAC models, the transgenic EAC model demonstrates T cell tolerance, activation and autoimmune responses^[29,32], facilitating the investigation of the mechanisms underlying bladder autoimmune pathogenesis.

To investigate the role of Treg cells in bladder autoimmunity, we established an autoimmune environment through crossbreeding of URO-OVA mice with OT-II mice that expressed the CD4⁺ T cell receptor (TCR) specific for the I-A^b/OVA₃₂₃₋₃₃₉ epitope^[33,34]. To further facilitate the analysis of Treg cells, we generated URO-OVA^{GFP-Foxp3}/OT-II mice that expressed green fluorescent protein (GFP)-fused forkhead box protein P3 (Foxp3), a Treg cell lineage specification factor^[35,36], enabling direct identification of Treg cells based on GFP fluorescence^[37]. By using these transgenic EAC models, we have found that CD4⁺ Treg cells play an important role in immunological homeostasis and the control of bladder autoimmune inflammation.

MATERIALS AND METHODS

Mice

URO-OVA mice [C57BL/6 (B6) genetic background] were previously developed in our laboratory^[29]. B6 mice were obtained from the National Cancer Institute/Frederick Cancer Research Animal Facility (Frederick, MD). OT-II mice (B6 genetic background), a line originally developed by Barnden *et al.*^[33,34], were

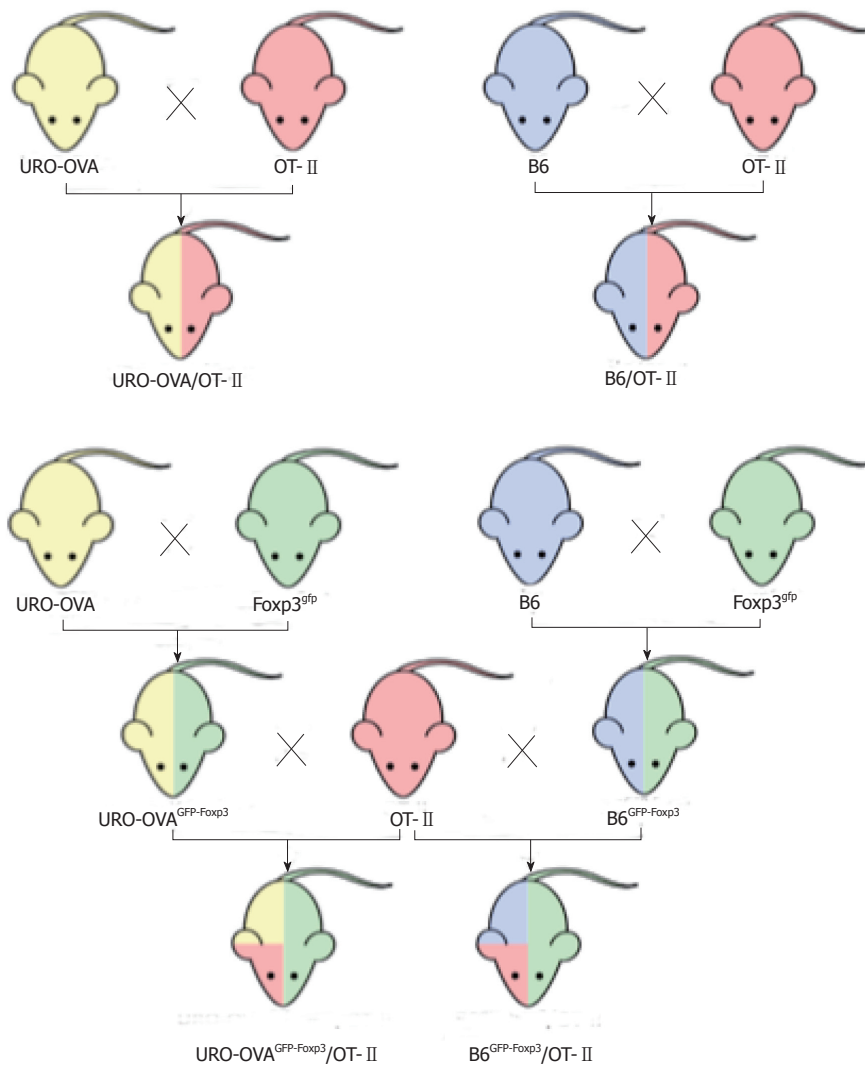


Figure 1 Animal crossbreeding. URO-OVA/OT-II mice were generated through crossbreeding of URO-OVA mice with OT-II mice. B6/OT-II mice were generated through crossbreeding of B6 mice with OT-II mice. URO-OVA^{GFP-Foxp3} mice were generated through crossbreeding of URO-OVA mice with Foxp3^{GFP} mice. B6^{GFP-Foxp3} mice were generated through crossbreeding of B6 mice with Foxp3^{GFP} mice. Both URO-OVA^{GFP-Foxp3} and B6^{GFP-Foxp3} mice were further crossed with OT-II mice to generate URO-OVA^{GFP-Foxp3}/OT-II and B6^{GFP-Foxp3}/OT-II mice, respectively. URO: Urothelium; OVA: Ovalbumin.

obtained from Dr. Ratliff (Purdue Cancer Center, West Lafayette, IN). As shown in Figure 1, URO-OVA/OT-II mice were generated through crossbreeding of URO-OVA mice with OT-II mice and B6/OT-II mice were generated through crossbreeding of B6 mice with OT-II mice, respectively. Foxp3^{GFP} mice, a line developed by Fontenot *et al.*^[37], were obtained from Dr. Rudensky (University of Washington, Seattle, WA). URO-OVA^{GFP-Foxp3} mice were generated through crossbreeding of URO-OVA mice with Foxp3^{GFP} mice (Figure 1), while B6^{GFP-Foxp3} mice were generated through crossbreeding of B6 mice with Foxp3^{GFP} mice (Figure 1). All progeny mice were selected for transgenic OVA by tail genotyping and for GFP-positive CD4⁺ T cells by flow cytometry. Both URO-OVA^{GFP-Foxp3} and B6^{GFP-Foxp3} mice were further crossed with OT-II mice to generate URO-OVA^{GFP-Foxp3}/OT-II and B6^{GFP-Foxp3}/OT-II mice, respectively (Figure 1). Male OT-II mice and their derived mice were used because only

the Y chromosome carries the transgenic CD4⁺ TCR specific for the I-A^b/OVA₃₂₃₋₃₃₉ epitope. Mice were housed in a pathogen-free facility at the University of Iowa Animal Care Facility. All procedures involving animals were reviewed and approved by the University of Iowa Institutional Animal Care and Use Committee.

***In vitro* CD4⁺ T cell response to OVA**

Splenocytes were prepared from OT-II, B6/OT-II and URO-OVA/OT-II mice as described previously^[32], resuspended in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 units/mL of penicillin and 100 µg/mL of streptomycin, and seeded in 96-well plates at a density of 4×10^5 cells/200 µL per well. Cells were cultured in the absence or presence of OVA₂₅₇₋₂₆₄ peptide (10 µg/mL) or OVA₃₂₃₋₃₃₉ peptide (10 µg/mL) for 3 d at 37 °C in a humidified incubator with 5% CO₂. Culture supernatants were then collected and

analyzed for IFN- γ by enzyme-linked immunosorbent assay (ELISA) with paired antibodies (Endogen; clones: R4.6A2 and XMGI.2; Woburn, MA).

***In vitro* Treg cell suppression assay**

OT-II splenocytes were prepared as described previously^[32], resuspended in the above-mentioned culture medium, seeded in 96-well plates at a density of 3×10^5 cells/200 μ L per well, and cultured in the absence or presence of OVA₃₂₃₋₃₃₉ peptide (10 μ g/mL) for 3 d at 37 °C in a humidified incubator with 5% CO₂. To evaluate the effect of Treg cells, OT-II splenocytes were also incubated at a 1:1 ratio with GFP-positive (Foxp3⁺) CD4⁺ T cells sorted from the spleens of URO-OVA^{GFP-Foxp3}/OT-II mice using FACS Aria (BD Biosciences; San Jose, CA). As control, GFP-negative CD4⁺ T cells were collected and incubated with OT-II splenocytes at a 1:1 ratio. Proliferation was assessed by pulsing the cells with 1 μ Ci of [methyl-³H]thymidine (Amersham; Piscataway, NJ) per well for the last 18 h and then assayed for thymidine incorporation by liquid scintillation counting. Culture supernatants from a parallel plate were collected after a 3-d incubation period and analyzed for IFN- γ by ELISA as described above.

***In vivo* Treg cell depletion assay**

Monoclonal antibodies (mAb) specific for CD25 (clone: PC61) and *glucocorticoid-induced tumor* necrosis factor receptor (GITR; clone: DTA-1) were prepared from hybridomas provided by Dr. Ratliff through ammonium sulfate precipitation and protein-A/G affinity chromatography as described previously^[38]. URO-OVA^{GFP-Foxp3}/OT-II mice were injected intraperitoneally (i.p.) with 500 μ g of PC61 or 250 μ g of DTA-1 every other day beginning at 6 wk of age and sacrificed for analysis at 10 wk. The bladders were then collected and processed for histological hematoxylin and eosin (H and E) staining and analysis of inflammatory factor mRNAs by reverse transcriptase-polymerase chain reaction (RT-PCR).

Bladder histological analysis

The standard paraffin-embedded histological sections of the bladder were prepared and stained with H and E solution as described previously^[29-32]. Bladder inflammation was scored in a blinded manner based on cellular infiltration in the lamina propria and interstitial edema as follows: 1+ (mild infiltration with no or mild edema); 2+ (moderate infiltration with moderate edema); 3+ (moderate to severe infiltration with severe edema). Statistical analysis was performed using Student's *t* test with SPSS11.0 software.

Flow cytometric analysis

In various experiments single-cell suspensions of the thymus, spleen, bladder draining lymph nodes (BLNs)

and bladder were prepared by mechanical disruption as described previously^[29,32]. Briefly, cells were washed with staining buffer [1% FBS, 0.09% (w/v) NaN₃ in Mg²⁺ and Ca²⁺ free PBS], stained with a FITC-, PE- or PE-Cy5-labeled antibody (eBioscience, San Diego, CA) to various surface markers including CD4 (clone: RM4-5), CD44 (clone: IM7), CD45RB (clone: C363.16A), CD62L (clone: MEL-14), CD69 (clone: H1.2F3), and OT-II CD4⁺ TCR clonal phenotype V α 2 (clone: B20.1) and V β 5 (clone: MR9-4) at 4 °C for 15 min, fixed in 2% formalin, and analyzed using a FACScan equipped with CellQuest (BD Biosciences). For GFP analysis, the FITC channel was used. Post-acquisition analysis was carried out using FlowJo software (Tree Star, Ashland, OR).

RT-PCR analysis

RT-PCR was used to analyze mRNAs expressed by bladder infiltrating Treg cells and the inflamed bladders of URO-OVA^{GFP-Foxp3}/OT-II mice. Total RNAs were extracted using Qiagen RNeasy Kit (Valencia, CA) from FACS-sorted bladder infiltrating CD4⁺ T cells (both GFP positive and negative cells) and the bladders of mice untreated or treated with depleting mAbs. Three microgram of total RNAs were used for cDNA synthesis using Invitrogen Superscript III RNase H Reverse Transcriptase (Carlsbad, CA) and oligo dT according to the manufacturer's instructions. Two microlitre of the cDNA products were further processed for PCR amplification using sequence-specific primer pairs and Invitrogen Taq DNA polymerase. The following primer pairs were used: 5'-agccgaagcgactactat-3' and 5'-agccgtgtattccgtctct-3' for transforming growth factor (TGF)- β (357 bp); 5'-tgctgtcttactgactgg-3' and 5'-gctcactgctgtcttcta-3' for interleukin (IL)-10 (397 bp); 5'-tcaacagtttgatggcaag-3' and 5'-ctgccgtgcattgtagtta-3' for FGL2 (468 bp); 5'-tgagtgctcatgctctgtg-3' and 5'-atcctcagctgacaactgcac-3' for GITR (583 bp); 5'-cgctacacactgcatcttg-3' and 5'-aaattcaaatagtgctggcaga-3' for interferon (IFN)- γ (522 bp); 5'-ctgatgctgggtgacaaccac-3' and 5'-gccactccttctgtgactcc-3' for IL-6 (505 bp); 5'-gtccccaaggatgagaag-3' and 5'-aagtagacctgccggactc-3' for tumor necrosis factor (TNF)- α (520 bp); 5'-agtgtcagtgtgtgggttg-3' and 5'-gccttgacgaaggtgtgagt-3' for nerve growth factor (NGF; 218 bp); and 5'-agcttgctcatcaacggaag-3' and 5'-gtcttctgggtggcagtgat-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 364 bp). PCR cycle numbers were initially optimized to achieve desirable discrepancies between the experimental groups. PCR was then performed for GAPDH with 30 cycles, IFN- γ , TNF- α and NGF with 36 cycles, and other molecules with 40 cycles. The cycling condition consisted of denaturing at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 1 min. DNA fragments were run on a 1% agarose gel, stained with ethidium bromide, and imaged by EpiChemi digital image system (Upland, CA).

