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A close-up portrait of a middle-aged man with dark, slightly graying hair, looking directly at the camera with a serious expression. He is wearing a dark jacket. In the background, to the left, is a brass-colored mechanical device with a circular gauge and various pipes. The background wall is light-colored with vertical lines.

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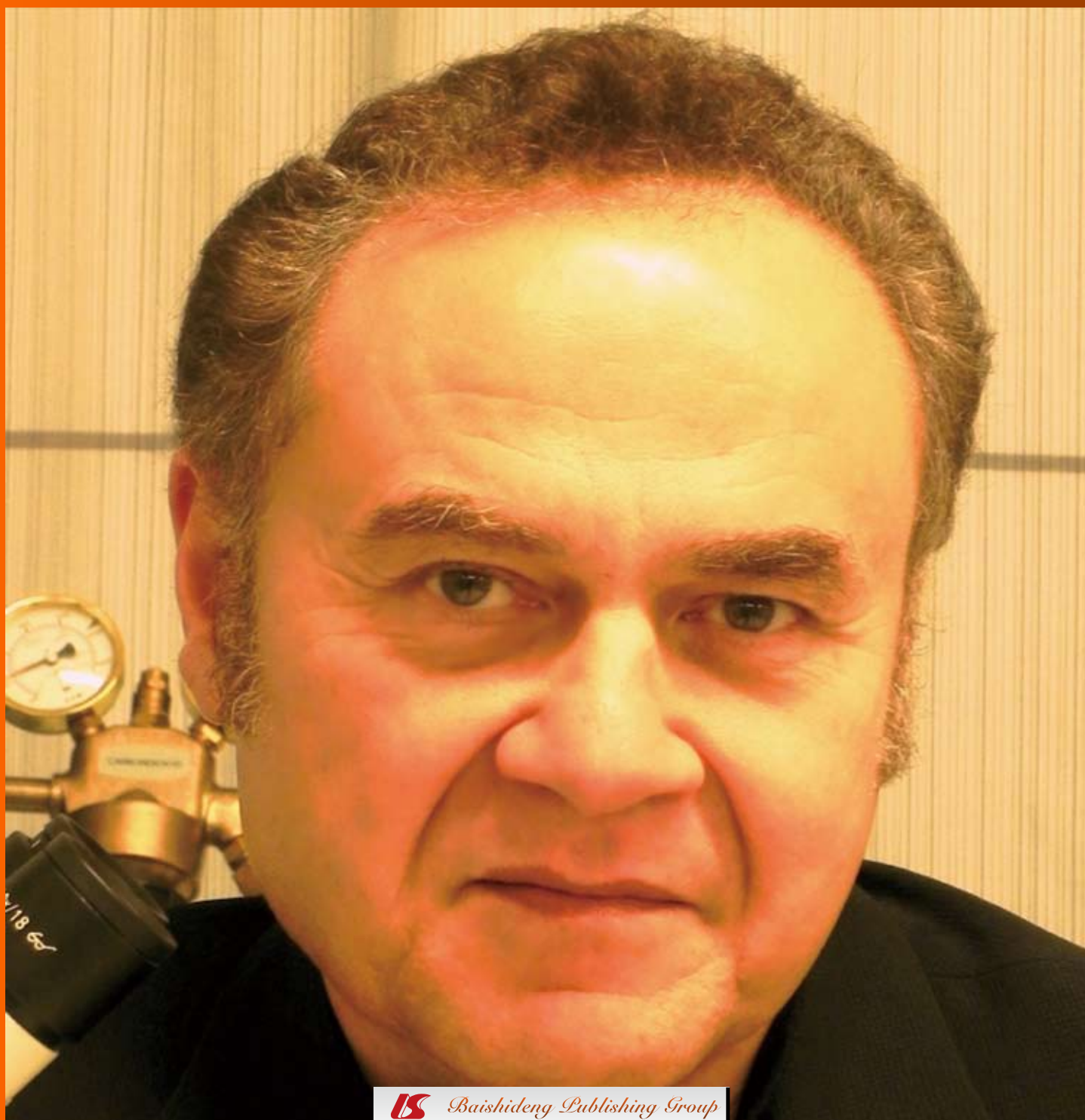
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Natural killer reprogramming in cutaneous T-cell lymphomas: Facts and hypotheses

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INTRODUCTION

Cutaneous T-cell lymphomas (CTCL) are a heterogeneous group of lymphoproliferative disorders involving primarily the skin. The most common subtypes of CTCL are mycosis fungoides (MF) and Sézary syndrome (SS). MF is characterized by a slowly progressing skin invasion by clonally derived mature CD4⁺ T-lymphocytes, these malignant cells residing primarily in the infiltrating skin lesion. SS is a more aggressive leukemic and erythrodermic form of CTCL involving malignant CD4⁺CD45RO⁺ T-cells. Despite the fact that MF and SS are classified as distinct disease entities, their clinical relationship is still a matter of debate as they share common features and similarities suggesting that they might be variants of the same disease spectrum^[1-3]. Patients with transformed MF can have blood findings characteristic of SS and can sometimes develop typical SS^[4]. On the other hand, the majority of patients diagnosed with early-stage MF will never progress to advanced-stage disease. The finding that MF and SS arise from two distinct functional T-cell subsets, central memory for SS (CCR7⁺L-selectin⁺ CD27⁺CCR4⁺CLA⁺) vs effector memory T cells for MF (CCR7⁻L-selectin⁻ CD27⁻CCR4⁺CLA⁺), if confirmed, favors the notion that they should be considered as separate lymphomas^[5].

Abstract

To better understand the pathogenesis of Sézary cells, distinguish them from reactive skin-infiltrating T-cells and improve disease treatment, efforts have been made to identify molecular targets deregulated by the malignant process. From immunophenotypic analysis and subtractive differential expression experiments to pan-genomic studies, many approaches have been used to identify markers of the disease. During the last decade several natural killer (NK) cell markers have been found aberrantly expressed at the surface of Sézary cells. In particular, KIR3DL2/CD158k, expressed by less than 2% of healthy individuals CD4⁺ T-cells, is an excellent marker to identify and follow the tumor burden in the blood of Sézary syndrome patients. It may also represent a valuable target for specific immunotherapy. Other products of the NK cluster on chromosome 19q13 have been detected on Sézary cells, raising the hypothesis of an NK reprogramming process associated with the malignant transformation that may induce survival functions.

The prognosis of MF and SS depends on the type and extent of skin lesions and extracutaneous disease. This is reflected in the TNMB classification of MF/SS defined by the International Society for Cutaneous Lymphomas, involving evaluation of the skin (T), lymph nodes (N), visceral organs (M) and blood (B)^[6]. SS is thus defined as meeting T4 plus B2 criteria, where T4 refers to a confluence of erythema covering at least 80% of the body surface area and B2 a high blood tumor burden^[6]. Peripheral blood studies are important for establishing the diagnosis and staging for SS. While determining the tumor mass by histological examination of blood smears, with Sézary cells defined by a cerebriform nuclear morphology, is widely used and valuable, flow cytometry analysis of T-cell blood subsets provides a more objective and reproducible means to quantify and track blood involvement in patients with MF/SS. For example, a CD4:CD8 ratio higher than 10 is observed in about 80% of patients with SS, whereas loss of CD7 ($CD4^+CD7^- \geq 30\%$) or CD26 ($CD4^+CD26^- \geq 40\%$) are found in about half of the SS patients^[7-9]. However, although it is possible to show using V β -specific TCR antibodies that clonally expanded cells in SS may have these immunophenotypes, loss of CD7 or CD26 among CD4⁺ T-cells can also be found in benign inflammatory erythroderma or even healthy blood. SS is considered as a clonal expansion of a T-cell subset and the analysis of T-cell clonality by PCR amplification of TCR- γ or - β chain genes can allow the detection of a dominant T-cell clone in the peripheral blood in most SS patients. However, a T-cell clonality can also be detected in 34% of cases with benign inflammatory erythroderma^[10]. Therefore the identification of a predominant T-cell clone might reflect a reactive rather than a neoplastic T-cell clone. The evaluation of other potential Sézary cell markers is consequently important for the diagnosis, prognosis and follow-up of SS. Among the proposed potential markers, several belong to the natural killer (NK) cell lineage, raising the question of a hypothetical NK-cell reprogramming mechanism occurring in the transformation of some CTCL. This editorial will focus on that provocative question.

THE NK RECEPTOR KIR3DL2/CD158K ON SS LYMPHOCYTES

Malignant T cells in MF and SS produce and respond to various cytokines in their microenvironment. Among them, interleukin (IL)-7 is sufficient to enhance the proliferation of healthy skin resident T-cells and is necessary to sustain an *in vitro* proliferation of malignant T-cells from SS or MF^[11-13]. The observation that IL7-transgenic mice develop cutaneous lymphomas at high frequency further illustrates the role of this cytokine in inducing the proliferation of skin infiltrating lymphocytes^[14]. This allowed us to develop T-cell lines derived from circulating Sézary cells as attested by their expression of TCR-V β and TCR β -VDJ sequences identical to the *in vivo*

tumor cells^[15,16]. Such long-term cultured cell lines have been valuable tools to study Sézary cells and were used to initially describe their expression of KIR3DL2/CD158k^[17].

The cell surface receptor KIR3DL2/CD158k belongs to the killer immunoglobulin-like receptor (KIR) family and is normally expressed by minor subsets of circulating NK cells and cytotoxic CD8⁺ T-lymphocytes. The KIRs display a clonally distributed expression in human NK cells and KIR3DL2/CD158k is only expressed on a few percentage of circulating blood lymphocytes^[18]. The KIR nomenclature is based on the biochemical structure of the receptors. Thus, they may have 2 (2D) or 3 (3D) extracellular immunoglobulin domains associated with a long (L) or short (S) cytoplasmic tail, responsible for an inhibiting or activating signaling activity respectively. KIRs recognize mainly determinants shared by a group of HLA class-I allotypes. The KIR3DL2/CD158k is an inhibitory receptor with specificity for HLA-A3 and -A11^[18] and has been reported recently to also recognize CpG oligodeoxynucleotides^[19].

Our group has identified KIR3DL2/CD158k as a new phenotypic marker for circulating Sézary cells^[17,20,21]. Despite the lack of commercially available anti-CD158k antibodies other groups have confirmed these observations^[8,22]. The proportion and absolute count of CD158k⁺ lymphocytes strongly correlate with the percentage and absolute count of atypical cells determined by cytomorphology^[20]. Interestingly, CD158k⁺ cells can be detected even in SS patients with low tumor burden^[20,22]. The CD4⁺CD158k⁺ cells found in the blood were shown to correspond to the malignant clonal cell population as assessed by the immunoscope technique^[21]. In the skin, KIR3DL2/CD158k transcripts were found to be significantly overexpressed in SS compared to erythrodermic inflammatory diseases^[23]. The only occasional expression of KIR3DL2/CD158k on rare CD4⁺ T-cells from healthy individuals makes it a valuable positive marker to identify malignant Sézary cells, even when present at low levels, and to monitor the tumor cell load during therapy. In some cases however, CD158k expression may not identify all the neoplastic T-cells, due to clonal evolution during tumoral progression^[24]. This raises the question of whether the appearance of CD158k is a relatively late event in the SS pathogenesis, occurring when genetic deregulation increases, or if it parallels the early oncogenic events. No definitive answer can be given but one may note that in normal T-cells KIR expression occurs after T-cell activation, and that in MF no CD158k⁺ T-cells are detected in the skin at the patch-plaque stage but can be found in patients at the transformed stage, favoring the acquisition of KIR3DL2/CD158k expression as a late event^[25]. It remains to understand what can be the consequences of this expression on the tumor cell biology in terms of proliferation or survival. CD158k/KIR3DL2 is an inhibitory receptor that upon engagement mediates an inhibitory signaling cascade through the ITIM domains located within its cytoplasmic tail. One can specu-

late that it may down regulate TCR-mediated signaling, in line with the reported hyporesponsiveness of Sézary cells to an anti-CD3 mAb stimulation^[12]. This may be seen as an advantage for tumor cells to resist to antigen receptor-mediated cell death associated to chronic antigenic stimulation, as observed on normal T-cells. However, as what was recently observed, KIR may act differently on Sézary cells, and behave as co-activating receptors through a JNK-dependent pathway^[26]. Clearly the exact function of KIR3DL2/CD158k in T-cells from SS patients has still to be defined. In a lower proportion of patients KIR3DL2 is not the only KIR expressed by Sézary cells. In particular, a significant expression of CD158a/KIR2DL1 and CD158b/KIR2DL2/3 can be observed in less than 10% of patients^[22,26].

OTHER NK RECEPTORS ON SS CELLS

An abnormal expression of other NK receptors has been observed at the surface of Sézary cells. The CD85j/Ig-like transcript 2 (ILT2) receptor belongs to a family of receptors homologous to the KIR, encompassing both inhibitory forms recruiting SHP-1 phosphatase and short-tailed activating forms^[27-29]. ILT2 is an inhibitory receptor specific for an $\alpha 3$ -domain epitope shared by many MHC class Ia and Ib molecules and the class I-like protein UL18 of human cytomegalovirus^[30]. It is expressed by myeloid cells, B lymphocytes and some NK and CD8⁺ T-cells with memory phenotype^[31]. Most circulating CD4⁺ lymphocytes fail to express ILT2 at their cell surface, whereas the molecule is in fact present in the cytoplasm of all T-cells^[32]. As for KIR, its action on circulating CD8⁺ T-cells is to reduce antigen driven activation-induced cell death without affecting proliferation and survival induced by cytokines and particularly IL-7^[31]. In SS, circulating malignant Sézary cells may be distinguished from non malignant reactive CD4⁺ autologous T-cells through the detection of ILT2 at the cell surface^[33]. In addition these receptors are functional as they can inhibit an anti-CD3 mAb-induced signaling and therefore perpetuate the survival of SS malignant cells by protecting them from CD3/TCR engagement induced apoptosis. Of note, in the skin, MF cells lack expression of ILT2^[33].

Another essential NK cell marker reported at the surface of Sézary cells is Nkp46/NCR1 that is not detected on normal circulating CD4⁺ T-cells^[34]. Nkp46/NCR1, together with Nkp44/NCR2 and Nkp30/NCR3, forms the family of activating natural cytotoxicity receptors(NCR)^[35]. These receptors are normally confined to NK cells, and their engagement induces strong activation of NK-mediated cytotoxicity. However, umbilical cord blood CD8⁺ T-cells, when stimulated for a long period of time with IL-15, expressed Nkp30 and Nkp44, although only Nkp30 was functional to induce cytotoxicity^[36]. Whereas Nkp30 and Nkp46 expression are constitutive, Nkp44 is acquired upon activation of NK cells. Nkp46 mediates signal transduction through

its association with the ITAM-bearing molecules CD3 ξ or Fc ϵ R1 γ , that become tyrosine phosphorylated upon receptor cross-linking. Nkp46 was detected at the surface of malignant Sézary cells in the absence of external stimulus. This expression, that parallels the one of KIR3DL2/CD158k, is specific to Sézary cells as it is not detected on cells isolated from MF or inflammatory erythroderma patients^[34]. In NK cells, Nkp46 acts as a full receptor and its engagement triggers their natural cytotoxicity against target cells. In Sézary cells however, its triggering does not induce CD3 ξ tyrosine phosphorylation and fails to initiate the activating events leading to cell proliferation. In fact, when brought to close proximity to the CD3/TCR, Nkp46 prevents the phosphorylation of CD3 chain, resulting in an overall inhibition of the TCR-mediated activation pathway. As mentioned above, SS cells are usually hyporesponsive to CD3/TCR-mediated triggering, which can be seen as a way to escape to antigen receptor-mediated cell death associated to chronic antigenic stimulation of T-cells. One can speculate that whereas KIR can mediate proliferation through JNK activation pathway, Nkp46 may downregulate TCR signaling to promote survival. Could such a behavior reflect a perversion of normal functions when placed in an ectopic environment?

NK REPROGRAMMING IN T-CELL TRANSFORMATION

Cell transformation and tumoral progression is generally associated with a reprogramming of the cell differentiation program. Celiac disease (CD) is a chronic inflammation of the small intestine secondary to gluten intolerance. This leads to a chronic activation of the intraepithelial lymphocytes (IEL), that are tissue specialized CD8⁺ cytotoxic T-lymphocytes, and to the alteration of the intestinal mucosa and the progression towards enteropathy-associated T-cell lymphoma^[37]. IL-15 production, which is greatly increased in the mucosa of patients with CD, has an important role in the disease process^[38]. Subjects expressing HLA-DQ2/DQ8, that form stable complexes with gluten peptides, elicit exacerbated response of DQ2/DQ8-restricted CD4⁺ T-cells leading to villous atrophy and malabsorption. However, despite the expansion of IEL in the mucosa, gluten-specific IEL are rare or absent^[39]. In fact, in CD patients, a massive expansion of few IEL cytotoxic T-cell clones that have undergone a genetic reprogramming under the IL-15 stimulation occurs, that essentially convert them into NK-like cells capable of cytotoxicity independently of a CD3/TCR signaling^[40]. This reprogramming consists in the aberrant expression on these IEL of a panoply of normally restricted cytolytic NK lineage receptors, such as NKG2C, Nkp44, Nkp46, or KIR. Such reprogramming has also been reported in the cytotoxic T-cells from cytomegalovirus-seropositive patients^[41]. This raises the question whether NK reprogramming may underlie the transformation of chronically stimulated T-cells.

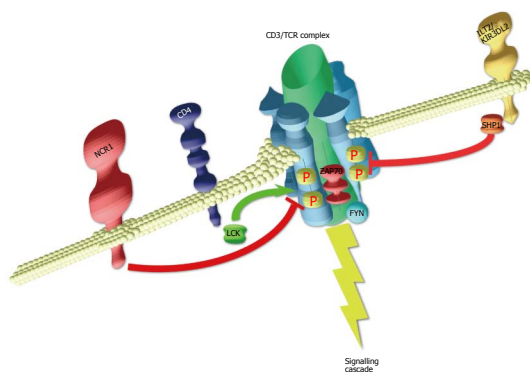


Figure 1 Tuning CD3/T cell receptor signalling threshold by natural killer receptors on Sézary tumoral cells. Ig-like transcript 2 (ILT2) or KIR3DL2 receptors on Sézary cells lower the threshold of T-cell receptor activation through the activation of the SHP1 phosphatase. Natural cytotoxicity receptors (NCR)1 (NKp46) receptors associate with CD3zeta chains and, when in close proximity, the NCR1 prevents the phosphorylation of CD3zeta chains of the T cell receptor complex. These mechanisms can be seen as a way to escape antigen-receptor mediated cell death associated with chronic T-cell stimulation in Sézary syndrome.

In SS, the aberrant expression of NK-cell lineage receptors such as KIR, ILT2 or NKp46 has been observed, that are all encoded in the NK cluster region on chromosome 19q13. Although the Sézary cells do not acquire cytotoxic capacity, signaling capacity through these receptors were observed in the malignant SS cells, suggesting an NK-like differentiation process. Chronic stimulation through antigen or allergen has been proposed to play a role in SS and MF^[42]. With this perspective, Figure 1 illustrates how NK receptors may interfere with T-cell stimulation in Sézary cells, tuning the signalling threshold of the CD3/TCR, preventing the tumoral cells from activation-induced cell apoptosis. Of note a high level of T-cell stimulating cytokines is present in the skin, such as IL7. Elevated levels of IL-15, an important cytokine for NK reprogramming in CD, have been reported in SS^[43,44]. Could there be a concerted aberrant expression of NK markers at the surface of Sézary cells, playing an important role in the pathobiology and tumoral progression of SS? Future work will tell us the truth, but that track is worth to be followed.

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Eotaxin-2 blockade ameliorates experimental autoimmune encephalomyelitis

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Abstract

AIM: To study the effect of blocking the eo-2 pathway on the development and severity of experimental autoimmune encephalomyelitis (EAE).

METHODS: We produced mAb directed against eo-2, named D8. MOG35-55 induced-EAE mice were daily intravenously injected with either 25 μ g or 100 μ g D8, or with vehicle control alone [phosphate-buffered saline (PBS)], starting from day 0 post immunization and were monitored for EAE clinical score ($n = 10$ in each group). Mice were sacrificed on day 58 and their sera were assessed for the presence of anti-myelin oligodendrocyte glycoprotein (anti-MOG) antibodies autoantibodies, as well as for the profile of pro-inflammatory cytokines and chemokines. Histological analysis of brain sections was performed by hematoxylin and eosin staining.

RESULTS: Daily treatment of EAE induced mice with D8 significantly decreased the severity of EAE symptoms. Treatment with both concentrations of D8 ameliorated EAE symptoms compared to PBS treated mice, starting from day 42 post immunization (0.89 ± 0.35 in D8 25 μ g and D8 100 μ g treated groups *vs* 2.11 ± 0.38 in the PBS treated group, $P = 0.03$). A significant improvement in EAE clinical score compared to total IgG treated mice was observed with the higher concentration of D8 (0.81 ± 0.38 in D8 100 μ g treated group *vs* 2.11 ± 0.31 in IgG1 treated group, on day 56 post immunization, $P = 0.04$). D8 treated mice with EAE did not significantly exhibit lower sera levels of anti-MOG autoantibodies compared to IgG-treated mice. However, they expressed lower sera levels of the pro-inflammatory cytokines: tumor necrosis factor (7.8 ± 0.2 pg/mL in D8 100 μ g treated mice *vs* 19.9 ± 3.4 pg/mL in IgG treated mice, $P = 0.005$) and interferon-gamma (1.4 ± 0.6 pg/mL in D8 100 μ g treated mice *vs* 3.6 ± 0.4 pg/mL in IgG treated mice, $P = 0.02$), as well as reduced levels of the chemokine macrophage chemoattractant protein-1 (27.2 ± 3.1 pg/mL in D8 100 μ g treated mice *vs* 63.7 ± 12.3 pg/mL in IgG treated mice, $P = 0.03$). These findings indicate that blocking the eo-2 pathway in EAE may affect not only eosinophil infiltration into the central nervous system (CNS), but also have an effect on monocytes and T cells, but not humoral, mediated responses. Histological analysis of the brains of D8 treated mice with EAE support that this treatment decreases immune cells infiltrates in the CNS.