Statistical analysis

Statistical analysis was performed using two-tailed Student's *t*-test with SPSS11.0 software. *P* < 0.05 was considered statistically significant.

RESULTS

Constitutive expression of urothelial OVA causes clonal deletion of OVA-specific CD4⁺ T cells in URO-OVA/OT-II mice

URO-OVA/OT-II mice (F1 generation), a crossed line of URO-OVA mice with OT-II mice, expressed self-Ag OVA on the urothelium and the TCR (V α 2V β 5) specific for I-A^b/OVA₃₂₃₋₃₃₉ epitope on CD4⁺ T cells. URO-OVA/OT-II mice showed T cell tolerance in potentially autoreactive OVA-specific CD4⁺ T cells. Compared to control B6/OT-II mice (F1 generation) that expressed the same OVA-specific CD4⁺ TCR but no urothelial OVA, URO-OVA/OT-II mice showed severe reduction in CD4⁺V α 2⁺ cells, CD4⁺V β 5⁺ cells, and V α 2⁺V β 5⁺ cells in the thymus (Figure 2, top panel; 1% vs 25% for all 3 populations). The severe population reduction was also observed in the spleen (Figure 2, middle panel; 4% vs 19% for CD4⁺V α 2⁺ cells, 1% vs 19% for CD4⁺V β 5⁺ cells, and 2% vs 18% for V α 2⁺V β 5⁺ cells) and the BLNs (Figure 2, bottom panel; 7% vs 55% for CD4⁺V α 2⁺ cells, 3% vs 50% for CD4⁺V β 5⁺ cells, and 9% vs 43% for V α 2⁺V β 5⁺ cells). However, this population reduction was incomplete, suggesting the presence of additional regulatory mechanism(s) in the control of autoreactive CD4⁺ T cells in URO-OVA/OT-II mice.

Deletion-escaped OVA-specific CD4⁺ T cells are responsive to OVA and gain activation in URO-OVA/OT-II mice

We next investigated whether OVA-specific CD4⁺ T cells that had escaped from clonal deletion retained OT-II CD4⁺ T cell responsiveness to OVA. Splenocytes were prepared from URO-OVA/OT-II mice and incubated with OVA₃₂₃₋₃₃₉ peptide specific for the OT-II CD4⁺ TCR for 3 d *in vitro*. Cells were also incubated with OVA₂₅₇₋₂₆₄ peptide as control. Splenocytes from age-matched OT-II and B6/OT-II mice were included for comparison. As expected, cells from both OT-II and B6/OT-II mice produced similar levels of IFN- γ in response to OVA₃₂₃₋₃₃₉ peptide stimulation (Figure 3). Interestingly, cells from URO-OVA/OT-II mice also produced IFN- γ in response to OVA₃₂₃₋₃₃₉ peptide stimulation (*P* < 0.001), although the level was 2-3 fold less than those of OT-II and B6/OT-II cells. This reduced IFN- γ production suggested that the autoreactivity of OVA-specific CD4⁺ T cells was compromised in URO-OVA/OT-II mice. However, despite the reduction of autoreactivity, OVA-specific CD4⁺ T cells gained activation *in vivo*. Compared to B6/OT-II mice, CD4⁺ T cells from the BLNs of URO-OVA/OT-II mice showed up-regulated expressions of CD44 and CD69 and down-regulated expressions of CD45RB and CD62L

(Figure 4). In addition, the bladders of URO-OVA/OT-II mice contained 6-15 fold more infiltrating CD4⁺, V α 2⁺ and V β 5⁺ cells than those of B6/OT-II mice (Figure 5A). Further analysis revealed that the majority of bladder infiltrating CD4⁺ T cells were V α 2⁺ and V β 5⁺ cells (Figure 5B), suggesting that they were OT-II CD4⁺ T cells. These observations indicated that endogenous OVA-specific CD4⁺ T cells retained the ability to respond to self-Ag OVA, gained activation in the BLNs, and infiltrated into the bladders in URO-OVA/OT-II mice. Interestingly, despite T cell activation and bladder infiltration, URO-OVA/OT-II mice developed no bladder histopathology, further suggesting the presence of additional regulatory mechanism(s) in these mice.

Bladder infiltrating CD4⁺ T cells consist of Treg cells in URO-OVA^{GFP-Foxp3}/OT-II mice

Since URO-OVA/OT-II mice contained activated OVA-specific CD4⁺ T cells but failed to develop bladder inflammation, we hypothesized that Treg cells might play an important role in the control of autoreactive CD4⁺ T cells in these mice. To facilitate the analysis of Treg cells, we crossed URO-OVA mice with Foxp3^{gfp} mice, a Foxp3^{gfp} allele knock-in line that expresses GFP-fused Foxp3^[36], to generate URO-OVA^{GFP-Foxp3} mice. As control, B6^{GFP-Foxp3} mice were generated in parallel. To investigate the role of Treg cells in bladder autoimmunity, we further crossed URO-OVA^{GFP-Foxp3} mice with OT-II mice to generate URO-OVA^{GFP-Foxp3}/OT-II mice. As control, B6^{GFP-Foxp3}/OT-II mice were generated through crossbreeding of B6^{GFP-Foxp3} mice with OT-II mice. Similar to URO-OVA/OT-II mice, URO-OVA^{GFP-Foxp3}/OT-II mice showed severe but incomplete reduction in OVA-specific CD4⁺ T cell population in the thymus, spleen and BLNs compared to B6^{GFP-Foxp3}/OT-II mice (data not shown). Also, similar to the bladders of URO-OVA/OT-II mice, the bladders of URO-OVA^{GFP-Foxp3}/OT-II mice showed increased infiltrating CD4⁺ T cells compared to B6^{GFP-Foxp3}/OT-II mice (Figure 6A). However, like URO-OVA/OT-II mice, URO-OVA^{GFP-Foxp3}/OT-II mice developed no bladder histopathology.

Analysis of bladder infiltrating CD4⁺ T cells revealed an increased number of GFP-positive (Foxp3⁺) cells in URO-OVA^{GFP-Foxp3}/OT-II mice compared to B6^{GFP-Foxp3}/OT-II mice (Figure 6B). Further analysis of bladder infiltrating CD4⁺ T cells in URO-OVA^{GFP-Foxp3}/OT-II mice indicated that the majority of the cells were GFP positive (Foxp3⁺) cells (Figure 7A; 64% vs 36%). These GFP-positive (Foxp3⁺) CD4⁺ T cells were functionally active, as they expressed increased CD44 and CD69 and decreased CD45RB and CD62L compared to GFP-negative (Foxp3⁻) CD4⁺ T cells (Figure 7B). Consistently, these GFP-positive (Foxp3⁺) CD4⁺ T cells expressed increased levels of Treg cell effector molecule TGF- β , IL-10 and FGL2 mRNAs and Treg cell marker GITR mRNA compared to GFP-negative (Foxp3⁻) CD4⁺ T cells (Figure 7C). These observations suggested that Treg cells were actively involved in bladder autoimmune responses in URO-OVA^{GFP-Foxp3}/OT-II mice.

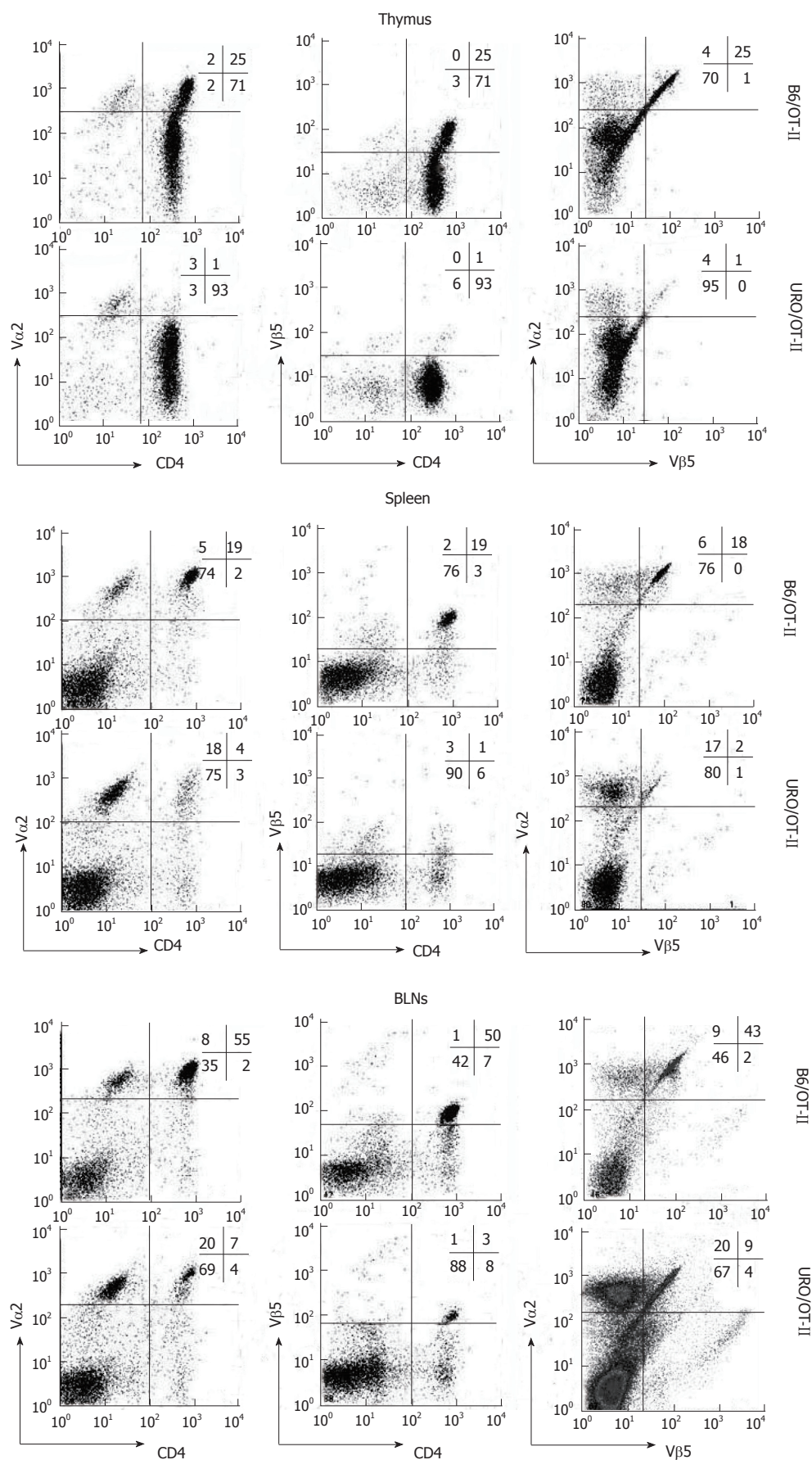


Figure 2 Clonal deletion of OT-II CD4⁺ T cells in urothelium-ovalbumin/OT-II mice. Cells from the thymus (top panel), spleen (middle panel), and BLNs (bottom panel) of URO-OVA/OT-II mice (8 wk) were analyzed for surface CD4, Vα2 and Vβ5 by flow cytometry. Age-matched B6/OT-II mice were included for comparison. Gate was set on lymphocytes according to scatter criteria. Percentages of single- and double-positive cells are indicated. Results are representative of 3 separate experiments consisting of 4-6 mice per group. URO/OT-II: Urothelium-ovalbumin/OT-II mice.