CONCLUSION: Taken together, these findings suggest a role for eo-2 in EAE pathogenesis and consequently may support a therapeutic potential of anti-eo-2 neutralizing mAb in multiple sclerosis.

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Key words: Multiple sclerosis; Experimental autoimmune encephalomyelitis; Eotaxin-2; Neutralizing mono-

clonal antibodies

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INTRODUCTION

Experimental autoimmune encephalomyelitis (EAE) is a T helper cell type 1 (Th1) mediated demyelinating disease of the central nervous system (CNS) that serves as an animal model for multiple sclerosis (MS)^[1-3]. EAE can either be induced by active immunization with whole myelin or a variety of myelin antigens plus adjuvant, or by passive transfer of encephalitogenic T cells. During induction of EAE, T cells sensitized to myelin antigens migrate across the blood-brain barrier (BBB) into surrounding white matter^[4], re-encounter antigen and become stimulated to release proinflammatory cytokines^[5] and chemokines^[6], for which there is compelling evidence for roles in lesion pathogenesis, including dysfunction of the BBB, demyelination, axonal injury and neurodegeneration^[3].

Chemokines are chemoattractants produced under pathological conditions by tissue elements and infiltrating leukocytes^[7], which were found to be involved, not only in leukocyte trafficking, but also in leukocyte maturation and renewal of circulating leukocytes^[8]. During EAE, involvement and up-regulation of several CC chemokines, including macrophage inhibitory protein-1a (MIP-1a) and macrophage chemoattractant protein-1 (MCP-1), are well established^[9]. *In vivo* neutralization studies have shown a distinct role for MIP-1a in the pathogenesis of acute EAE and for MCP-1 in relapsing EAE^[10].

Eosinophil chemotactic protein 2 (eotaxin-2 or eo-2), also known as CC ligand 24 (CCL24) or myeloid progenitor inhibitory factor 2 (MPIF-2), is a CC chemokine which interacts with the CC chemokine receptor 3 (CCR3) to induce chemotaxis in eosinophils^[11]. This chemokine was also found to be strongly chemotactic for basophils and resting T lymphocytes, and slightly chemotactic for neutrophils^[12]. Eo-2 mRNA is expressed in activated T lymphocytes, GM-CSF treated macrophages^[12] and dermal fibroblasts^[13], indicating a possible route for cross-talk between activated T lymphocytes and macrophages with eosinophils.

The role of eo-2 in eosinophils-mediated classic disorders, such as asthma^[14], chronic bronchitis^[15] and allergic reactions^[16], has been well established. However, it should be noted that the eo-2 receptor CCR3 expression is not restricted to eosinophils but it is also expressed on other inflammatory cells, such as monocytes^[17], mast cells^[18], peripheral memory T cells^[19], Th2 lymphocytes^[20] and immature dendritic cells^[21]. This emphasizes the complexity of the eo-2/CCR3 system and raises

the possibility of eo-2/CCR3 system involvement in a wide range of inflammatory and autoimmune disorders, far exceeding its role in allergy and atopy. Indeed, it has been previously shown that CCR2, CCR3 and CCR5 expression is elevated in MS CNS tissue compared to control CNS tissue, suggesting that the eo-2/CCR3 system might also be involved in MS pathogenesis^[22].

We have recently demonstrated that treatment of adjuvant-induced arthritis (AIA), a commonly used animal model of rheumatoid arthritis (RA), with our developed D8 anti-eo-2 neutralizing mAb was effective in ameliorating AIA, both as a preventive treatment given before development of arthritis and as a therapeutic agent given at the time of the initial manifestation of arthritis^[23].

The aims of the current study were: to evaluate the effect of blocking the eo-2 pathway on the development and severity of EAE; to study the effect of this treatment on humoral-mediated response in our EAE model, *i.e.*, sera levels of anti-myelin oligodendrocyte glycoprotein antibody (anti-MOG) autoantibodies; and on the levels of the cytokines: interleukin-6 (IL-6), interferon- γ (IFN- γ), tumor necrosis factor (TNF), IL-12p70 and MCP-1.

MATERIALS AND METHODS

Production of monoclonal antibodies directed against eo-2

We have produced several clones of monoclonal antibodies (mAbs) against eo-2, according to standard protocols. Briefly, Balb/C mice were immunized with 20 μ g of eo-2 (Peprotech, Rocky Hill, NJ, United States) followed by 4 additional boosts. After confirming the presence of polyclonal anti-eo-2 Abs in the sera, mice were sacrificed and their spleens were hybridized with a NS/0 myeloma line, followed by clonal screening for binding to eo-2. The hybridomas were then grown in serum-free media for 2-3 wk and media collected and loaded onto 100 kDa centricons (Biological Industries, Beit Haemek, Israel) for antibody concentration. D8 refers to the anti-eo-2 mAb clone which was selected to treat the mice with EAE. The cross-reactivity of D8 between human and murine eotaxin-2 [5 μ g eotaxin-2 diluted in phosphate-buffered saline (PBS)], with Kd of 0.77 mg and 4 mg, respectively, was determined.

EAE induction

EAE was induced in 6-8 wk C57BL/6 female mice (Harlan Laboratories, Jerusalem, Israel) by subcutaneous immunization on days 0 and 7 at two sites with 200 μ g/mouse myelin-oligodendrocyte glycoprotein peptide (MOG₃₅₋₅₅, synthesized by Sigma-Aldrich) in 100 μ L PBS. The peptide was emulsified in an equal volume of Complete Freund's Adjuvant (CFA, from DIFCO) containing 500 μ g *Mycobacterium tuberculosis* H37RA (MT, from DIFCO)^[24]. Mice were maintained at the local animal facility and all procedures were performed under the supervision and guidelines of the Animal Welfare Committee.

Treatment of EAE-induced mice with anti eo-2 neutralizing mAb

EAE-induced mice were injected daily intraperitoneally with either 25 µg or 100 µg D8, or with vehicle control only (PBS), starting from the day of immunization (day 0). Animals were monitored for symptoms of EAE and scored as follows: 0, no disease; 1, tail paralysis; 2, hind limb weakness; 3, hind limb paralysis; 4, hind limb plus forelimb paralysis; and 5, moribund/death.

ELISA for detection of anti-MOG autoantibodies

Mice were sacrificed on day 58 and their sera were assessed for the presence of anti-MOG autoantibodies. For this purpose, a flat-bottom 96-well plate (Greiner bio-one) was coated with 10 µg/mL MOG35-55 peptide (Sigma-Aldrich) in carbonate buffer (0.05 mol/L NaHCO₃, pH 9.5) overnight at 4 °C. The next day, the plate was blocked with 2% bovine serum albumin (BSA, Sigma-Aldrich) in PBS for 1 hour at room temperature. To detect serum antibodies, sera were diluted 1/25 in PBS with 0.5% BSA. The diluted sera were then added to the plates (100 µL/well in duplicates) and incubated for 2 h at room temperature. Bound antibodies were detected using 1/8000 diluted horseradish-peroxidase (HRP) conjugated goat anti-mouse IgG secondary antibody (Santa-Cruz Biotechnology, United States). 3,3',5,5'-Tetramethylbenzidine (TMB) reagent (Chemicon-Millipore) was used as a substrate solution and the reaction was halted by the addition of 1 mol/L H₂SO₄. Absorbance at 450 nm was measured using a Thermo Max ELISA reader (Molecular Devices microplate reader, United States).

Assessment of pro-inflammatory cytokines profile

Sera of EAE-induced mice were assessed for the presence of IL-6, IFN-γ, TNF-α, IL-12p70 and MCP-1 using the BD™ Cytometric Bead Array (CBA) Mouse Inflammation Kit, according to the manufacturer's instructions (BD Biosciences, United States). Briefly, test samples or recombinant standards of the cytokines were incubated with beads coated with capture antibodies specific for IL-6, IFN-γ, TNF, IL-12p70 and MCP-1 proteins and PE-conjugated detection antibodies to form sandwich complexes. Samples were analyzed on a FACScan flow cytometer, using CellQuest software (Becton Dickinson).

Histological assessment

EAE-induced mice and their healthy C57BL/6 littermates brains were removed, snap-frozen and kept at -80 °C until examination. Brains were sectioned at 8 µm and stained with hematoxylin and eosin.

Statistical analysis

Two-tailed Student's *t* test was performed when 2 groups were compared. The 1-way analysis of variance (ANOVA), followed by Tukey's test for multiple comparisons, was carried out for statistical analysis of the clinical

course of EAE. *P* < 0.05 was considered statistically significant. Results are expressed as mean ± SEM unless otherwise specified in the text.

RESULTS

Anti-eo-2 neutralizing mAb treatment ameliorates the clinical course of progressive EAE

Monoclonal antibodies against human eo-2 were developed in our laboratory. As previously described^[24], of our newly-developed monoclonal antibodies, D8 was selected for *in vivo* treatment since it has been demonstrated to possess neutralizing activity, *i.e.*, to inhibit adhesion of murine and rat splenocytes as well as human peripheral blood mononuclear cells (PBMCs) to fibronectin, to inhibit their migration towards vascular endothelial growth factor (VEGF) and to reduce adhesion of HEK cells stably transfected with CCR3 to eo-2 (data not shown), indicating that D8 interferes with the CCR3/eo-2 binding interaction.

A moderate model of monophasic (progressive) EAE was achieved by immunization of C57BL/6 mice with two following subcutaneous injections of MOG35-55 peptide, emulsified in CFA, with an interval of 1 wk^[25]. EAE-induced mice were injected daily intraperitoneally with 25 µg or 100 µg D8, starting from day 0 post immunization. EAE mice treated with total mouse IgG, or with vehicle control only (PBS) served as negative controls. As shown in Figure 1, all EAE-induced mice started to display clinical symptoms on days 14-17 post immunization. As expected from this monophasic model, a gradual increase in clinical score was observed in PBS-treated EAE mice until a maximal average score of 2.44 was observed on day 45, which remained constant until day 58. A similar trend, although more moderate, of a gradual increase in EAE severity, was also observed in total IgG treated mice until a maximal average score of 2.22 was observed on day 58, indicating that total IgG treatment did not significantly affect EAE severity. Interestingly, though initially both D8 doses (25 µg and 100 µg) exhibited a higher average clinical score in comparison to the total IgG treated group, this trend was inverted on day 32, from which both D8 treated groups exhibited an improved average score compared to PBS and IgG treated mice. Treatment with both concentrations of D8 led to a significant improvement in EAE clinical score compared to PBS treated mice. This significant effect was first observed on day 42, in which D8 25 µg and D8 100 µg treatment led to a decline of 57.9% in average clinical score (0.89 ± 0.35 in D8 25 µg and 2.11 ± 0.38 in IgG treated group, *n* = 10 in each group, *P* = 0.03), and remained constant until day 58.

However, a significant improvement in EAE clinical score compared to total IgG treated mice was observed only with the higher concentration of D8 on day 56, in which treatment with D8 100 µg led to a decline of 61.5% in average clinical score (0.81 ± 0.38 in D8 100 µg treated

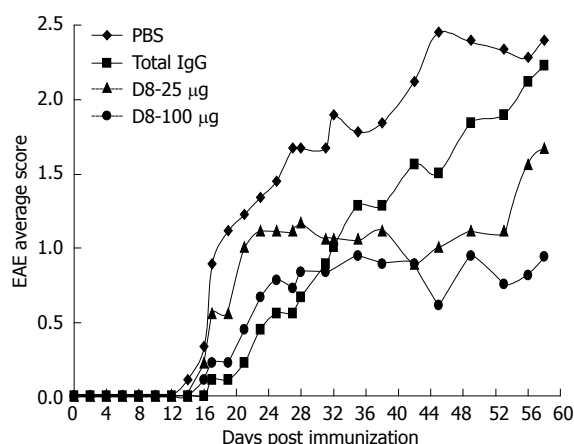


Figure 1 Anti-eo-2 neutralizing mAb treatment effect on progressive experimental autoimmune encephalomyelitis clinical course. Progressive experimental autoimmune encephalomyelitis (EAE) was induced in female C57BL/6 mice by immunization with two following subcutaneous injections of MOG₃₅₋₅₅ peptide, emulsified in CFA, with an interval of 1 wk. EAE-induced mice were injected daily intraperitoneally with 25 µg or 100 µg D8, mouse IgG, or with vehicle control only (PBS) starting from day 0 post immunization, and monitored for EAE clinical score. A significant improvement in EAE clinical score compared to total IgG treated mice was observed only with the higher concentration of D8 on day 56, in which treatment with D8 100 µg led to a decline of 61.5% in average clinical score ($n = 10$ in each group, $P = 0.04$, one way ANOVA).

group vs 2.11 ± 0.31 in PBS treated group, $n = 10$ in each group, $P = 0.04$). Thus, it can be concluded that treatment with both concentrations of D8 ameliorated EAE severity, although it appears that treatment with the higher concentration of D8 (100 µg) is more effective.

Anti-eo-2 neutralizing mAb treatment does not significantly affect anti-MOG antibody response

In contrast to other models, MOG₃₅₋₅₅ protein elicited EAE is also characterized by a pathogenic antibody response. Although anti-MOG antibodies cannot induce EAE on their own, they strongly enhance T cell and macrophage-initiated demyelination and may augment disease severity^[25,26]. Since it has been previously demonstrated that the severity of EAE might correlate with the presence of MOG-specific autoantibodies, our next purpose was to examine the effect of anti-eo-2 neutralizing mAb treatment on serum levels of anti-MOG autoantibodies. As demonstrated in Figure 2, although treatment with 25 µg D8 led to a significant decrease of 55.6% in the level of anti-MOG IgG antibodies compared to PBS treatment, as detected in EAE-induced mice sera on day 58 ($n = 9$ in each group, $P = 0.007$), no significant effect in the level of anti-MOG IgG antibodies was seen in both D8 treated groups compared to the IgG treated group, indicating that the clinical anti-eo-2 neutralizing mAb treatment effect in EAE is probably not mediated through the humoral anti-MOG antibodies response.

Anti-eo-2 neutralizing mAb treatment decreases Th1-mediated response

We next examined the effect of anti-eo-2 neutralizing

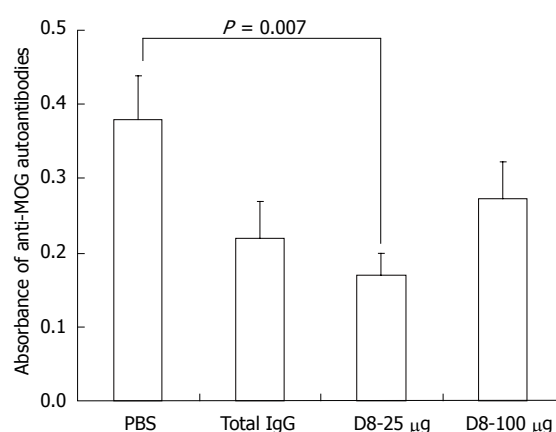


Figure 2 The effect of anti-eo-2 neutralizing mAb treatment on the level of anti-MOG₃₅₋₅₅ autoantibodies in experimental autoimmune encephalomyelitis sera. Experimental autoimmune encephalomyelitis (EAE) mice daily treated with PBS, total mouse IgG, 25 µg or 100 µg D8, were sacrificed on day 58 and their sera were assessed for the presence of anti-myelin oligodendrocyte glycoprotein (anti-MOG) autoantibodies using ELISA. No significant effect in the level of anti-MOG IgG antibodies was accepted in both D8 treated groups compared to the IgG treated group (values presented are $A_{450 \text{ nm}}$, $n = 9$ in each group).

mAb treatment on serum levels of the cytokines IL-6, IFN- γ , TNF- α , IL-12p70 and the chemokine MCP-1. As shown in Figure 3, treatment of EAE-induced mice with D8 100 µg led to a significant decrease of 57.3% in serum levels of MCP-1 compared to IgG treatment (27.2 ± 3.1 pg/mL in D8 100 µg treated mice vs 63.7 ± 12.3 pg/mL in IgG treated mice, $P = 0.03$), a decrease of 61.2% in serum levels of IFN- γ (1.4 ± 0.6 pg/mL in D8 100 µg treated mice vs 3.6 ± 0.4 pg/mL in IgG treated mice, $P = 0.02$) and a reduction of 60.8% in levels of TNF- α (7.8 ± 0.2 pg/mL in D8 100 µg treated mice vs 19.9 ± 3.4 pg/mL in IgG treated mice, $P = 0.005$). Although a similar trend for reduction of IL-12p70 sera levels was accepted in D8 100 µg treated mice vs total IgG treated mice, this effect was found to be non significant (3.9 ± 1.5 pg/mL in D8 100 µg treated mice vs 8.6 ± 4.2 pg/mL in IgG treated mice, $P = \text{not significant}$). Serum levels of IL-6 did not seem to be affected by D8- 100 µg treatment.

Anti-eo-2 neutralizing mAb treatment decreases cellular infiltration into the CNS

Histopathological analysis of EAE-induced mice brains, treated with either D8 100 µg or with IgG, and their healthy C57BL/6 littermates, demonstrates that the extent of cellular infiltration in the D8 100 µg treated group is very mild compared with the IgG treated group (Figure 4).

DISCUSSION

Although eosinophils have been observed in the spinal fluid of MS patients^[27,28], their role in MS pathology has been poorly investigated. Gladue *et al.*^[29] reported that EAE treatment with the specific LTB₄ receptor antagonist CP-105,696 selectively inhibited eosinophils recruitment into the spinal cord, without inhibition of

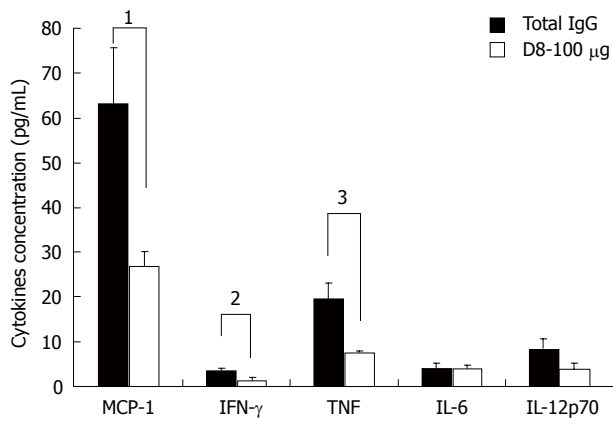


Figure 3 Anti-eo-2 neutralizing mAb treatment effect on pro-inflammatory cytokines profile. Experimental autoimmune encephalomyelitis (EAE) mice sera from total IgG and 100 μg D8 groups were assessed for the presence of interleukin (IL)-6, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, IL-12p70 and macrophage chemoattractant protein (MCP)-1 using the BD™ Cytometric Bead Array Mouse Inflammation Kit. Treatment of EAE-induced mice with D8 100 μg led to a significant decrease of 57.3% in serum levels of MCP-1, 61.2% in serum levels of IFN-γ and 60.8% in levels of TNF-α, compared to IgG treatment ($n = 6$ in each group, $^1P = 0.03$, $^2P = 0.02$, $^3P = 0.005$, two-tailed Student's *t*-test).

lymphocyte infiltration into the CNS, and concomitantly prevented EAE symptoms. This finding led to the hypothesis that the role of eosinophils in EAE may have been underestimated in previous studies and that blockade of eosinophil infiltration into the CNS may represent a potential therapeutic target in MS, in addition to the well known strategy of restraining activated T cells and monocytes.

In this current study, we blocked the eo-2 pathway directly involved in eosinophil migration in EAE-induced mice, by our developed specific D8 anti-eo-2 neutralizing mAb. Treatment with D8 significantly ameliorated EAE clinical score in a trend of a dose-dependent manner. Whereas the trend of an improved clinical score in both D8 treated groups *vs* PBS treated group was observed during the whole experiment, ameliorated EAE symptoms in both D8 treated groups *vs* IgG treated group was seen only from day 32. This finding could imply that although the initial beneficial effect of D8 is probably not specific, a specific effect of blocking the eo-2 pathway, mediated by the mAb D8 occurs in later stages of the disease.

Theoretically, the clinical beneficial effect of blocking the eo-2 pathway in EAE could be explained merely by inhibiting eosinophil infiltration into the CNS^[29]. We hypothesized that this therapeutic effect of D8 involves an expanded immune reaction and might also be mediated *via* restraining T cells and monocyte responses since MS is rarely associated with eosinophilia.

Although we found that treatment of EAE-induced mice with D8 did not significantly affect the humoral response, as examined by the level of anti-MOG IgG autoantibodies in mice sera, it had a significant impact on T cell and monocyte mediated responses, *i.e.*, the level of

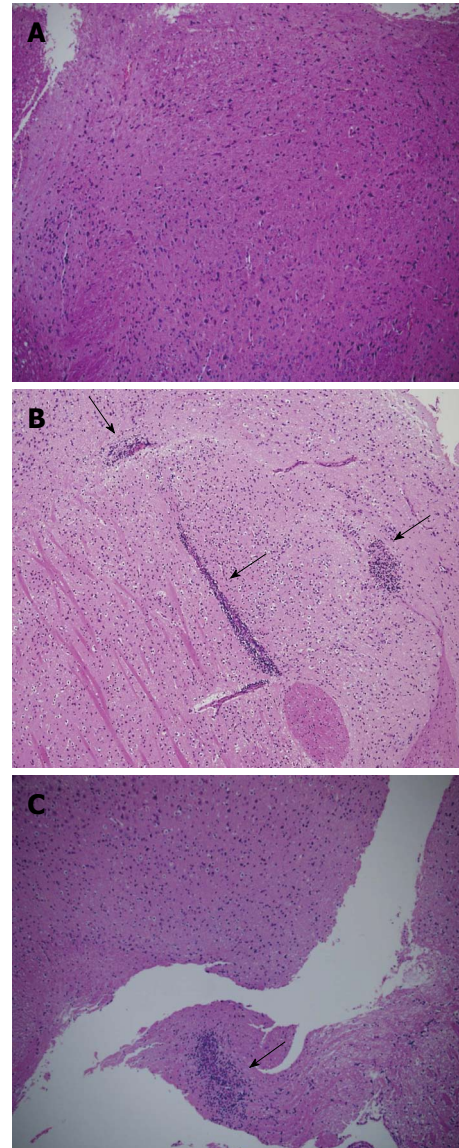


Figure 4 The extent of cellular infiltration in anti-eo-2 mAb treated experimental autoimmune encephalomyelitis mice is low compared with PBS treated experimental autoimmune encephalomyelitis mice and their healthy littermates. A: Hematoxylin and eosin staining of representative brain sections from healthy C57BL/6 mice; B: PBS treated experimental autoimmune encephalomyelitis (EAE) mice; C: EAE mice treated with 100 μg D8. Lower extent of cellular infiltration in D8 100 μg treated group is observed compared with the IgG treated group. Arrows indicate inflammatory infiltration. Magnification × 200.

the proinflammatory (Th1 type) cytokines TNF-α and IFN-γ in the sera. Histological examination of EAE-induced murine brains confirmed that D8 treatment inhibited immune cell infiltration into the CNS. Since eosinophils tend to appear in the lower area of the spinal cord in EAE near the cauda equina^[29], it can be assumed that the reduced cellular infiltrates in EAE mice treated with D8 brains is a result of reduced T cells and monocyte infiltration into the CNS.