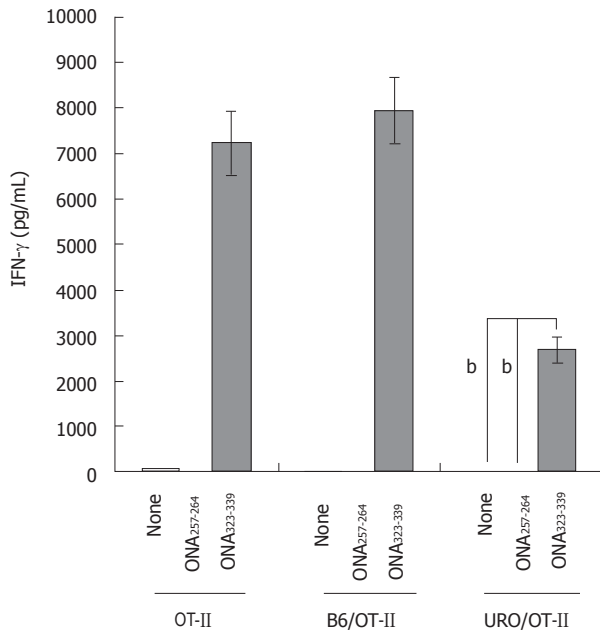


Figure 3 Deletion-escaped OT-II CD4⁺ T cells retain responsiveness to ovalbumin. Splenocytes from URO-OVA/OT-II mice (8 wk) were cultured alone or in the presence of OVA₂₅₇₋₂₆₄ peptide (10 μg/mL) or OVA₃₂₃₋₃₃₉ peptide (10 μg/mL) for 3 d, followed by ELISA analysis of IFN-γ production in culture supernatants. Splenocytes from age-matched OT-II and B6/OT-II mice were included for comparison. Data are presented as the mean ± SD from duplicate determinations. ^b*P* < 0.001 compared with non-stimulated or OVA₂₅₇₋₂₆₄-stimulated splenocytes (two-tailed Student's *t* test). URO/OT-II: Urothelium-ovalbumin/OT-II mice.

Treg cells from URO-OVA^{GFP-Foxp3}/OT-II mice are suppressive to OVA-specific CD4⁺ T cells

To determine whether Treg cells found in URO-OVA^{GFP-Foxp3}/OT-II mice were suppressive, we prepared GFP-positive (Foxp3⁺) CD4⁺ T cells from the spleens of URO-OVA^{GFP-Foxp3}/OT-II mice. GFP-negative (Foxp3⁻) CD4⁺ T cells were prepared for comparison. The purity of both cell types was > 95%. Responder OT-II splenocytes were incubated with or without OVA₃₂₃₋₃₃₉ peptide in the presence or absence of GFP-positive (Foxp3⁺) or GFP-negative (Foxp3⁻) CD4⁺ T cells at a 1:1 ratio for 3 d *in vitro*, followed by analysis of cell proliferation and IFN-γ production (Figure 8). Compared to OT-II cells incubated with OVA₃₂₃₋₃₃₉ peptide alone, OT-II cells incubated with OVA₃₂₃₋₃₃₉ peptide in the presence of CD4⁺Foxp3⁺ cells showed similar high levels of proliferation and IFN-γ production. However, when incubated with OVA₃₂₃₋₃₃₉ peptide in the presence of CD4⁺Foxp3⁺ cells, OT-II cells showed significantly reduced levels of proliferation (*P* < 0.001) and IFN-γ production (*P* < 0.05). These observations indicated that CD4⁺ Treg cells were suppressive, suggesting their importance in the control of bladder autoimmunity in URO-OVA^{GFP-Foxp3}/OT-II mice.

Depletion of CD4⁺ Treg cells results in spontaneous development of bladder autoimmune inflammation in URO-OVA^{GFP-Foxp3}/OT-II mice

To determine whether CD4⁺ Treg cells played an

Table 1 Summary of bladder histological inflammation

	Bladder histologic score ^b			
	-	+	++	+++
Anti-CD25 (<i>n</i> = 12)	10	2	0	0
Anti-GITR (<i>n</i> = 12)	1	3	6	2

^b*P* < 0.001 compared between two groups (two-tailed Student's *t* test). GITR: Glucocorticoid-induced tumor necrosis factor receptor.

inhibitory role in bladder autoimmune inflammation, we depleted CD25⁺ cells or GITR⁺ cells in URO-OVA^{GFP-Foxp3}/OT-II mice. Mice were injected i.p. with anti-CD25 mAb (PC61) or anti-GITR mAb (DTA-1) every other day beginning at 6 wk and sacrificed for analysis at 10 wk. Depletion of CD4⁺ Treg cells was verified by flow cytometric analysis of splenocytes showing the lack of GFP-positive (Foxp3⁺) CD4⁺ T cells. Interestingly, depletion of CD25⁺ cells led to the development of bladder histopathology in only 2 of 12 mice (score: +), whereas depletion of GITR⁺ cells led to the development of bladder histopathology in 11 of 12 mice (score: + for 3 bladders, ++ for 6 bladders, and +++ for 2 bladders) (Table 1 and Figure 9A, *P* < 0.001). Consistently, the bladders of mice treated with anti-GITR mAb expressed increased levels of IFN-γ, IL-6, TNF-α and NGF mRNAs compared to the bladders of mice treated with anti-CD25 mAb (Figure 9B). Indeed, the latter bladders showed no clear increase in the mRNA expressions compared to the bladders of non-treated mice. These observations indicated that depletion of GITR⁺ cells but not CD25⁺ cells resulted in spontaneous development of bladder inflammation in URO-OVA^{GFP-Foxp3}/OT-II mice.

DISCUSSION

The role of Treg cells in bladder autoimmunity has not been identified due to the lack of a proper animal model. In this study we used transgenic EAC models to investigate the role of Treg cells and found that CD4⁺ Treg cells played an important role in the control of bladder autoimmune inflammation. Acquisition of autoreactive CD4⁺ T cells was not sufficient to cause bladder inflammation; however, depletion of CD4⁺ Treg cells led to spontaneous development of bladder inflammation in the transgenic EAC models.

We generated URO-OVA/OT-II mice to investigate bladder autoimmunity, because CD4⁺ T cells are preferentially induced in IC/BPS compared to CD8⁺ T cells^[14,22,39-41]. The ability of OT-II CD4⁺ T cells to induce bladder inflammation was previously demonstrated in URO-OVA mice^[32]. To facilitate the analysis of Treg cells, we generated URO-OVA^{GFP-Foxp3} mice that expressed the GFP-Foxp3 fusion protein. We further crossed URO-OVA and URO-OVA^{GFP-Foxp3} mice with OT-II mice to establish an autoimmune environment in mice. Constitutive expression of urothelial OVA resulted in clonal deletion

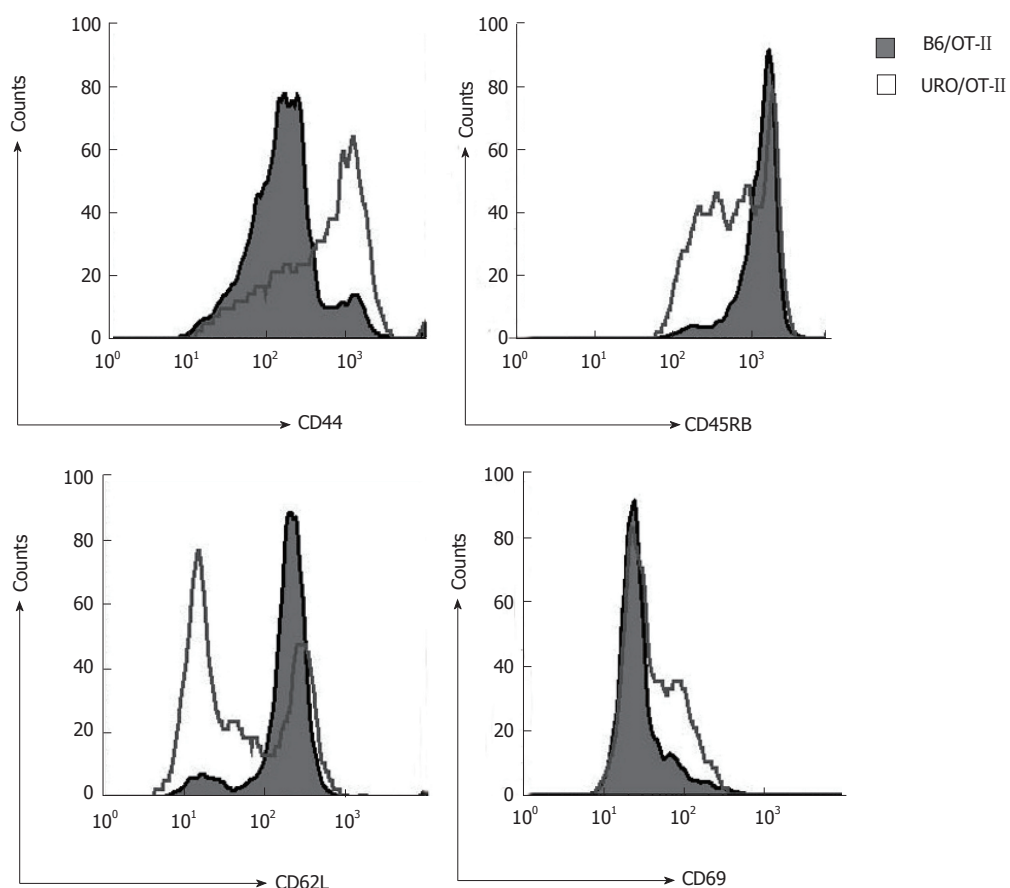
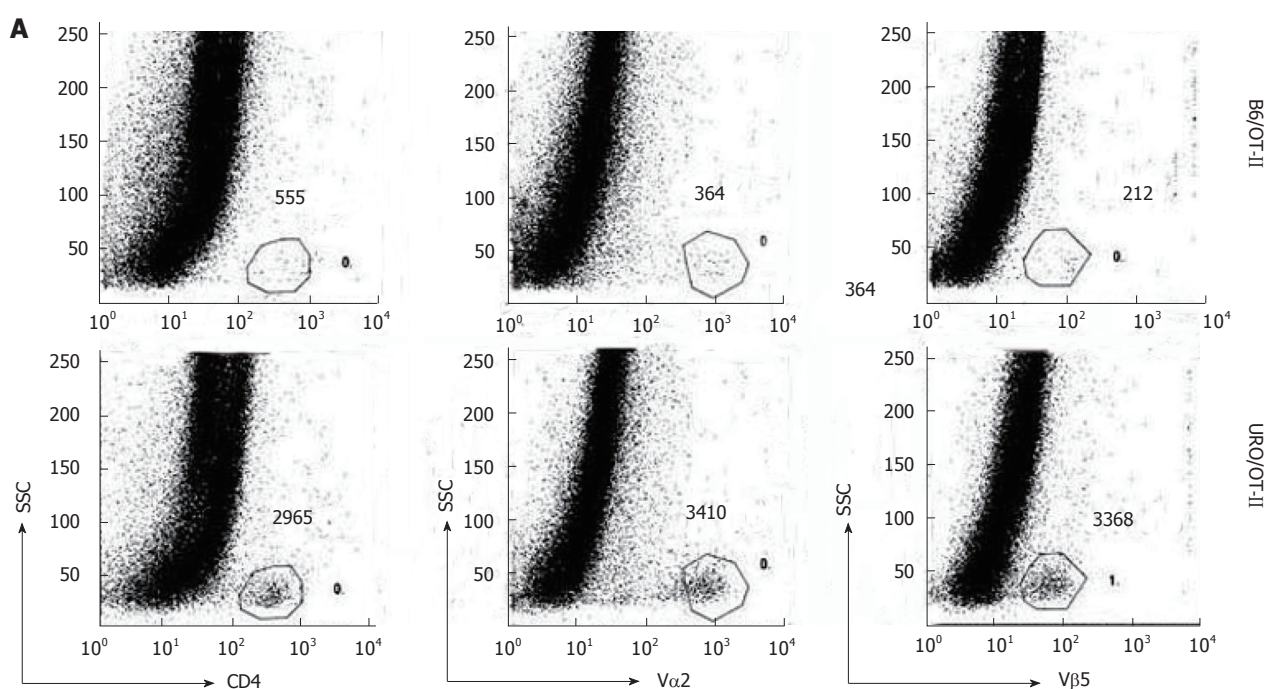


Figure 4 OT-II CD4⁺ T cells gain activation in the bladder draining lymph nodes of urothelium-ovalbumin/OT-II mice. BLN cells of URO-OVA/OT-II mice (8 wk) were analyzed for surface CD44, CD45RB, CD62L, and CD69 by flow cytometry. Age-matched B6/OT-II mice were included for comparison. Gate was set on CD4⁺ T cells. Results are representative of 3 separate experiments consisting of 5 mice per group. Filled histograms: B6/OT-II mice; Gray line histograms: URO-OVA/OT-II mice. URO/OT-II: Urothelium-ovalbumin/OT-II mice; BLN: Bladder draining lymph nodes.