How might blocking the eo-2 pathway affect monocyte infiltration into the CNS? The answer is probably concealed in the complex cross-talk between different chemokines. Indeed, we found that by blocking the eo-2

pathway directly involved in eosinophil chemotaxis, the level of MCP-1, primarily involved in monocytes chemotaxis, significantly diminished. This finding is not surprising since it has been previously demonstrated that peripheral blood monocytes express and secrete both bioactive eo-2 and MCP-1 constitutively, and that both of these chemokines production in monocytes stimulated with LPS is regulated by IL-4^[30]. Thus, a reciprocal regulation mechanism might exist in which the level of each of these CC chemokines might be influenced by the other.

The role of MCP-1 in EAE pathogenesis has been well established. It has been previously demonstrated that C57BL/6 MCP-1-null mice exhibit markedly reduced clinical and histological EAE after active immunization and do not develop clinical disease after receiving encephalitogenic T cells from wild-type animals. Moreover, disruption of the MCP-1 gene led to an attenuated Th1 pathogenic response and additionally increased the Th2 protective response^[31].

The correlation between IL-6 and TH17 responses, in general as well as specifically in EAE, has been previously described^[32,33]. Since we did not detect lower sera levels of IL-6 in D8 treated EAE mice, we do not believe that eo-2 blockade mode of action is mediated *via* restriction of TH17 pathogenic responses. Nevertheless, this aspect remains open and should be further investigated. Moreover, given the well recognized protective role of IL-10, TGF- β and IL-4 in EAE, as well as the putative role of the pro-inflammatory cytokines, IL-17 and IL-23, in EAE induction^[34-38], the effect of eo-2 blockade on the levels of these cytokines in the sera should be further studied.

Our results imply that the main mode of action of eo-2 blockade is mediated *via* the restriction of cellular responses rather than affecting humoral responses. Therefore, we did not focus in this study on the effect of D8 treatment on the humoral responses and the effect of D8 on IgG sub-classes, such as IgG1 and IgG2a, remains unclear.

Taken together, although the exact mode of action of eo-2 blockade should be further characterized, our results indicate that eo-2 plays a critical role in EAE pathogenesis and that blocking the eo-2 pathway ameliorates EAE, either by direct inhibition of eosinophil infiltration into the CNS or by indirect impact on MCP-1 level, involved in monocyte infiltration into the CNS. Herein, these findings support a therapeutic potential of anti-eo-2 neutralizing antibody in EAE, as well as motivation for a continuing effort to study the role of the eo-2 pathway in MS.

COMMENTS

Background

Multiple sclerosis (MS) is a chronic, inflammatory, demyelinating disease that affects the central nervous system (CNS). Although it is still unclear how exactly MS initiates, it is well recognized that autoreactive T cells generated in the systemic compartment migrate into the CNS where they persist and induce an

inflammatory cascade, which includes recruitment of macrophages and activation of local microglia. The recruitment of inflammatory cells into the CNS is mediated by chemokines. Eosinophil chemotactic protein 2 (eotaxin-2 or eo-2) is known to induce chemotaxis, primarily in eosinophils. Nonetheless, authors have previously demonstrated that our developed neutralizing mAb against eo-2, named D8, was effective in ameliorating other inflammatory diseases not classically eosinophil mediated, such as adjuvant-induced arthritis (AIA).

Research frontiers

Experimental autoimmune encephalomyelitis (EAE) is the most commonly used animal model for testing new therapeutic agents in the field of MS. Progressive EAE, which resembles the progressive pattern of MS in humans, is induced by immunization of C57BL/6 mice with the autoantigen MOG₃₅₋₅₅. The research hot spot was to examine the effect of inhibiting eo-2 with the neutralizing mAb, D8, on the development and severity of EAE.

Innovations and breakthroughs

Although eosinophils have only rarely been associated with MS pathogenesis, we have demonstrated that direct blockage of the eo-2 pathway may possess therapeutic properties in EAE. This effect was found to be mediated by restricting cell-mediated responses, *i.e.*, reducing T cells and monocyte infiltration into the CNS, but not substantially affecting humoral responses. Restriction of cell-mediated responses may be derived from the observed reduced levels of pro-inflammatory cytokines, tumor necrosis factor (TNF)- α and interferon (IFN)- γ , as well as diminished levels of the chemokine macrophage chemoattractant protein (MCP)-1.

Applications

The results suggest that blockage of the eo-2 pathway by D8 may represent a new therapeutic strategy for MS. Moreover, these results raise the need for further research in order to gain a better insight of the role of eosinophils in MS pathogenesis.

Terminology

A neutralizing antibody is an antibody which neutralizes or inhibits the biological activity of its antigen. Cell-mediated response is an immune response that does not involve antibodies but rather involves the activation of macrophages, antigen-specific T-lymphocytes and the release of various cytokines in response to an antigen. Humoral-mediated response is the aspect of immunity that is mediated by secreted antibodies.

Peer review

The authors have previously shown that blocking the eo-2/CCR3 interaction by anti-eo-2 neutralizing mAb (D8) improves the therapeutic outcome of inflammatory diseases such as AIA. In this study, the authors took similar approaches to test this D8 mAb in another autoimmune model, EAE. They found that daily treatment of MOG₃₅₋₅₅ induced-EAE mice with anti-eo-2 neutralizing mAb (D8) significantly decreased the severity of EAE in a dose-dependent manner. While D8 treated EAE mice did not show lower sera levels of anti-MOG autoantibody, they expressed lower levels of the pro-inflammatory cytokines, such as TNF- α , IFN- γ and the chemokine MCP-1, in the serum. They also found that blocking the eo-2 pathway by D8 affects the infiltration of eosinophils, monocytes and T cells into the CNS. These data are expected as the authors found similar results in AIA.

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WJI covers a wide range of subjects including: (1) autoimmune diseases such as type 1 diabetes, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, thyroiditis, myasthenia gravis, in both humans and animal models of disease, with an interest on aspects including the etiology, pathogenesis, mechanisms of disease induction, maintenance and progression; (2) tumor immunology including immunosurveillance, immunoediting and immunotherapies in animal models and in humans; (3) clinical immunology in humans and animal models including mechanisms of disease, regulation and therapy and immunodeficiencies; (4) innate immunity including cell subsets, receptors and soluble mediators, complement and inflammation; (5) adaptive immune mechanisms and cells including soluble mediators and antibodies; (6) immune cell development, differentiation, maturation; (7) control mechanisms for immune cells including immune tolerance and apoptosis; (8) immune cell interactions and immune cell receptors; (9) immunological methods and techniques; (10) immune cell activation including cell signaling pathways, biochemical and pharmacologic modulation studies; (11) infection; (12) different modalities of vaccination including gene therapy; (13) hypersensitivity and allergy; (14) transplantation.

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Cell transplantation therapy using pluripotent stem cells

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Core tip: The 2012 Nobel Prize in Physiology or Medicine was awarded jointly to Sir John B Gurdon and Shinya Yamanaka "for the discovery that mature cells can be reprogrammed to become pluripotent". Induced pluripotent stem (iPS) cells derived from healthy and sick patients could be a useful source for drug discovery, while healthy iPS cells could serve as a unique and highly promising source for future cell transplantation therapies and regenerative medicine.

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Abstract

The 2012 Nobel Prize in Physiology or Medicine was awarded jointly to Sir John B Gurdon and Shinya Yamanaka "for the discovery that mature cells can be reprogrammed to become pluripotent". Professor John B Gurdon who pioneered the field of somatic cell nuclear transfer was the first to show that a nucleus of a mature cell can be transplanted into an enucleated egg and give rise to a living organism. His pioneering "cloning" technique paved the way for genome reprogramming and has led to subsequent cloning of different animal species. Professor Shinya Yamanaka revolutionized the field of stem cell production by showing that the introduction of four selected genes into cells transform them into induced pluripotent stem cells that resemble embryonic stem cells and serve as promising cells for future regenerative medicine.

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Key words: Stem cells; Induced pluripotent stem cells; Somatic cell nuclear transfer; Nobel Prize

COMMENTARY ON HOT TOPICS

One of the most fascinating fields of the present biomedical research relies on the development of new technologies that enable reprogramming of mature cells and induction of their de-differentiation into young or embryonic cells that can later form many different cell types. The cells formed in this process, termed induced pluripotent stem (iPS) cells, represent a promising solution for cell-based therapy and regenerative medicine.

The pioneering work of two eminent scientists in this field has recently been recognized by the Nobel committee, who decided to jointly award the 2012 Nobel Prize in Physiology or Medicine to Sir John B Gurdon and Shinya Yamanaka "for the discovery that mature cells can be reprogrammed to become pluripotent".

The two new Nobel Laureates, Professor John B Gurdon from the Wellcome Trust-Cancer Research United Kingdom Institute for Cell Biology and Cancer (that was renamed the Gurdon Institute, in his honor), at the University of Cambridge, United Kingdom, and Professor Shinya Yamanaka, from the Center for iPS Cell Research and Application at Kyoto University, Japan made groundbreaking discoveries by demonstrating that

specialized somatic cells that are fully differentiated, can be reprogrammed to become pluripotent immature cells. These newly formed cells are capable of differentiating into all types of cells that generate the body's tissues and organs. The findings of Gurdon and Yamanaka have revolutionized our understanding of how cells and organisms develop.

John B Gurdon pioneered the field of somatic cell nuclear transfer. He was the first to demonstrate, in the early sixties, that cell specialization is a reversible process. In a classic experiment, Gurdon *et al*^[1] transplanted a nucleus from a mature *Xenopus laevis* intestinal cell into an enucleated *Xenopus laevis* egg and found that the modified egg cell developed into a normal tadpole. The phenotype of the tadpole was identical to that of the nucleus donor. Gurdon^[2,3] demonstrated therefore that a nucleus of a differentiated somatic cell could undergo reprogramming and acquire a state of totipotency. He further demonstrated that the adult frogs arising from the nuclei-transplanted egg were healthy and capable of reproduction^[2,3]. This experiment indicated that the DNA of the mature cell included the entire genetic information required for the development of all cells in the frog.

The discoveries by Gurdon *et al*^[1] paved the way for additional somatic cell nuclear transfer experiments that were performed in different species, with "Dolly the sheep" being the most famous cloned animal. Dolly was produced by Wilmut *et al*^[4] who took a nucleus from an adult mammary gland cell and transferred it into an unfertilized and enucleated egg. Dolly represented a true clone since the genomic DNA of her cells, including the DNA microsatellites, were identical to that of her parent DNA, which served as the cell nucleus donor. Dolly lived a normal life and gave birth to six normal and healthy lambs. She was euthanized in 2003 because of severe arthritis and progressive lung disease. Although it was suspected that Dolly, as well as other cloned animals were destined to age prematurely, intensive health screening did not reveal abnormalities in Dolly that could result from advanced aging.

Many cell cloning attempts were performed in the past few years in a wide range of species (including mouse^[5], rabbit^[6], cat^[7], horse^[8], ferret^[9], and other species), using the somatic cell nuclear transfer method with variable degrees of success. These studies further substantiated Gurdon's conclusions that the genome of adult cells contains the entire information necessary to generate living organisms.

A significant leap in the stem cell technology progress was made in 2006 by Yamanaka *et al* who were the first to induce de-differentiation of mature cells and their conversion into immature cells that resemble embryonic stem (ES) cells. Shinya Yamanaka and his graduate student, Kazutoshi Takahashi, first compiled a large set of genes that are known to be expressed in stem cells and are suspected of accounting for the pluripotency of the human ES cells. By ectopic expression of a set of 24 individual genes in adult fibroblast, the researchers succeeded in obtaining cells that

appeared to have the morphology of human ES cells. The researchers then repeated the experiment multiple times, using subsets of these genes, in a search for a minimal number of genes that were sufficient for reverting the somatic cells into ES cells.

In this remarkable breakthrough study, published in 2006 by Takahashi *et al*^[10], the researchers reported their success in converting mouse embryonic and adult fibroblasts into pluripotent stem cells by introducing a set of only four regulatory genes into the fibroblasts under ES cell culture conditions. These regulatory genes, namely octamer-binding transcription factor 3/4, sex determining region Y-box 2, v-myc myelocytomatosis viral oncogene homolog (avian), and Krüppel-like factor-4, encode transcription factors that are required and sufficient for the maintenance of the essential properties of normal ESCs. Yamanaka's reprogrammed cells, termed "induced pluripotent stem cells", exhibited the morphology and growth properties of ES cells and expressed ES cell marker genes. Upon their transplantation into nude mice, the iPS cells formed polyclonal tumors representing tissues from all three germ layers.

In a following publication by Takahashi *et al*^[11], the authors demonstrated that the technology for iPS cell generation could be applied to human cells. In this study, the researchers succeeded in reprogramming adult human dermal fibroblasts to iPS cells with the same four factors they have previously used in the preparation of mouse iPS. The researchers demonstrated that the human fibroblast-derived iPS cells were similar to human ES cells in just every parameter tested, including morphology, growth behavior, surface markers, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity. The pluripotent human iPS cells were capable of differentiating into cell types representing all three germ layers both *in vitro* and *in vivo*. Transplantation of the iPS cells into the dorsal flank of immune compromised mice resulted in the formation of teratomas and histological examination of these tumors revealed tissues of endodermal-, mesodermal- and ectodermal-origin.

Yamanaka's discoveries have completely changed our view of cell development, differentiation and maturation. The powerful technique he created for the reprogramming of human cells serves as a new platform for studies of fundamental questions in normal cell development. Within 6 years after its initial description, a large number of patient-specific iPS cell models were generated and characterized. Furthermore, a large number of patient-specific iPS cells prepared in the last few years from diseased human tissues generated enormous interest due to their potential use as physiological human disease models that can be studied *in vitro*. Studies of patient-specific iPS cells may help decipher the molecular basis of human diseases, identifying disease-related genes and proteins and determining their role in modifying cell behavior. In addition, these cells may contribute to the identification of new and novel drug targets and establish the basis for the design of new therapeutic approaches.

The pioneering work of cell reprogramming, significantly contributed to the understanding of the cellular and molecular mechanisms involved in cell reprogramming processes. Furthermore, iPS cells derived from healthy and sick patients could be a useful source for drug discovery, while healthy iPS cells could serve as a unique and highly promising source for future cell transplantation therapies and regenerative medicine.

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Role of the clinical immunology laboratory in disease monitoring

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Abstract

Immunological investigations provide useful information to guide diagnosis of several disorders. Many such tests are also commonly repeated at intervals, in an effort to facilitate disease monitoring. In general however, immunology test results are often slow to alter. Furthermore, audit activity has indicated that repeated testing accounts for a substantial workload in many immunology services, which may waste resources and compromise the efficient completion of necessary tests. Consequently, the need and appropriate minimum interval between repeated testing requires critical evaluation. In this review, the clinical utility of repeated performance of several common immunology investigations has been evaluated, based upon published evidence. In some cases (*e.g.*, paraprotein quantification, or measurement of anti-glomerular basement membrane antibodies), repeated testing provides vital clinical information and can be justified on a frequent and individualized basis. However, many other investigations provided by immunology services provide less

valuable information when used to aid disease monitoring rather than diagnosis. It is hoped that the data summarized here will facilitate a more evidence-based approach to repeated testing. Such information may also assist with the local implementation of demand management strategies based upon setting of minimum retesting intervals for these investigations.

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Key words: Clinical immunology; Immune monitoring; Retest; Test interval

Core tip: Immunological investigations provide useful information to guide diagnosis of several disorders. Many such tests are also commonly repeated at intervals, in an effort to facilitate disease monitoring. Here, the evidence underlying the need and appropriate minimum interval between repeated testing has been critically evaluated.

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INTRODUCTION

The clinical immunology laboratory serves an important role in the diagnosis of several disorders. Equally, periodic immunological testing can contribute to the monitoring of disease status. However, such tests are expensive, relatively slow to undergo change and are commonly repeated. In two recently published audits, up to 30% of tests were unnecessarily repeated within a 3-mo time window^[1,2]. Most clinical immunology laboratories experience a continued year on year increase in test requests. For example, combined data from two large National

Health Service Trusts has indicated that requests for serological screening for coeliac disease had doubled over a 5-year interval^[3]. Consequently, as part of their demand management strategy, some services have imposed minimum retesting intervals in order to reduce misuse and unnecessary investigation. However, the evidence base for the implementation of such protocols is scattered throughout several publications and is variable in quality. In this review, the role of several immunology tests in disease monitoring has been examined. The overall goal was to provide an evidence base to support the need for repeated testing and, where possible, to provide guidance on intervals that may be appropriate between retesting.

RHEUMATOLOGY AND NEPHROLOGY

Anti-nuclear antibody

Testing for anti-nuclear antibodies (ANA) plays an important role in the diagnosis of several rheumatological and other conditions. However, the major limitation of ANA testing is the high frequency of false positive results. Up to 30% of healthy individuals have ANA at a serum dilution (titre) of 1:40, while 5% remain positive at a titre of 1:160^[4]. This finding emphasises the importance of performing this test only in individuals who are suspected to have an ANA-associated illness. Once a diagnosis has been established, repeat testing for ANA in positive individuals is generally not useful unless clinical features change. This is because there is no evidence to indicate that alteration in ANA titre (or immunofluorescence pattern) provides useful information to guide the management of any illness^[4-6]. In summary therefore, there is no clear indication to repeat a positive ANA unless the result is not compatible with the clinical presentation, in which case the test should not have been requested in the first instance.

Because ANA-associated illnesses are often dynamic, it is sometimes reasonable to retest individuals with such a diagnosis where ANA testing has been negative. In general, the presence of ANA generally precedes diagnosis in relevant rheumatological conditions^[7]. However, in systemic lupus erythematosus (SLE) for example, ANA may occasionally be undetectable at the onset of symptoms ("ANA negative SLE"). While many factors may account for this finding, it has been estimated that up to 18% of such ANA negative SLE patients will "seroconvert" over the course of their illness^[6,8], providing a rationale for retesting in highly selected cases.

Anti-double stranded DNA antibody

The only disorder for which the detection of anti-double stranded (ds)DNA antibodies is diagnostically useful is SLE. The level or titre of these antibodies is dynamic and can provide useful information in monitoring the activity of SLE. A rise in anti-dsDNA antibodies often precedes SLE exacerbation by a few weeks^[9]. High titres have been identified in lupus nephritis and their levels tend to rise and fall in direct proportion to disease activ-

ity^[10]. However, the relationship between titre of anti-dsDNA antibodies and disease activity in SLE is not absolute. Thus, an increment in the titre of anti-DNA antibody does not predict with certainty that a subsequent SLE flare will occur^[11]. The converse is also true in that SLE patients may have a disease flare without a prior increase in anti-dsDNA antibody levels^[11]. Two recently conducted prospective case series have highlighted the limitations of anti-dsDNA antibody testing in mirroring disease status^[12,13]. Consequently, alterations in the level of these antibodies should never be used in isolation to guide disease management. Against this background, the optimum frequency of retesting of anti-dsDNA antibodies is unknown^[11]. However, since the half-life of most immunoglobulin G (IgG) sub-classes is approximately 3 wk, the minimum period required to detect significant alterations of antibody titres should be at least an interval of 4 or more weeks^[5,6,14,15].

Antibody against extractable nuclear antigens

Antibodies against extractable nuclear antigens (ENA) (e.g., SSA/Ro, SSB/La, Sm and/or U1-RNP) are commonly used to support the diagnosis of several conditions, notably SLE, Sjogren's syndrome and mixed connective tissue disease. The presence of these antibodies tends to be stable over time so that if the test is positive, repeat testing is generally not helpful^[16,17]. Furthermore, alterations in titre of these antibodies do not predict the need for changes in disease management^[18]. Similarly, anti-centromere and anti-scl70 antibodies tend to remain stable over time and do not correlate with disease activity^[19]. Occasionally however, it is recognised that autoantibodies within the above groups may either emerge or recede over time in patients with rheumatological conditions^[8], providing a rationale for selected retesting.

Rheumatoid factor

Rheumatoid factor (RF) is a generic term for antibodies that react with the Fc portion of the IgG molecules. The presence of RF in both children and adults with rheumatoid arthritis (RA) correlates with a more aggressive and erosive disease course^[20]. Recent studies have indicated that RF tends to be stable over time other than a trend to reduction in levels^[21-23]. Consequently, there seems little point in repeating this test over time.

Anti-cyclic citrullinated peptide antibodies

Anti-cyclic citrullinated peptide (CCP) antibodies are directed against citrulline residues, formed as a result of the post-translational modification of arginine. The presence of anti-CCP antibodies is a highly specific diagnostic marker of RA, correlating like RF with more aggressive disease. Current (2010) American College of Rheumatology guidelines include the use of anti-CCP antibodies in the classification of rheumatoid arthritis. In further similarity to RF, recent data indicate that anti-CCP antibodies remain stable over a period of up to 5 years. Consequently, re-testing over this period is not supported

by evidence^[21-23]. Furthermore, repeated measurement of anti-CCP-antibodies does not help to evaluate disease activity^[24].