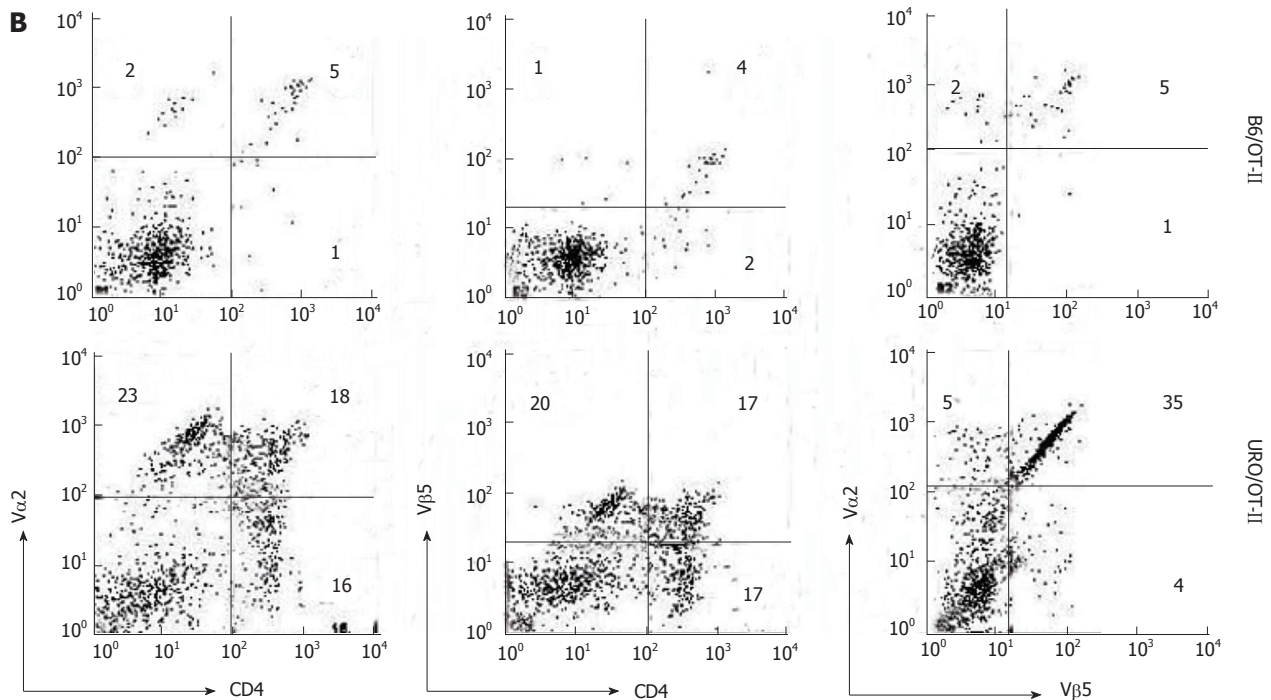


Figure 5 OT-II CD4⁺ T cells infiltrate into the bladders of urothelium-ovalbumin/OT-II mice. Bladder single-cell suspensions were prepared from URO-OVA/OT-II mice (8 wk) and analyzed for surface CD4, Vα2 and Vβ5 by flow cytometry. Age-matched B6/OT-II mice were included for comparison. Gate was set on lymphocytes according to scatter criteria. Total numbers of CD4⁺, Vα2⁺ and Vβ5⁺ cells per bladder are indicated in (A) and percentages of single- and double-positive cells per bladder indicated in (B). Results are representative of 3 separate experiments consisting of 5-8 mice per group. URO/OT-II: Urothelium-ovalbumin/OT-II mice.

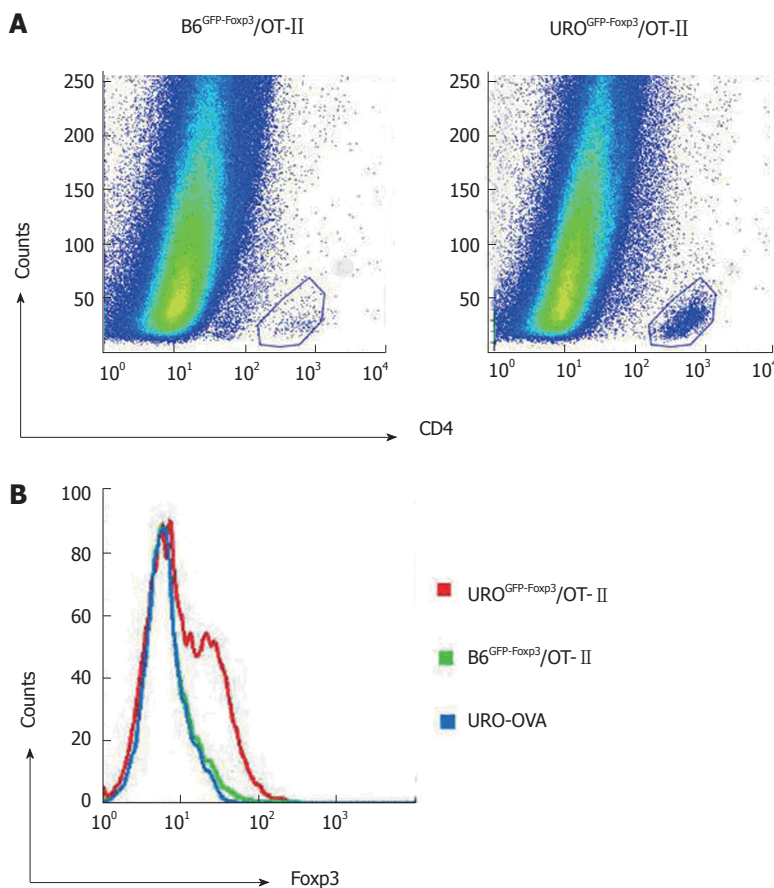


Figure 6 Bladder infiltrating CD4⁺ T cells consist of Treg cells in urothelium-ovalbumin^{GFP-Foxp3}/OT-II mice. Bladder single-cell suspensions were prepared from URO-OVA^{GFP-Foxp3}/OT-II mice (8 wk) and analyzed by flow cytometry. Age-matched B6^{GFP-Foxp3}/OT-II mice were included for comparison. A: Flow cytometric analysis of bladder infiltrating CD4⁺ T cells. Gate was set on lymphocytes according to scatter criteria; B: Flow cytometric analysis of bladder infiltrating GFP-positive CD4⁺ T cells (*i.e.*, Foxp3⁺CD4⁺ T cells). Gate was set on CD4⁺ T cells. Results are representative of 3 separate experiments consisting of 6 mice per group. URO^{GFP-Foxp3}/OT-II: Urothelium-ovalbumin^{GFP-Foxp3}/OT-II mice.

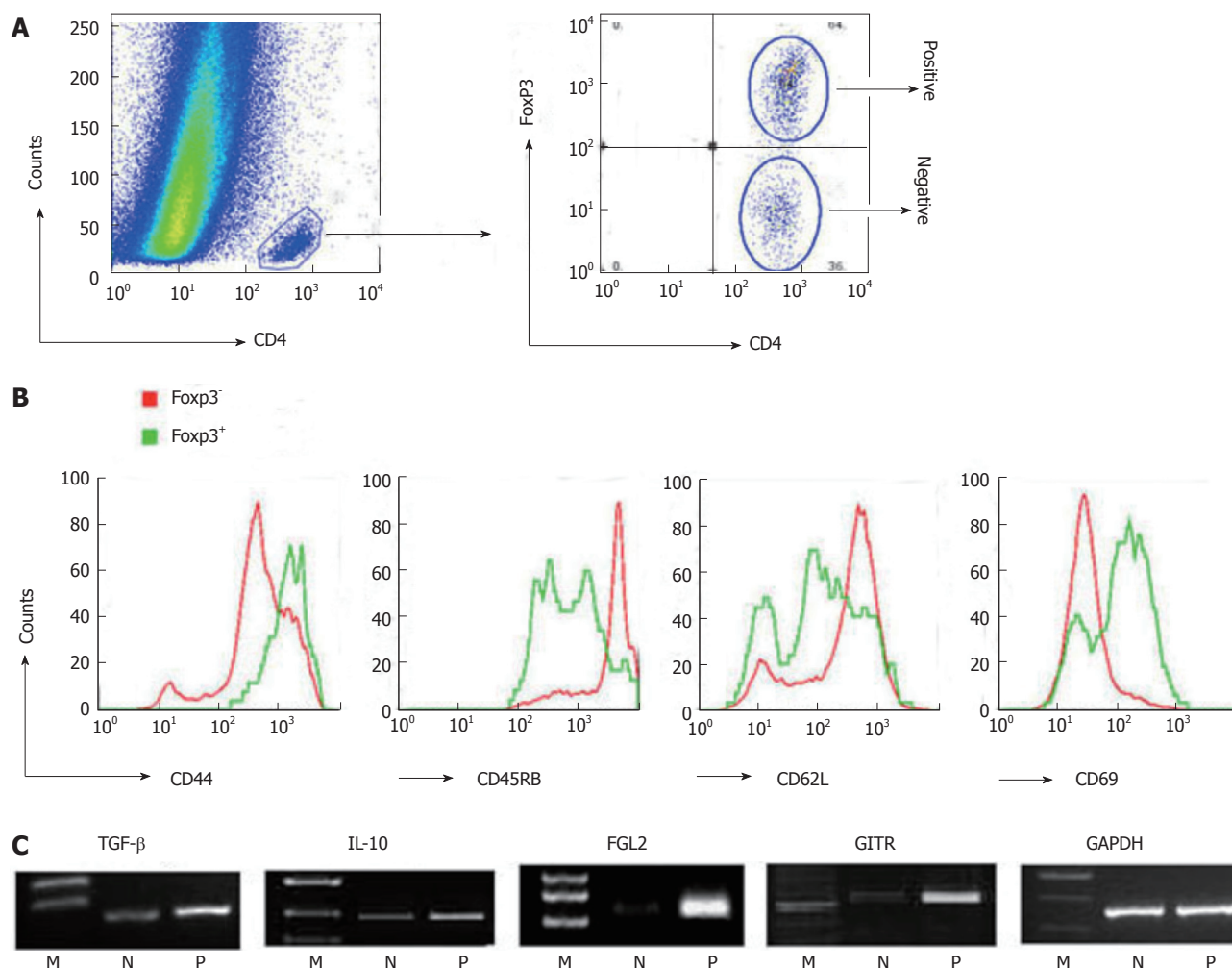


Figure 7 Bladder infiltrating CD4⁺ Treg cells are functionally active and express inhibitory effector molecules in urothelium-ovalbumin^{GFP-Foxp3}/OT-II mice. Bladder single-cell suspensions were prepared from URO-OVA^{GFP-Foxp3}/OT-II mice (8 wk) and analyzed by flow cytometry or sorted for GFP-positive (Foxp3⁺) and GFP-negative (Foxp3⁻) CD4⁺ T cells. A: Bladder infiltrating CD4⁺ T cells consist of both GFP-positive (Foxp3⁺) and GFP-negative (Foxp3⁻) populations by flow cytometry. Gate was set on lymphocytes according to scatter criteria; B: Flow cytometric analysis of surface CD44, CD45RB, CD62L and CD69 on bladder infiltrating GFP-positive (Foxp3⁺) and GFP-negative (Foxp3⁻) CD4⁺ T cells. Gate was set on CD4⁺ T cells; C: RT-PCR analysis of TGF- β , IL-10, FGL2 and GITR mRNAs in bladder infiltrating GFP-positive (Foxp3⁺) and GFP-negative (Foxp3⁻) CD4⁺ T cells. GAPDH was used as an internal control. M: Marker; N: GFP-negative (Foxp3⁻) CD4⁺ T cells; P: GFP-positive (Foxp3⁺) CD4⁺ T cells; URO^{GFP-Foxp3}/OT-II: Urothelium-ovalbumin^{GFP-Foxp3}/OT-II mice; RT-PCR: Reverse transcriptase-polymerase chain reaction; TGF: Transforming growth factor; GITR: Glucocorticoid-induced tumor necrosis factor receptor.

of autoreactive CD4⁺ T cells in both URO-OVA/OT-II and URO-OVA^{GFP-Foxp3}/OT-II mice. However, this clonal deletion was incomplete, as a tiny population of autoreactive CD4⁺ T cells was observed in both central and peripheral compartments. Such incomplete clonal deletion of autoreactive T cells has been observed in our previously reported autoimmune cystitis model (URO-OVA/OT-I mice)^[29] and others' organ-specific transgenic inflammation models^[42-44].