Anti-phospholipid antibodies

Anti-phospholipid antibodies are a heterogeneous group of antibodies with reactivity against cell surface phospholipids and/or associated cofactor proteins. These antibodies may be found in patients with the anti-phospholipid syndrome (APS). Commonly, the diagnosis of APS is based upon the revised Sapporo classification criteria (also called the Sydney criteria)^[25,26] and requires one clinical (thrombotic or pregnancy-associated morbidity) and one laboratory criterion. The laboratory requirements for diagnosis of APS include either the presence of (1) lupus anticoagulant; (2) IgG and/or immunoglobulin M (IgM) anti-cardiolipin antibodies; or (3) IgG and/or IgM antibodies against β_2 glycoprotein I. However, false positive antibodies may be found in several circumstances, including infection and even in normal individuals. Consequently, in patients with initial positive results, the test must be repeated and found positive on a second occasion, after a minimum interval of 12 wk. Experience indicates that in the majority of cases, repeat testing does not confirm the presence of these antibodies, highlighting the importance of such confirmatory testing^[27].

In light of these considerations, it is recommended that the minimum re-testing interval for all three tests is set at 12 wk. Although evidence is limited, it appears that anti-phospholipid antibodies are generally stable for over 2 years from diagnosis and are not influenced by commonly used therapies such as warfarin, aspirin and/or hydroxychloroquine^[28]. Poor assay standardisation makes comparison of serial results from different assays challenging and the relationship of titre to disease activity in SLE is also contentious^[28]. Consequently, the role of repeated testing beyond the diagnostic phase would appear to be limited.

Anti-neutrophil cytoplasmic antibody

Anti-neutrophil cytoplasmic antibody (ANCA) detection is a valuable tool for diagnosing small-vessel vasculitis, including granulomatosis with polyangiitis (GPA, Wegener's granulomatosis), microscopic polyangiitis, eosinophilic GPA (Churg-Strauss syndrome) and primary pauci-immune crescentic glomerulonephritis. International guidelines for ANCA testing recommend screening for ANCA by indirect immunofluorescence microscopy, followed by enzyme-linked immunosorbent assay (ELISA) testing of positive samples^[29,30]. Positive immunofluorescence staining may be cytoplasmic (c-ANCA) or peri-nuclear (p-ANCA) in distribution. Commonly, but not always, c-ANCA positivity is associated with ELISA reactivity against proteinase 3 (PR3) while p-ANCA correlates with binding of myeloperoxidase (MPO). Initially, it was reported that ANCA quantification could reliably predict relapse of necrotising vasculitides and thereby guide treatment decisions^[31,32]. More recently however, evidence from sev-

eral sources has indicated that this application of ANCA testing is not sufficiently robust to be clinically useful in the majority of cases^[33-35]. This viewpoint has been confirmed in a recent meta-analysis^[36]. For individual patients in whom an association between PR3-ANCA levels and disease activity has been established, serial ANCA testing may have some predictive value for subsequent relapse^[37]. It is well recognised that PR3-ANCA positive patients are more likely to relapse than those with MPO-ANCA, indicating the need for closer monitoring of the former group^[38]. Furthermore, there is evidence that in PR3-ANCA positive patients, it is safer to progress from cyclophosphamide-based remission induction therapy to azathioprine maintenance therapy when ANCA have reverted to negativity^[39]. However, once clinical remission has been achieved, renal transplantation is equally successful, regardless of ANCA levels^[40]. Taken together, this indicates that serial ANCA measurement may be useful in some selected cases but that a straightforward role linking test results to disease activity is lacking.

Complement C3 and C4

In SLE, serum C3 and C4 levels commonly fall close to the time of a flare and return to higher concentrations after a minimum of several weeks of appropriate treatment. However, these findings are not uniform. Some patients have disease flares without a reduction in C3 or C4 levels. In keeping with this, a recent 6-year prospective study demonstrated that C3, C4 and anti-dsDNA antibody measurements had a satisfactory negative predictive value for lupus nephritis but that positive predictive value was inadequate^[12]. Furthermore, a second prospective study has highlighted the inadequacy of C3, C4 and anti-dsDNA antibody testing in monitoring disease activity in lupus^[13]. There are a number of reasons why reductions in C3 and C4 in particular may not necessarily indicate disease flare. For example, low levels of C4 are commonly seen in inactive SLE due to inherited partial C4 deficiency, which is common in SLE^[41]. Furthermore, C4 may be reduced even during the inactive stage of SLE. Nonetheless, serial C3 and C4 measurements may be useful in disease monitoring in individual patients. Furthermore, it has been suggested that C3 and C4 levels should be monitored monthly during pregnancy in patients with lupus^[42].

C1q antibodies

Anti-C1q antibodies bind to C1q, which is the first component of the classical complement pathway. These antibodies have been found in patients with SLE in addition to several other inflammatory rheumatological conditions, notably hypocomplementaemic urticarial vasculitis. In SLE, these antibodies are found more commonly in patients with lupus nephritis. Furthermore, antibody titre tends to be higher in association with active disease. Although disease flare may be seen in their absence, evidence is emerging to suggest that these antibodies provide one of the strongest biomarkers of active disease and serial measurement warrants further investigation^[12,43,44].

Anti-glomerular basement membrane antibody

Glomerular basement membrane (GBM) antibody disease is associated with auto-antibodies that bind the non-collagenous 1 domain of the $\alpha 3/\alpha 5$ chains of type IV collagen, found in the GBM and alveoli. Patients with anti-GBM antibodies commonly present with pulmonary renal syndrome and require intensive immunosuppression using cyclophosphamide, corticosteroids and plasmapheresis. In this context, frequent repeated antibody measurement can be used to guide therapy^[45]. Typically, plasmapheresis is performed every 1-2 d for at least 14 d or until anti-GBM antibodies are no longer detectable^[46]. In general, anti-GBM disease does not relapse and consequently does not require long-term immunosuppression. However, a proportion of patients also have ANCA and behave clinically more like patients with ANCA-associated vasculitis (e.g., with a propensity to relapse). In patients with anti-GBM disease who require renal transplantation, it is recommended that anti-GBM antibodies are undetectable for at least 6 mo previously in order to minimise the risk of disease recurrence^[46].

NEUROLOGY

Disorders of the neuromuscular junction

Myasthenia gravis (MG) is characterised by an immune-mediated failure of neuromuscular transmission. In part, the disease is mediated by auto-antibodies that bind to the postsynaptic nicotinic acetylcholine receptor (AChR) and muscle-specific receptor tyrosine kinase (MuSK) receptor complex. However, no studies have confirmed the usefulness of serial auto-antibody measurement in MG^[47].

Antibodies directed against the AChR are detected in approximately 80% of patients with MG and thus contribute importantly to diagnosis of this disease. Although individual patients may vary, neither the presence nor the level of AChR antibodies on serial testing correlates well in general with disease severity^[48,49] or response to immunosuppressive therapy^[50]. Nonetheless, about 15% of patients who are initially seronegative may become seropositive within 12 mo, warranting re-testing in this population^[51]. Furthermore, such seroconversion can be delayed for several years in some cases^[51].

About 70% of the AChR seronegative population has antibodies directed against MuSK. In this scenario, a correlation between antibody titre and disease severity has been observed^[52]. Patients with MuSK-specific antibodies tend to respond less well to acetylcholinesterase inhibition and often require immunosuppressive therapy. Consequently, it is possible that repeated measurement of these antibodies may be useful to aid disease monitoring, in conjunction with other parameters.

Anti-striated muscle antibodies may also be found in patients with myasthenia gravis and these correlate with increased risk of co-existing thymoma. The primary target of these antibodies is Titin. Emergence of these antibodies in a patient with myasthenia have also been linked to thymoma^[53].

Autoimmune “channelopathies”

Antibodies to the water channel, aquaporin-4, are a sensitive and highly specific serological marker for neuromyelitis optica. Furthermore, titres of aquaporin-4 antibodies correlate at the population level with disease activity^[54]. In the individual patient however, changes in antibody levels are not sufficiently predictive of disease relapse and the need to modify therapeutic approach^[55].

Antibodies that bind to neuronal voltage-gated potassium channels may be found in neuromyotonia, limbic encephalitis and Morvan’s syndrome. These conditions commonly respond to immunosuppressive therapy although they may occasionally signal the presence of underlying malignancy. A correlation has been observed in several cases between reduction in antibody titre and therapeutic response^[56], providing a rationale for repeated measurement of antibodies in these patients.

Antibodies that bind to neuronal voltage-gated calcium channels are associated with both paraneoplastic and idiopathic forms of the Lambert-Eaton myasthenic syndrome. They antibodies are not diagnostic however in that they also occur in other neurological and malignant disorders. Antibody titre does not correlate well with clinical and electrophysiological parameters of disease. Serial antibody titres may reflect clinical status, at least in some patients, although clinical remission may be accompanied by persistence of high titre antibody levels^[57,58].

Finally, a series of complex encephalitic conditions may be considered in this category of which the prototype is associated with antibodies to an ion channel, namely the N-methyl-D aspartate receptor. Other targets include the glycine, GABA_B and AMPA receptors. Once again, some cases may be tumour-associated (e.g., ovarian teratoma). Autoantibody concentrations are generally higher in serum than in CSF in these conditions. However, intrathecal synthesis of antibody also occurs, and the role of serological monitoring of these patients is presently unclear^[59].

Disorders associated with antibodies to glycolipid and glycoprotein-related saccharides

Several neurological disorders have been associated with this class of autoantibody (Table 1). Pre-eminent among these are anti-ganglioside antibodies. Molecular mimicry between lipooligosaccharides on the surface of infectious agents (most commonly *Campylobacter jejuni*) and ganglioside antigens on neural cells may induce cross-reactive antibody responses, targeted against ganglioside complexes. Alternatively, these antibodies may manifest as non-malignant IgM monoclonal gammopathies. Complement fixation by these antibodies appears to be an important contributor to disease pathogenesis^[60].

Although assays are not standardised, these antibodies have important diagnostic utility. Furthermore, several of these antibodies appear to have pathogenic relevance in these neurological conditions. In some cases, an association has been observed between antibody titre and disease activity^[61] or response to immunosuppressive

Table 1 A summary of clinically relevant antibodies targeted against glycolipid and glycoprotein-related saccharides

Neuropathy syndrome	Antibody target	Antibody isotype
Chronic Sensory-Motor demyelinating	Myelin-associated glycoprotein	IgM (monoclonal)
Chronic ataxic neuropathy	Sulfoglucuronylparagloboside	
Multifocal Motor neuropathy	GD1b, GQ1b	IgM (monoclonal)
Sensory neuropathy	GM1, GM2, GD1b	IgM (polyclonal or monoclonal)
Acute motor axonal neuropathy	Sulfatide	IgM (monoclonal or polyclonal)
Acute inflammatory demyelinating polyneuropathy	GM1, GD1a, GalNAc GD1a, GM1b	IgG
Miller-Fisher syndrome	Variable	Variable
Bickerstaff's brainstem encephalitis	GQ1b ("anti-GQ1b antibody syndrome"), GT1a	IgG
Acute ophthalmoparesis		
Ataxic Guillain-Barré syndrome		
Pharyngeal-cervical-brachial weakness	GT1a (GQ1b)	IgG
Amyotrophic lateral sclerosis	GM1	IgG

Modified from http://www.aetna.com/cpb/medical/data/300_399/0340.html (Accessed December 8, 2012). IgM: Immunoglobulin M; IgG: Immunoglobulin G.

therapy^[62]. Once again however, the temporal relationship between antibody levels and disease status is not straightforward. To further complicate matters, IgG antibodies directed against some of these targets can be found in pooled immunoglobulin, which is commonly used to treat these disorders.