Due to urothelial OVA expression, deletion-escaped OVA-specific CD4⁺ T cells gained activation in the BLNs and infiltrated into the bladders in URO-OVA/OT-II mice. These observations suggested that bladder urothelial OVA was antigenic and could access the immune system for CD4⁺ T cell activation. However, despite the CD4⁺ T cell activation and bladder infiltration, URO-OVA/OT-II mice developed no bladder inflammation. This observation differed from our previous observation

in URO-OVA/OT-I mice as these mice spontaneously developed bladder inflammation at 10 wk of age^[29,31]. This discrepancy might be attributed to differential expression levels of I-A^b vs H2-K^b on the bladder urothelium, which directly influences Ag recognition by autoreactive CD4⁺ and CD8⁺ T cells, respectively. Alternatively, the presence of different numbers of deletion-escaped autoreactive T cell subsets might lead to different autoimmune responses in these mice. However, despite this discrepancy, Treg cells appeared to play a predominant role in the control of bladder autoimmune responses. To support this, splenocytes from URO-OVA/OT-II mice showed a substantially reduced ability to produce IFN- γ in response to OVA₃₂₃₋₃₃₉ peptide stimulation *in vitro*, suggesting that the autoreactivity of OVA-specific CD4⁺ T cells was greatly compromised in these mice. Also, depletion of Treg cells *in vivo* by anti-GITR mAb has been observed

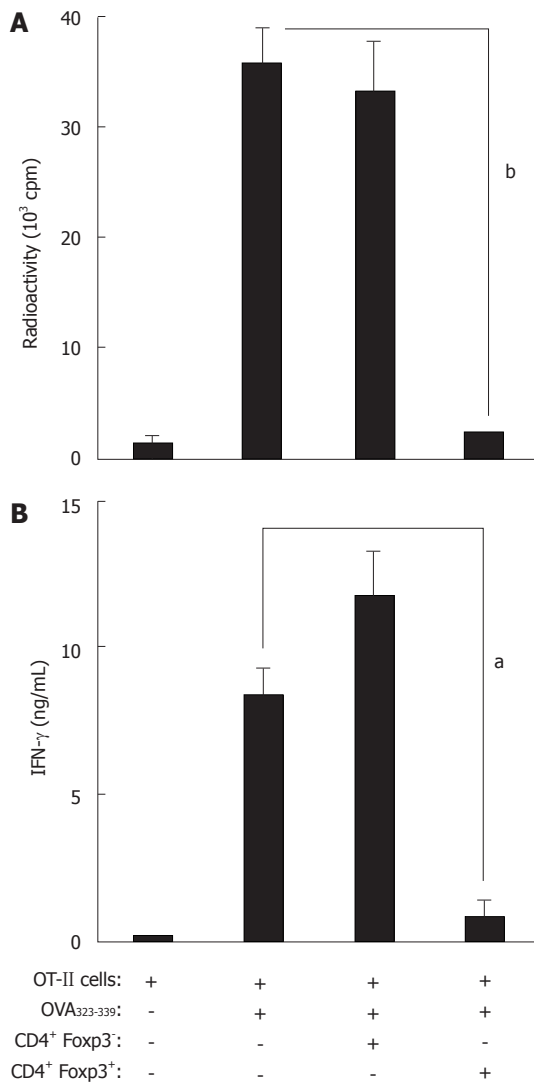


Figure 8 Treg cells from urothelium-ovalbumin^{GFP-Foxp3}/OT-II mice are suppressive to ovalbumin-specific CD4⁺ T cells. **A:** OT-II splenocytes were incubated alone or in the presence of OVA₃₂₃₋₃₃₉ peptide (10 μ g/mL), GFP-positive (Foxp3⁺) CD4⁺ T cells (at a 1:1 ratio), and/or GFP-negative (Foxp3⁻) CD4⁺ T cells (at a 1:1 ratio) sorted from URO-OVA^{GFP-Foxp3}/OT-II mice for 3 d. Proliferation was assessed by labeling the cultures with 3H-thymidine for the final 18 h. Data are presented as the mean $\pm$ SD from triplicate cultures. ^b*P* < 0.001 compared with OT-II cells stimulated with OVA₃₂₃₋₃₃₉ peptide alone (two-tailed Student's *t* test); **B:** Culture supernatants from a parallel plate were collected after 3-d incubation and analyzed for IFN- γ by ELISA. Data are presented as the mean $\pm$ SD from duplicate cultures. ^a*P* < 0.05 compared with OT-II cells stimulated with OVA₃₂₃₋₃₃₉ peptide alone (two-tailed Student's *t* test). URO^{GFP-Foxp3}/OT-II: Urothelium-ovalbumin^{GFP-Foxp3}/OT-II mice.

to result in spontaneous development of bladder inflammation in URO-OVA^{GFP-Foxp3}/OT-II mice. Therefore, Treg cells appeared to counteract autoreactive CD4⁺ T cells for the induction of bladder inflammation in these mice. However, our observations cannot exclude the possibility that other cell types with regulatory activities may contribute to the control of bladder autoimmune responses, since the bladders of mice depleted of GITR⁺ cells showed varying degrees of inflammation.

By monitoring GFP for Foxp3⁺ cells we observed a considerable number of CD4⁺Foxp3⁺ T cells, along

with CD4⁺Foxp3⁺ T cells, in the bladders of URO-OVA^{GFP-Foxp3}/OT-II mice. Compared to CD4⁺Foxp3⁺ T cells, CD4⁺Foxp3⁺ T cells expressed increased levels of Treg cell effector molecule TGF- β , IL-10 and FGL2 mRNAs as well as Treg cell marker GITR mRNA. In addition, CD4⁺Foxp3⁺ T cells exhibited an activated phenotype with up-regulated expressions of CD44 and CD69 and down-regulated expressions of CD45RB and CD62L. Such Treg cell activation *in vivo* has been observed in other animal models^[45,46]. Moreover, we have observed the inhibitory effect of CD4⁺Foxp3⁺ T cells on OVA-specific CD4⁺ T cells in co-culture assays and the spontaneous development of bladder inflammation in URO-OVA^{GFP-Foxp3}/OT-II mice after depletion of GITR⁺ cells. All these observations support the important role of CD4⁺ Treg cells in the control of bladder autoimmune responses in the transgenic EAC models.

As direct evidence for the role of Treg cells in the control of bladder autoimmunity, URO-OVA^{GFP-Foxp3}/OT-II mice spontaneously developed bladder inflammation after depletion of GITR⁺ cells. Interestingly, mice depleted of CD25⁺ cells failed to develop clear bladder inflammation. This phenomenon might result from the elimination of CD25-expressing autoreactive CD4⁺ T cells, together with CD4⁺CD25⁺ Treg cells, by anti-CD25 mAb (PC61). Our observation was consistent with previous studies demonstrating that anti-GITR mAb (DTA-1) but not PC61 was effective in the control of cancer in diverse animal models^[47-49]. These studies revealed the differential activities of DTA-1 and PC61, *i.e.*, DTA-1 specifically depleted Treg cells whereas PC61 depleted both CD25⁺ effector T cells and Treg cells. In addition, studies have also shown that DTA-1 co-stimulates conventional effector T cells while disabling Treg cells^[50,51].

The origin of CD4⁺ Treg cells in URO-OVA^{GFP-Foxp3}/OT-II mice is unknown. It is generally accepted that naturally-occurring Treg cells specific for self-Ag presented by the thymic epithelium are positively selected in the thymus and then colonize in secondary lymphoid organs^[52-55]. It has also been shown that peripheral CD4⁺CD25⁻ naïve T cells can be converted into CD4⁺CD25⁺ Treg cells under certain circumstances^[56-58]. In the presence of a physiologically low level of cognate self-Ag, resting autoreactive Treg cells can gain activation in the draining lymph nodes and then enter circulation^[45,59]. Therefore, it is possible that in the transgenic EAC models the urothelial self-Ag OVA is transported to the BLNs and presented to OVA-specific CD4⁺ Treg cells as well as effector CD4⁺ T cells by Ag-presenting cells. This Ag presentation activates both autoreactive CD4⁺ T cell types, leading to proliferation in the BLNs and infiltration into the bladders. However, because of the co-presence of Treg cells *in situ*, effector CD4⁺ T cells are suppressed and cause no bladder inflammation. This assumption is supported by our observations that URO-OVA^{GFP-Foxp3}/OT-II mice spontaneously develop bladder inflammation after depletion of GITR⁺ cells. The origin of Treg cells

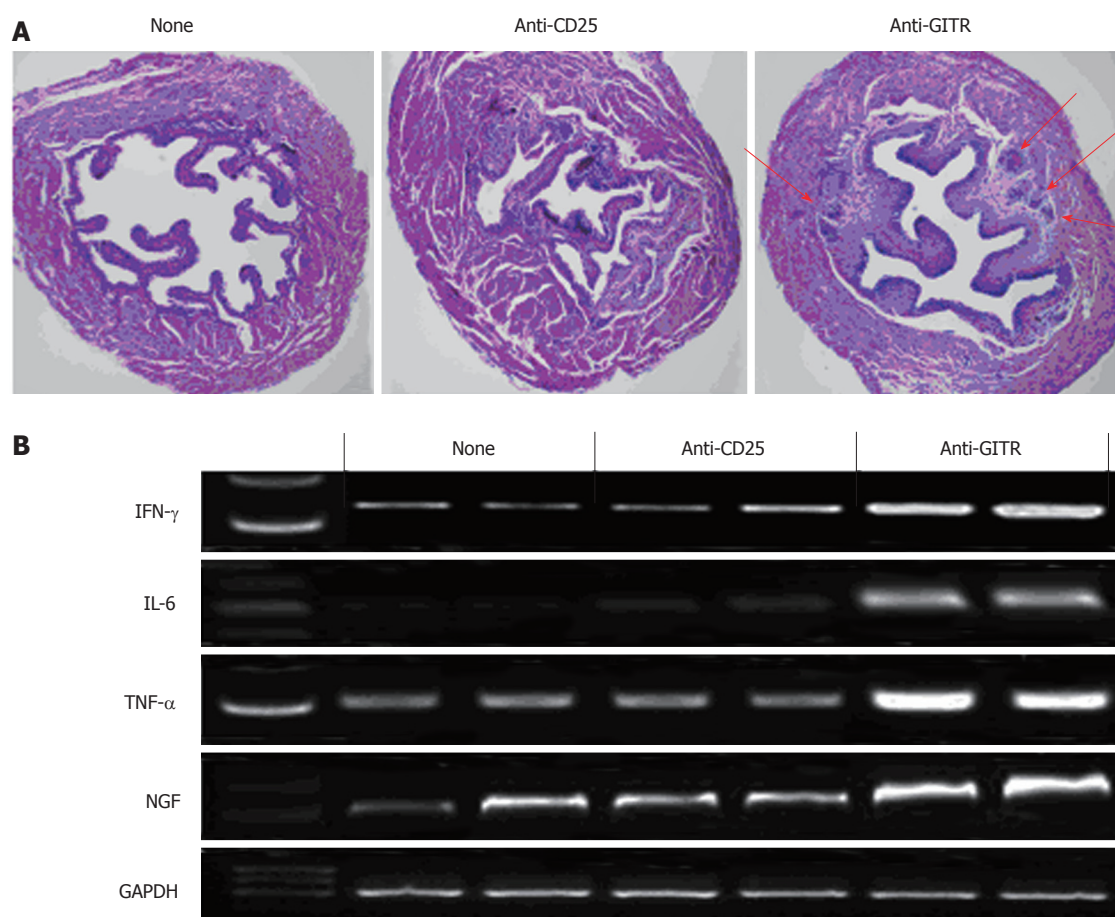


Figure 9 Depletion of CD4⁺ Treg cells results in bladder autoimmune inflammation in urothelium-ovalbumin^{GFP-Foxp3}/OT-II mice. URO-OVA^{GFP-Foxp3}/OT-II mice were treated with anti-CD25 or anti-GITR mAb every other day beginning at 6 wk of age and sacrificed for analysis at 10 wk. A: Bladder histological H and E staining. The slides are representative of 12 bladders for each of anti-CD25 and anti-GITR mAb treated groups. Cellular infiltration is indicated by red arrows. The bladder of an untreated mouse is included for comparison. The summary of bladder histological inflammation is shown in Table 1; B: RT-PCR analysis of IFN- γ , IL-6, TNF- α and NGF mRNA expressions in the bladders of mice treated with anti-CD25 or anti-GITR mAb. GAPDH was used as an internal control. The bladders from untreated mice are included for comparison. URO^{GFP-Foxp3}/OT-II: Urothelium-ovalbumin^{GFP-Foxp3}/OT-II mice; RT-PCR: Reverse transcriptase-polymerase chain reaction; IFN: Interferon; TNF: Tumor necrosis factor; NGF: Nerve growth factor.

and the mechanisms underlying Treg cell action are interesting topics in bladder autoimmunity research and warrant further investigation.

In summary, we have demonstrated that CD4⁺ Treg cells play an important role in immunological homeostasis and the control of bladder autoimmune inflammation in the transgenic EAC models. This study, together with our previous studies^[29,32], sheds light on the cellular mechanisms of bladder autoimmunity. Clear understanding of bladder autoimmune responses will add to future development of novel therapies for bladder inflammatory diseases that contain an autoimmune component in the pathophysiology such as IC/BPS in subgroups of patients.

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COMMENTS

Background

Interstitial cystitis/bladder pain syndrome (IC/BPS) is one of the most refractory diseases in urology today. Since the etiology of IC/BPS remains elusive, current treatments are largely empirical, often dissatisfactory, and vary in efficacy. Therefore, effort to identify the mechanisms of the disease for therapeutic development is greatly needed. Evidence suggests that autoimmune inflammation may cause IC/BPS in subgroups of patients. However, the role of Treg cells in immunological homeostasis and the control of bladder autoimmune inflammation has not yet been identified.

Research frontiers

Rodent models of experimental autoimmune cystitis (EAC) have been actively used in IC/BPS research for identifying the importance of bladder autoimmunity in the disease pathology.

Innovations and breakthroughs

This is the first study demonstrating that Treg cells specific for bladder epithelial Ag play an important role in immunological homeostasis and the control of CD4⁺ T cell-mediated bladder autoimmune inflammation.

Applications

The authors have demonstrated the presence of Treg cells in the developed

transgenic EAC models. The authors have also demonstrated that depletion of Treg cells causes bladder autoimmune inflammation in the transgenic EAC models. The results suggest that loss of functional Treg cells may contribute to IC/BPS pathology in subgroups of patients.

Terminology

IC/BPS is a chronic and debilitating inflammatory condition of the urinary bladder characterized by the hallmark symptom of pelvic pain in the absence of other identified etiologies for the symptom. IC/BPS patients also frequently have voiding dysfunction such as increased urinary frequency and urgency. This urologic condition is significant and severely affects quality of life. The etiology of IC/BPS is currently unknown and may involve multiple causes. Increasing evidence suggests that autoimmune inflammation may be causative in subgroups of IC/BPS patients.

Peer-review

The manuscript describes the role of regulatory T cells in IC/BPS model mice. The experiments are well designed and the results are clearly presented.

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Myeloid derived suppressor cells in breast cancer: A novel therapeutic target?

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Abstract

The relationship of the immune system and tumour cells is complex; although recognised that the immune system can protect the host against tumour development, the immune system also facilitates tumour progression through immune suppression. Pro-inflammatory mediators associated with chronic inflammation are responsible for the expansion and activation of myeloid derived suppressor cells (MDSCs); a heterogeneous group of cells that originates from myeloid progenitor cells but does not complete the final stages of differentiation. A causal relationship between chronic inflammation and tumour progression relies on the accumulation and maintenance of MDSCs as its linchpin; responsible for immunosuppression through the down-regulation of anti-tumour responses. MDSCs cause immunosuppression through a number of mechanisms; inhibiting the proliferation of CD4⁺ and CD8⁺ T cells, blocking natural killer cell activation and limiting dendritic cell maturation and function. As well as using various mechanisms to inhibit adaptive and immune responses, MDSCs also have non-immunological functions that aid tumour spread; including directly promoting tumour proliferation and metastasis by having an important role in tumour angiogenesis, secretion of matrix metalloproteinases and induction of epithelial-mesenchymal transition. Breast cancer is the most common cancer among women in the United Kingdom with 44540 new cases of invasive carcinoma in 2013 and results in the second highest cancer mortality rate in women, with 11600 deaths in 2012. Considering this, the need for novel therapeutic interventions is higher than ever. This review summarises the rationale for the targeting of MDSCs in breast cancer as a realistic avenue to increase survival from breast cancer.

Key words: Breast cancer; Immune cells; Treatment; Suppression; Survival

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Core tip: Breast cancer is the most common cancer among women in the United Kingdom; there were 44540 new cases of invasive carcinoma in 2013. The incidence rate is 169.8:100000, an increase of 5.5% in 10 years. Despite significant advances in the detection and treatment of breast cancer, breast cancer still results in 11600 deaths a year; the second highest number of cancer deaths in women. With increased appreciation of the sustaining importance of cells in the tumour microenvironment, in particular myeloid derived suppressor cells, their targeting is being considered as a novel treatment approach.

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INTRODUCTION

Breast cancer is the most common cancer among women in the United Kingdom; there were 44540 new cases of invasive carcinoma in 2013. The incidence rate is 169.8:100000, an increase of 5.5% in 10 years^[1]. Despite significant advances in the detection and treatment of breast cancer, breast cancer still results in 11600 deaths a year; the second highest number of cancer deaths in women^[2,3]. With increased appreciation of the sustaining importance of cells in the tumour microenvironment, in particular myeloid derived suppressor cells, their targeting is being considered as a novel treatment approach.

Myeloid derived suppressor cells (MDSCs) originate from myeloid progenitor cells in the bone marrow but do not complete the final stages of differentiation. They are immune modulatory cells which expand in cancer and go on to block tumour immunity and facilitate tumour progression^[4]. MDSCs can be identified by surface markers and their functional ability to suppress the immune system^[5,6], with the surface marker expression on human MDSCs being CD33⁺CD11b⁺HLA-DR^{low/neg}. The cells can also be identified through the absence of markers that would usually be found on mature lymphoid and myeloid cells^[7,8]. Due to different factors involved in cell development and activation, the expression of surface markers is not limited to those mentioned above; there is a large diversity in MDSCs found in different cancers (CD11b⁺CD33⁺ in non small cell lung carcinoma; CD11b⁺CD33⁺LIN⁺HLA-DR⁻ in Colon, breast, renal, lung, pancreatic, gastric and oesophageal carcinoma; CD11b⁺CD14⁺CD15⁺ in melanoma; CD11b⁺CD33⁺CD14⁺ in head and neck squamous cell carcinoma^[8,9]). This is because the factors of stimulation originate from the tumour cells themselves and a particular phenotype of MDSCs is produced that depends on the inducer molecules released from the tumour^[6]. Therefore, despite

MDSCs having recognisable surface markers, most of these are not unique and the most effective way to identify MDSCs is by their immunosuppressive function^[8].

INFLAMMATION, MDSCS AND CANCER

Chronic inflammation is linked to an increased risk of cancer, with experiments showing that high levels of pro-inflammatory mediators cause an increase in tumour development^[10-12]. There are several mechanisms for this; induction of cell proliferation, increased angiogenesis, triggering of genetic changes and expansion and activation of MDSCs^[4,5,10]. Inflammation-associated molecules, such as vascular endothelial growth factor (VEGF) and granulocyte macrophage colony-stimulating factor (GM-CSF), have been associated with the accumulation of MDSCs^[11] and pro-inflammatory cytokines have been linked to MDSC induction^[10]. These findings indicate that the link between inflammation and cancer is the induction of MDSCs and their inhibition of tumour immunity. Bunt *et al.*^[10] developed a causal relationship between chronic inflammation and tumour progression: Proliferation of tumour cells induces an inflammatory microenvironment consisting of pro-inflammatory factors, causing induction, accumulation and maintenance of MDSCs which leads to immune suppression; facilitating the survival and proliferation of the tumour cells. As well as inflammatory mediators being involved in the activation of MDSCs, MDSCs themselves produce pro-inflammatory factors. These factors, for example S100A8, S100A9 and interleukin (IL)-6, maintain the inflammatory environment and in turn maintain the MDSC population^[6].

UPREGULATION OF MDSCS

MDSCs are up-regulated by a variety of pro-inflammatory mediators, including S100A8/S100A9, IL-1 β , IL-6 and prostaglandin E₂ (PGE₂); leading to immune suppression and proliferation of malignant cells.

S100A8 and S100A9, members of the S100 family of calcium binding proteins, act as inflammatory mediators. Elevated levels of these proteins in serum indicate inflammation and/or recurrent infection with up-regulation found in many cancer patients^[4], leading to the hypothesis that S100A8/9 contribute to the recruitment of MDSCs. Not only do MDSCs have receptors for S100A8/9 but also have an ability to synthesise and secrete these proteins, allowing a positive feedback loop to ensure maintenance of the MDSC population^[4]. When the binding of S100A8/9 was blocked, there was a reduction in MDSC accumulation indicating that these pro-inflammatory mediators are indeed involved in the accumulation of MDSCs^[4]. When tissue sections from 101 invasive ductal carcinoma cases were studied, co-expression of the two S100 proteins was associated with poor tumour differentiation, vessel invasion and node metastasis, indicating that in invasive ductal carcinoma, over-expression of the S100A8/A9 complexes was

associated with poor prognosis^[12].

IL-1 β is important in mediating inflammatory responses, enhancing the accumulation of MDSCs and promoting tumour progression. When the IL-1 receptor is knocked out in murine models, there is a delay in MDSC accumulation and reduced tumour progression, indicating the importance of the interaction between IL-1 β (inflammation) and MDSCs^[10,13]. When IL-1 receptor knock-out mice were inoculated with IL-6-producing tumour cells, there was partial restoration of MDSC accumulation and tumour progression, indicating that IL-6 has a role as a downstream mediator of this IL-1 β -induced process^[13]. The stimulation of a variety of cell types by IL-1 β leads to an increased production of PGE₂, VEGF and GM-CSF; molecules all associated with the induction of MDSCs. As MDSCs do not have a receptor for IL-1 β , it is therefore likely that these molecules could therefore be the downstream mechanism for IL-1 β -induced immune suppression^[10].

IMMUNE SUPPRESSIVE ACTIVITIES OF MDSCS

MDSCs play an important role in tumour progression through the down-regulation of anti-tumour responses. As the tumour develops, the accumulation of MDSCs in the blood and lymphoid tissue is increased with patients newly diagnosed with breast cancer having double the number of circulating MDSCs compared to healthy volunteers^[14]. There is a correlation between increasing levels of circulating MDSCs, clinical stage of breast cancer and decreased survival rate; the highest level of MDSCs are found in those with metastatic disease, stage IV^[14]. MDSCs cause suppression through a number of mechanisms; inhibiting the proliferation of CD4⁺ and CD8⁺ T cells, blocking natural killer cell activation, limiting dendritic cell (DC) maturation and function and impacting on antigen presentation^[4,7,13].

Inhibition of T cell-mediated anti-tumour immunity - biochemical switches

The presence of MDSCs in cancer patients strongly correlates with the absence of antigen-specific T cells, explaining the immunosuppression seen in sufferers^[15,16]. Inhibition of T cell-mediated anti-tumour immunity is due to the production of arginase, reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS), IL-10 and by isolating cystine and limiting availability of cysteine^[17].

The cell's requirement for the essential amino acid cysteine is increased during proliferation, differentiation and activation by an antigen. Cells usually synthesise this cysteine by the enzyme cystathionase, converting intracellular methionine to cysteine. Cells are also able to reduce cystine to cysteine in the cytoplasm after being imported *via* the Xc⁻ cystine/glutamate antiporter^[9,17]. T cells lack both cystathionase and a functional antiporter, therefore depend on antigen presenting cells (APCs) to provide cysteine which is exported *via* the ASC neutral amino acid transporter. APCs also release thioredoxin,

an enzyme that reduces extracellular cystine to cysteine which can then be taken up directly by T cells. MDSCs also express the antiporter and are able to import cystine, competing with APCs for extracellular cystine. Since MDSCs do not express the ASC transporter and are therefore unable to export cysteine, there is a reduced amount of exported cysteine available to be taken up by T cells. As the MDSC burden increases, there is less cystine available for APCs and less cysteine available for T cells to import. MDSCs also disrupt the function of thioredoxin. As a consequence, T cells are deprived of the essential cysteine required for activation and function^[9,17].

PGE₂ produced by tumour cells can induce up-regulation of arginase 1 in MDSCs, leading to increased consumption and reduced availability of arginine, an amino acid required by T cells for protein synthesis^[9]. This depletion of arginine impairs T cell signal transduction and function due to loss of T cell receptor signalling and the inhibition of the cell cycle in G₀ phase^[18]. A significant increase in arginase levels has been found in patients with renal cell carcinoma compared to controls, resulting in a decrease in arginine levels. By using specific inhibitors to block arginase activity, suppression is removed and allows development of an effective anti-tumour response^[18].

Indoleamine 2, 3 dioxygenase (IDO) is an enzyme that is responsible for the break-down of tryptophan, an essential amino acid for protein synthesis. Expression of IDO by MDSCs inhibits T cell proliferation and induces T cell apoptosis through depletion of tryptophan and production of cytotoxic metabolites^[9]. IDO expression was increased in MDSCs isolated from resected invasive ductal carcinoma tissue. This up-regulation requires phosphorylation of signal transducer and activator of transcription 3 (STAT3); increased levels of STAT3 phosphorylation is found in MDSCs. When an IDO inhibitor, 1-methyl-L-tryptophan, or a STAT3 antagonist was used, the MDSCs immunosuppressive activity on T cells was blocked, indicating that STAT3-dependent IDO expression mediates the immunosuppressive effects of MDSCs in breast cancer^[19]. IDO has also been found to accumulate in tumour-infiltrating DCs, further suppressing T cell activity^[3].

iNOS, an enzyme expressed in MDSCs, catalyses the reaction between L-arginine and oxygen to produce L-citrulline and nitric oxide (NO); NO can lead to T cell inhibition through various mechanisms. NO activates cyclic guanosine monophosphate-dependent protein kinase, leading to inhibition of IL-2 receptor signalling and in turn preventing activation of T cells. NO is also able to accelerate tumour growth and may lead to T cell apoptosis^[9,18].

As L-arginine is depleted by MDSCs, iNOS switches to production of O₂⁻, a superoxide anion which goes on to react with other molecules to form ROS and reactive nitrogen species, such as peroxynitrite. Peroxynitrite, which is produced when O₂⁻ reacts with NO, is able to diffuse through the cell membrane, acting as an

intracellular messenger and altering protein functions through nitration of amino acid residues. This strong oxidising agent has the ability to inhibit cytotoxic T cell responses by catalysing the nitration of the T cell receptor and in turn, preventing T cell-peptide-major histocompatibility complex interactions^[9,20]. Peroxynitrite also induces T cell apoptosis through the inhibition of phosphorylation events in the T cell signal transduction pathway^[9].

Hydrogen peroxide is formed from the ROS O_2^- and H^+ and has similar effects as peroxynitrite, impairing T cell receptor signalling, and also inducing T cell apoptosis by increasing the expression of CD95, a death receptor^[9].

Limiting DC function

DCs, as part of the innate immune system are the most important APCs for activation of naïve T cells. Since DCs are derived from the myeloid lineage, DC defects in cancer are likely due to abnormal or incomplete differentiation of DCs due to tumour-derived factors. The abnormal differentiation results in decreased production of mature DCs, accumulation of immature DCs that cannot produce cytokines, and increased production of MDSCs^[21]. The reduction in number of mature DCs is closely associated with an accumulation of MDSCs^[11,21]. Although immature DCs can process and present antigen, there are no co-stimulatory molecules. This fact, as well as a lack of stimulatory cytokines, may result in T cell tolerance^[21]. In cancer, MDSCs inhibit DC production of IL-12 by producing IL-10, decreasing T cell activation and leading to immune suppression and tumour development^[22].

MDSCS AS THERAPEUTIC TARGET

Apart from their immune suppressive function, MDSCs directly promote tumour proliferation and metastasis by furthering tumour angiogenesis^[4,7,23]. Tumour blood vessels are important for providing the blood supply required for the development of the tumour, its progression and metastasis. When tumours are injected with MDSCs, there is increased blood vessel growth and maturation^[24]. MDSCs are also able to incorporate themselves into the tumour endothelium, acquiring endothelial cell properties; expressing endothelial cell markers and leading to tumour vascularisation^[24,25]. Immunotherapies need to overcome the break MDSCs exert on the host's immune responses^[6,26]. Studies have shown that a reduction in the immunosuppressive function of MDSCs is required for the induction of anti-breast immune responses^[27], demonstrating that targeting MDSCs will increase effectiveness of immunotherapies.

Markowitz *et al.*^[26] reviewed studies to determine that there are four main ways that the immunosuppressive effects of MDSCs can be reversed: Forcing MDSCs to differentiate into more mature states; inhibiting expansion of MDSCs accumulating at tumour sites and blocking the function of MDSCs.

Forced MDSC differentiation

Treatment of cancer patients with all-trans retinoic acid (ATRA) resulted in a substantial decrease in the number of MDSCs and an improvement in immune responses without significant toxicity^[28]. ATRA delivers these results through differentiation of MDSCs to mature myeloid cells through inhibition of ROS production; ATRA acts to upregulate glutathione synthase, leading to an accumulation of glutathione^[29]. By determining the mechanism of action and the concentration of ATRA required for a response, there is potential for a therapeutic agent that could be tested further, perhaps in combination with cancer vaccines.

Inhibiting MDSC expansion

Therapeutics that prevent MDSC expansion include STAT3 inhibitors, tyrosine kinase inhibitors and amino-bisphosphonates. Cytotoxic agents such as gemcitabine, 5-FU and cisplatin may directly decrease MDSC accumulation^[26,30].

Inhibiting receptor tyrosine kinases: Sunitinib, an inhibitor of receptor tyrosine kinases involved in breast cancer growth and metastasis which bind VEGF and platelet-derived growth factor, reduces elevated levels of MDSCs in cancer patients^[31,32]. Preclinical studies using a breast cancer model showed that sunitinib, in combination with docetaxel, enhanced the anti-tumour activity of the alkaloid docetaxel and increased survival. In a phase II trial, sunitinib in combination with docetaxel, showed promising anti-tumour activity in patients suffering from advanced breast cancer without significant drug-drug interactions^[31].

In light of these results, Bergh *et al.*^[31] carried out a prospective, multicentre, randomised phase III trial designed to determine if progression-free survival was prolonged when patients were treated with sunitinib and docetaxel rather than docetaxel alone. Progression-free survival is the time that passes from the first day of treatment and the date on which the disease progresses. The results of this study contrasted those expected; despite improving the objective response rate, the treatment failed to prolong the progression-free survival^[31]. Objective response rate is the percentage of patients whose cancer shrinks or disappears after treatment.

Although this treatment regime is not recommended for clinical use, it does not rule out the possibility of using tyrosine kinase inhibitors to target MDSCs in breast cancer.

Inhibiting matrix metalloproteinase-9: MDSC expansion can also be acted against by inhibiting matrix metalloproteinase-9 (MMP-9); an enzyme secreted in high levels by MDSCs that is capable of breaking down extracellular matrix components and is responsible for tumour metastasis promotion and tumour vascularisation^[9]. Although MMP inhibitors have either

failed or showed toxicity when studied as a therapeutic intervention in clinical trials^[33], it has been discovered that amino-bisphosphonates have the ability to inhibit MMP-9; leading to impaired VEGF availability and therefore a reduction in tumour angiogenesis^[34]. Zoledronic acid is a potent amino-bisphosphonate that was developed to treat bony metastases in cancer patients; it also targets MDSCs by preventing the differentiation of immature myeloid cells into MDSCs and has been found to prolong disease-free survival in breast cancer patients when used as an adjuvant in combination therapy^[35-37]. In models of pancreatic cancer, zoledronic acid was effective in impairing intra-tumoural MDSC accumulation and depleting MDSCs, resulting in delayed tumour growth and an increased immune response. There was an increased recruitment of T cells to the tumour, showing that treatment with zoledronic acid improved the anti-tumour response. The mechanism of action is unclear for both of these effects, however, meaning that further work is required^[38]. The efficacy of a direct antitumour effect of bisphosphonates may be enhanced by the adjuvant administration of an anion transport blocker^[39].

Blocked function of MDSCs

Reactive oxygen species: ROS prevent T cell signalling through nitration of the T cell receptor. Breast tumours are sensitive to oxidative stress, typically expressing higher levels of ROS than normal cells due to increased intracellular ROS production, leading to dysregulation in the redox balance^[40]. Kusmartsev *et al.*^[41] demonstrated that *ex vivo* neutralisation of ROS in immature myeloid cells derived from spleens of tumour bearing mice led to differentiation of cells away from their MDSC to a macrophage phenotype. Therefore, inhibitors of free radical formation may be useful in the treatment of breast cancer.

NOV-002, a glutathione disulphide imitator, induces S-glutathionylation, the post-translational modification of cysteine residues through the addition of glutathione. This leads to inhibition of free radical formation^[40]. Drugs targeting S-glutathionylation effect cell signalling pathways, inhibit DNA repair, have inhibitory effects on tumour cell invasion, proliferation and survival^[40,42].

Since chemotherapy is generally less effective in HER-2 negative breast cancer, Montero *et al.*^[40] hypothesised that the addition of NOV-002, to inhibit ROS, to a standard chemotherapy programme would increase response rates to chemotherapy compared to if the patient was just being treated with chemotherapy alone. To test this hypothesis, thirty-nine women with newly diagnosed stage II-IIIc HER-2 negative breast cancer were enrolled in a clinical trial and received doxorubicin and cyclophosphamide, followed by docetaxel every three weeks, in conjunction with daily doses of NOV-002. Fifteen of the patients achieved a pathologic complete response (pCR) rate, a higher response than would be expected from chemotherapy alone. It was also found that patients who had lower levels of circulating MDSCs at their last cycle of chemotherapy had a significantly

higher likelihood of a pCR^[40]. However, since the study was of small size with no randomised control, addition of NOV-002 cannot be conclusively identified as a causal factor for the higher pCR rates. To determine this and if circulating MDSCs can be used to predict patient response, a much larger, randomised control trial needs to be carried out.

IL-12: IL-12 can override the activity of immune suppressing cells and promote anti-tumour responses, giving it the potential to be used as a therapeutic agent by modulating MDSC activity. IL-12 is the major cytokine responsible for the differentiation of T helper cells to promote cell-mediated immunity and also stimulates the proliferation of activated T cells^[43].

MDSCs decrease IL-12 production. When treating MDSCs with IL-12 *in vitro* and *ex vivo*, up-regulation of co-stimulatory molecules CD80 and CD86 on MDSCs was noted, indicating a change in cell phenotype with a new potential for the cells to activate T cells rather than suppress them. There was decreased percentage of MDSCs in the tumour microenvironment and an increased percentage of cytotoxic T cells of CD45⁺ hematopoietic cells. As a result of these benefits, IL-12 treatment resulted in an increase in overall survival and a reduction in metastasis in a mouse model of breast cancer^[43]. These findings identify IL-12 as having significant potential as a therapeutic agent in metastatic breast cancer with the benefit of being a single-cytokine based therapy that could work alone as well as in combination with another agent^[43].

Cysteine as chemo-preventative therapy

High levels of cysteine correlate with reduced breast cancer risk. There may be potential in cysteine or its derivatives as a chemo-preventative therapy against breast cancer to help to biochemically redress the balance of T cell vs MDSC activities. Although cysteine is commercially available as a dietary supplement, neural toxicity was observed in mice with high doses, meaning that cysteine may not be ideal for therapeutic use^[44]. N-acetylcysteine is a synthetic precursor of cysteine and glutathione and is already used as a mucolytic agent in humans, appearing safe even in high doses^[45]. It has been shown in rat models to be a promising candidate for cancer chemo-prevention^[46].

CONCLUSION

MDSCs play a crucial role in tumour progression, responsible for immunosuppression of the host's immune system *via* a multitude of mechanisms. The literature shows MDSCs have a significant role in the development and spread of cancer but it should be noted that research into the action of MDSCs specifically in breast cancer is limited, with most knowledge gained from murine models. Although useful, this knowledge has its limitations as murine responses cannot be assumed to correspond directly with human responses. Much

of the knowledge of MDSCs in human cancers comes from studies on renal cell carcinoma and colon cancer^[9]; again, whilst useful, MDSCs in breast cancer will differ in terms of surface markers and therefore may act slightly differently. Further clinical studies involving breast cancer patients need to be done to determine the true significance of the role that MDSCs have.

Since the level of circulating levels of MDSCs is a strong prognostic indicator^[47], incorporating some of these features into the traditional TNM classification and staging for breast cancers may be helpful in determining the scope of the patient's illness, prognosis and the most effective treatment options on a personalised basis.

Despite there being several potential mechanisms to target MDSCs, there are currently no established therapies to target these mechanisms; the heterogeneity of MDSCs remains a hurdle for MDSC targeted treatments. The lack of unique markers and limited analysis of tumour associated cells hampers progress in the field^[48]. It is exciting to consider MDSC targeted treatments for incorporation in therapeutic regimes of breast cancer, which could further involve immune checkpoint blockade inhibition or breast cancer vaccines^[49]. A significant technological advance in *ex vivo* differentiation of tumour specific MDSC has recently been made^[50].

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IgE regulates airway smooth muscle phenotype: Future perspectives in allergic asthma

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Abstract

The purpose of this commentary is to highlight the

emerging role of IgE on airway smooth muscle (ASM) cells function through activation of the high-affinity Fc receptor for IgE. We discuss the potential implications of IgE-mediated ASM sensitization in airway inflammation and remodeling, the hallmark features of allergic asthma.

Key words: IgE; Airway; Allergic asthma; Remodeling; Inflammation

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Core tip: In this commentary, we summarize the emerging role IgE in influencing airway smooth (ASM) muscle function in allergic asthma. Recent intriguing findings from ours and other groups suggest a potential proinflammatory and pro-remodeling role of IgE-mediated activation of its high-affinity Fc receptor, *i.e.*, FcεRI, on ASM tissue in allergic asthma.

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Airway remodeling is a hallmark feature of allergic asthma, characterized by thickening of the reticular basement membrane (RBM) and numerous structural alterations to the epithelium, submucosa, and airway smooth muscle^[1]. Among these, increased airway smooth muscle (ASM) mass, potentially due to yet poorly defined mechanisms is considered a major determinant of lung function. In addition, increased ASM mass is also believed to result in airway hyperresponsiveness (AHR), contributing to a decline in lung function in asthma^[1]. Interestingly, the ASM proliferation was shown to be a dynamic process in long-standing severe asthma^[2].

Thus, the mechanisms defining ASM proliferation are of significant value in pursuit of novel asthma therapeutics. While there is some evidence from childhood studies and primate models arguing that airway structural changes precede the inflammatory process^[3,4], a vast majority of data suggest that inflammation, particularly resulting from IgE-driven processes, initiates the airway structural alterations, culminating in a remodeled airway wall^[1]. IgE exerts its effector functions mainly through its receptors, the high-affinity Fc receptor (FcεRI), and the low-affinity receptor (FcεRII/CD23)^[5]. Given the central role of IgE in allergic inflammation, strategies blocking IgE or its interaction with Fc receptors have been developed to treat allergic asthma. The proof-of-concept comes from a clinically-approved humanized anti-IgE antibody (Omalizumab) that prevents the binding of free IgE to FcεRI and has shown tremendous clinical benefits in allergic asthma^[6].

The allergic cascade initiated by IgE establishes an inflammatory milieu in asthmatic airways, characterized by the abundance of eosinophils, mast cells, and Th2 cells that produce cytokines such as IL-4, IL-5 and IL-13^[7]. While IgE-mediated effector functions on mast cells and basophils, including the production of Th2 cytokines is known to drive airway remodeling^[8], it is often asked whether IgE can directly influence the ASM function and contribute to airway inflammation and/or remodeling? About a decade ago, we had first demonstrated that cultured primary human ASM cells from bronchus and trachea express FcεRI *in vitro*, and *in vivo* within the bronchial biopsies of allergic asthma patients. IgE and anti-IgE-mediated FcεRI cross-linking led to the release of Th2 cytokines (IL-4, IL-5 and IL-13) and CC chemokine (eotaxin-1/CCL11) in ASM cells^[9]. Furthermore, pro-asthmatic factors such as tumor necrosis factor (TNF) and IL-4 enhanced the abundance of FcεRI and amplified IgE-induced chemokine (eotaxin-1/CCL11, RANTES/CCL5, IL-8/CXCL8, and IP-10/CXCL10) release in ASM cells *via* transcriptional mechanisms^[10]. Complementary findings from another group showed that IgE stimulation of ASM cells from asthmatic patients exhibit *de novo* synthesis and release of higher levels of pro-asthmatic factors including IL-4, IL-6, IL-8/CXCL8 and TNF compared with the ASM cells obtained from chronic obstructive pulmonary disease patients or from healthy controls^[11]. Recently, we have also shown that IgE can induce the expression of smooth muscle myosin light chain kinase, a component involved in ASM contractile machinery^[12]. Interestingly, IgE induced a prominent Th2-favoring cytokine, thymic stromal lymphopoietin (TSLP) through the activity of spleen tyrosine kinase (Syk), and nuclear factor kappa B and activator protein-1^[13]. This data suggested that FcεRI activation on ASM may directly regulate the immune response within the airways *via* induction of TSLP. Notably, ASM cells express a functional TSLP receptor, which upon activation leads to chemoattractants (CCL11/eotaxin-1

and CXCL8/IL-8) and proinflammatory cytokine (IL-6) release^[14], induces ASM migration^[15], and enhances intracellular Ca²⁺ mobilization in ASM^[16]. Interestingly, mast cells also produce and respond to TSLP^[17,18], and it has been proposed that TSLP may bridge the ASM-mast cell cross-talk^[18]. We propose that the TSLP produced by ASM and/or mast cells *via* IgE/FcεRI pathway may act in an autocrine/paracrine manner on ASM which might in fact explain the "atopic" or "pro-allergic" state of ASM in allergic asthma that has been linked to the development of AHR in large epidemiological studies, particularly the *Tucson Respiratory Cohort Study*^[19]. Overall, these studies clearly suggest a pro-inflammatory role of IgE-FcεRI interaction in ASM cells and arguably may explain some of the benefits of Omalizumab in allergic asthma^[6].

Importantly, one of the most compelling findings was that IgE could induce *de novo* DNA synthesis and proliferation in primary human ASM cells^[20], and that this proliferative response was inhibited by anti-IgE Omalizumab pre-treatment^[21]. IgE-induced proliferation involved the activity of Syk, and intracellular signaling Erk 1/2, p38, and JNK MAPK, and Akt kinases. Lentivirus-shRNA-mediated experiments showed that STAT3 activity is indispensable for IgE-induced ASM cell proliferation^[20]. Parallel work by others showed that IgE stimulation of ASM cells from asthma patients enhanced the deposition of collagen type I and VII through the activation of Erk1/2 MAPK downstream of FcεRI; whereas IgE stimulated the deposition of collagen type III, and fibronectin through both FcεRI and FcεRII/CD23, as well as Erk1/2 and p38 MAPK pathways^[21]. These data suggest that IgE may directly induce pro-remodeling changes in ASM *in vivo*. Moreover, the finding that Omalizumab prevents IgE-induced remodeling effects^[21] rationalizes the use of anti-IgE strategies in the modulation of airway remodeling. Although MAPK such as Erk1/2 is known to interact with STAT3 in ASM cells^[22], it remains yet to be determined how IgE facilitates cross-talk among MAPK, STAT3 and other signaling pathways in pro-mitogenic signaling in ASM cells. This is even more interesting of a question since therapeutics targeting MAPK signaling, *e.g.*, p38 inhibitor, has already been in clinical trials of asthma and COPD^[23].

It is clear that IgE induction of ASM proliferation is a significantly important finding since IgE can gain access to the smooth muscle tissue (Figure 1), likely due to: (1) microvascular leakage and edema due to underlying inflammation^[24]; (2) local IgE expression or class switching in bronchial mucosa of asthmatic patients^[25,26]; and (3) potential trans-epithelial transport of IgE by FcεRII/CD23, similar to the intestinal compartment^[27]. *In vivo*, anti-IgE treatment of an ovalbumin-induced murine model of asthma significantly reduced the smooth muscle mass and extracellular matrix deposition, suggesting an IgE-induced pro-mitogenic effect on ASM cells^[28]. Most importantly, the addition of Omalizumab to conventional therapies in asthma patients showed a significant decrease in airway wall thickness^[29]; and reduced thick-

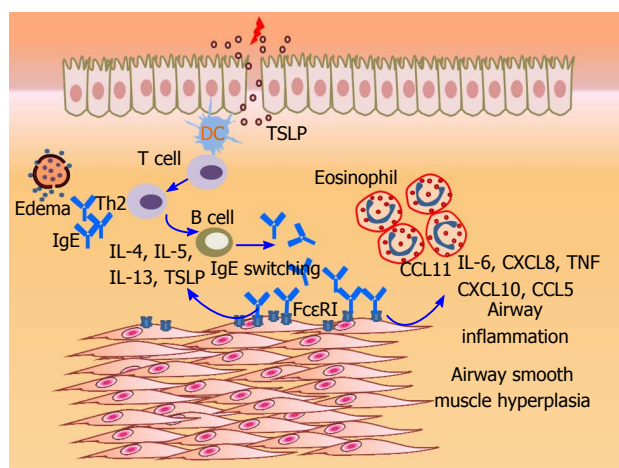


Figure 1 IgE sensitization-induced alterations in airway smooth muscle function. Owing to edema or local IgE class switching in airway mucosa, IgE likely gains access to the sub-epithelial region where smooth muscle resides. In addition to other sources (T cells, epithelium), IgE stimulates the production of key Th2 cytokines (IL-4, -5, and -13) and thymic stromal lymphopoietin in ASM that may promote Th2-biased immune response within the airways. In addition, IgE-induced production of chemokines facilitates the recruitment of inflammatory cells including eosinophils. Most importantly, IgE stimulates ASM proliferation (hyperplasia), ultimately contributing to the airway wall thickness observed in allergic asthma. Notably, anti-IgE such as Omalizumab (not shown) has been shown to limit IgE-induced ASM synthetic and proliferative responses. TSLP: Thymic stromal lymphopoietin; TNF: Tumor necrosis factor; ASM: Airway smooth muscle.

ness of RBM and eosinophil infiltration^[30]. Therefore, anti-IgE therapy is likely a promising tool in targeting airway remodeling in asthma.

Interestingly, while cross-linking of IgE bound to FcεRI is necessary to cause mast cell and basophil degranulation, compelling evidence from various studies indicate that IgE alone, in the absence of allergen or anti-IgE antibodies, can drive pro-survival effects in mast cells, basophils, and neutrophils, reviewed in^[31]. While we and others have already shown that IgE alone can induce ASM proliferation^[20,21], it remained unknown whether the pro-mitogenic response of IgE is further enhanced by allergens. Roth *et al.*^[32] recently showed that human ASM cells stimulated with serum from patients with allergies (with or without asthma) induced ASM proliferation and deposition of collagens and fibronectin compared to the serum from healthy controls. Very interestingly, however, the addition of allergens did not augment allergic serum-induced ASM cell proliferation. Authors confirmed that the pro-mitogenic effects of allergic serum were specific to IgE since Omalizumab pre-incubation of ASM cells prevented these effects^[32]. This is a very important aspect of how IgE sensitizes the airway mucosa without the presence of allergens. Given the early sensitization-induced predisposition to airway remodeling in murine studies^[33], and to asthma development in epidemiological studies^[19], it is conceivable that early sensitization of ASM may confer a distinctive persistent IgE-mediated hypercontractile ASM phenotype that can arise neonatally and persist into adult life.

Of note, mast cells are known to infiltrate the ASM tissue in asthma^[34], and their interaction with ASM is thought to be critical in modulating airway immunopathology in asthma^[35]. One may argue that the IgE-induced changes in ASM function may be an indirect effect of mast cell mediators following FcεRI cross-linking on mast cells. However, it does not appear to be the case since: (1) inhibition of Syk, an upstream FcεRI signaling protein, abolished the IgE effects on ASM synthetic and proliferative function^[10,13,20]; (2) no detectable contamination of mast cells (tryptase staining) was observed in ASM cell preparations^[9]; and (3) inhibition of IgE-induced ASM functions by anti-IgE Omalizumab^[11,32]. While the role of mast cells and their secreted mediators such as histamine in regulating ASM function may not be denied, the existing data indicates that IgE clearly can bind to ASM cells *via* Fc receptors and induce pro-asthmatic changes directly.

In summary, cumulative evidence suggests that the IgE-induced FcεRI activation leads to expression of multiple chemokines and proinflammatory cytokines in ASM which may recruit inflammatory cells and promote allergic airway inflammation. IgE may directly participate in driving Th2 immune response within the airways by inducing TSLP, and IgE may potentially contribute to airway remodeling by directly inducing ASM cell proliferation (Figure 1). Some of the potential questions for future research in this area include: (1) detailed investigation of how IgE gains access *in vivo* to the sub-epithelial compartment including ASM; (2) what are the mechanisms of IgE “sensitization” vs “cross-linking” modes of FcεRI activation in ASM cells, and whether they are similar to that in mast cells and basophils; (3) what is the relative contribution of FcεRI vs FcεRII/CD23 in determining the outcome of IgE-induced ASM activation; and (4) how IgE acts differentially on ASM vs infiltrating mast cells *in vivo*?

Overall, clinical and experimental^[11,28,32,36,37] evidence suggest that the strategies targeting IgE by anti-IgE have significant benefit in reducing airway wall remodeling. Therefore, a detailed understanding of the molecular events downstream of IgE-FcεRI interaction on ASM cells is needed to advance our knowledge of this pathway for developing better therapeutics in allergic asthma.

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