Antibodies associated with paraneoplastic disorders

Several antibodies have been described in paraneoplastic disorders, including anti-Hu, Ri, Yo, Tr, CV2/CRMP5, amphiphysin, recoverin and Ma1/Ma2^[63]. When used diagnostically, detection of these antibodies may be predictive of the presence of underlying malignancy. Some, but not all anecdotal reports have suggested that antibody levels may increase prior to emergence of clinical cancer relapse^[64]. Once again however, the role of repeated measurement of these antibodies in disease monitoring is not established.

Monitoring of paraproteins is discussed elsewhere in this review.

ENDOCRINOLOGY

Type 1 diabetes

Antibodies against islet cells and associated autoantigens (glutamate decarboxylase, islet antigen-2 and insulin) are commonly detectable in individuals with type 1 diabetes and in latent forms of this disease^[65]. Multiplicity of antibody positivity is associated with greater risk of disease development. However, in the absence of proven interventions to decrease disease risk, such use of these tests would appear unwarranted outside of research protocols. These antibodies may also be detected in a sub-group of patients with type 2 diabetes who have a greater risk of progression to require insulin. Once again however, there is no firm evidence that this information either leads to improved outcome or altered management. The presence of islet-reactive autoantibodies can be useful in categorising patients with features of both type 1 and type 2 diabetes, or in predicting risk of future diabetes in women

with gestational diabetes. In none of these situations however is there evidence to support repeat antibody measurement.

Steroid cell autoantibodies

Adrenal antibodies are found in individuals with established or evolving primary adrenal failure^[66], either alone or as part of one of the autoimmune polyglandular syndromes. In about 15% of cases, antibodies cross-react with other steroid hormone producing cells (*e.g.*, Leydig cells, theca cells, syncytiotrophoblast), indicating a high risk of progression to primary gonadal failure, particularly in women with high titre antibodies. The principal autoantigen recognized by adrenal antibodies is 21-hydroxylase whereas the latter "steroid cell antibodies" commonly react with 17 hydroxylase. While these antibodies have no pathogenetic role, their detection is useful both for diagnostic purposes and also in the identification of subjects at high-risk for future development of clinical adrenal failure.

There is no evidence to support retesting for these antibodies, either in the event of positive or negative results. In the context of polyglandular autoimmunity, periodic reassessment for the presence of several autoantibodies would appear to be justifiable. In this context, it has been suggested that in patients with primary adrenal failure, autoantibody testing is repeated every 2-3 years^[67]. The rationale underlying this proposed strategy is the detection of serological markers of other clinical manifestations of one of the associated autoimmune polyglandular syndromes, leading in turn to appropriate functional testing and diagnostic reclassification.

Thyroid autoantibodies

Antibodies with reactivity to thyroid peroxidase are commonly used markers of autoimmune thyroid disease, including Hashimoto's disease, Graves disease, autoimmune post-partum thyroiditis and sub-clinical autoimmune thyroid disease. However, antibody levels do not correlate with disease severity. Consequently, repeated

Table 2 Common clinical associations of paraproteins^[70]

Disorder	Diagnostic features
Monoclonal gammopathy of undetermined significance	All three criteria must be met: Serum monoclonal protein < 30 g/L Clonal bone marrow plasma cells < 10% Absence of end-organ damage ("CRAB"), e.g., hypercalcemia, renal insufficiency, anaemia and bone lesions due to the plasma cell disorder
Smouldering myeloma	Both criteria must be met: Serum monoclonal protein (IgG or IgA) > 30 g/L and/or clonal bone marrow plasma cells > 10% Absence of "CRAB", as defined above
Multiple myeloma	All three criteria must be met: Clonal bone marrow plasma cells > 10% Presence of serum and/or urinary monoclonal protein (except in patients with true non-secretory multiple myeloma) Evidence of "CRAB", as defined above
Waldenström's macroglobulinaemia	Both criteria must be met: IgM monoclonal gammopathy and 10% bone marrow infiltration (usually intertrabecular) by lymphoplasmacytic cells (morphology/immunophenotype) ¹
IgM monoclonal gammopathy of undetermined significance	All three criteria must be met: Serum IgM monoclonal protein < 30 g/L Bone marrow lymphoplasmacytic infiltration < 10% No evidence of anemia, constitutional symptoms, hyperviscosity, lymphadenopathy or hepatosplenomegaly
Smoldering Waldenström's macroglobulinemia	Both criteria must be met: Serum IgM monoclonal protein > 30 g/L and/or bone marrow lymphoplasmacytic infiltration > 10% No evidence of end-organ damage such as anemia, constitutional symptoms, hyperviscosity, lymphadenopathy or hepatosplenomegaly due to a lymphoplasma cell proliferative disorder

¹Note that other clonal B-cell disorders may be associated with paraproteins and may require careful clinical, morphological and immunophenotypic assessment for diagnosis (e.g., chronic lymphocytic leukaemia, mantle cell lymphoma, etc.). IgM: Immunoglobulin M; IgG: Immunoglobulin G; CRAB: Hypercalcemia, renal insufficiency, anaemia and bone lesions.

measurement of these antibodies in general has no clinical value^[68].

HAEMATO-ONCOLOGY

Monitoring of serum and urinary paraproteins

A paraprotein or M (monoclonal) protein is a clonal intact immunoglobulin or light chain that is produced in excess. Paraproteins can be detected in multiple myeloma, Waldenström's macroglobulinaemia, other clonal B-cell/plasma cell disorders or, more commonly, as a manifestation of monoclonal gammopathy of undetermined significance (MGUS). When intact, paraproteins are best detected in the serum. By contrast, light chain paraproteins (also known as Bence Jones proteins) are often easier to detect in urine, owing to their smaller size. Paraproteins are generally detected and/or quantified using one of four assay systems. In serum, this is achieved by agarose gel electrophoresis or capillary zone electrophoresis. By contrast, light chain paraproteins are traditionally detected by urine electrophoresis but may also be detected and quantified using the recently developed free light chain assay. This is a sensitive, latex-enhanced immunonephelometric test that quantitates free κ and λ light chains, generally in serum samples^[69]. Finally, in some carefully selected cases, paraprotein quantification is best achieved by measurement of the relevant immunoglobulin isotype, although this approach requires caution.

The role of paraprotein measurement in the diagnosis of haematological malignancy/MGUS is beyond the scope of this review and the reader is referred to

a number of recent articles for a more comprehensive discussion of this area^[70,71]. The common causes of paraproteins are listed in Table 2, together with relevant diagnostic features. In the discussion that follows, the role of these assays in disease monitoring is considered.

Changes in paraprotein levels are the principal indicators used to evaluate therapeutic response in patients with multiple myeloma, except in those patients with oligo- or non-secretory disease. Owing to the limitations of the assays involved, measurable disease has been defined as a serum paraprotein of ≥ 10 g/L or a urine paraprotein of ≥ 200 mg/d^[72]. In serum, reductions in paraprotein levels of at least 25% and 50% respectively are considered minimal and partial responses. By analogy, a reduction in urinary paraprotein (per day) of at least 50% and 90% respectively are classified as minimal and partial responses^[72]. A "very good partial response" is defined as the situation where a serum/urine paraprotein is detectable by immunofixation but not on electrophoresis or where a 90% or greater reduction in serum paraprotein has occurred, together with a urine paraprotein level of < 100 mg/d. A complete response requires the demonstration that a previously identified paraprotein is no longer present when sought using immunofixation electrophoresis and that bone marrow plasma cells are < 5% with resolution of any plasmacytomas. A "stringent complete remission" also requires that the serum free light chains (see below) have normalised and clonal bone marrow cells are absent.

If the paraprotein level is 15 g/L or lower and the patient is asymptomatic with no other laboratory or clinical abnormalities, MGUS is probable. In patients with

low-risk MGUS, paraproteins can be rechecked in 6 mo, and then once every two years or only at the time of symptoms for evidence of progression. All other subsets of patients need to be rechecked in 6 mo, and then yearly thereafter^[73]. Patients with smouldering multiple myeloma (Table 2) need more frequent follow up than those with MGUS; at least every 3-4 mo and especially with IgA paraproteins^[73].

Patients who are treated with plasmapheresis may require frequent paraprotein measurements in addition to serum or plasma viscosity to guide this therapy.

Monitoring of serum free light chains

The serum free light chain assay quantifies circulating free κ and λ immunoglobulin light chains. The assay is useful in three circumstances. First, measurement of free light chains is useful as a component of screening for paraprotein-related illness. Second, baseline free light chain levels provide valuable prognostic information in virtually all paraprotein-related conditions, from MGUS through to myeloma. Third, and of greatest relevance to this review, repeated measurement of free light chains can be useful in monitoring of these disorders under the following selected circumstances.

In the absence of a serum or urine paraprotein that is “measurable” according to the definitions described above, myeloma can be considered as “oligosecretory”. In this setting, the International Myeloma Working Group has recommended that the serum free light chain assay can be used to monitor disease, provided that the paraprotein (*e.g.*, involved free light chain) is ≥ 100 mg/L and that the free light chain κ/λ ratio is abnormal. Using this threshold for “measurable disease, a 50% decrease in paraprotein level indicates partial response^[72]. A recent consensus statement has recommended the additional use of this assay for monitoring of other oligosecretory plasma cell disorders, including amyloidosis AL and the majority of patients with “non-secretory” myeloma^[74]. The free light chain ratio is not a useful monitoring tool in this setting owing to treatment-related suppression of the disease-unrelated light chain. It is also important to emphasise that the free light chain assay is not recommended as a replacement for 24-h urine protein electrophoresis for monitoring of myeloma patients with measurable urinary paraproteins.

There is no clear evidence to guide appropriate retesting intervals. However, rapid changes are possible in particular since the half-life of serum free light chains (2 to 4 h) is much shorter than intact immunoglobulins (8-21 d). In myeloma kidney, recovery of renal function in patients requires an early substantial reduction in serum free light chain concentrations, warranting frequent retesting^[75], perhaps at monthly or quarterly intervals.

Caution is warranted in the interpretation of repeated measurement of free light chains. There can be significant lot-to-lot variation between batches of polyclonal free light chain antiserum. Indeed, the κ/λ ratio can even “double” artifactually in a patient with a stable disease^[76].

Thus, as in the case for 24-h urine protein measurements, a change of up to 50% in the involved free light chain concentration may not necessarily be significant. Furthermore, antigen excess errors can cause highly spurious trends in repeat measurements, which sometimes can lead to the erroneous conclusion that disease progression is occurring^[77].

$\beta 2$ -microglobulin

$\beta 2$ -microglobulin concentration at presentation is the best prognostic indicator for multiple myeloma^[78]. Half-life is only 1-3 h providing a rationale for frequent repeated measurement in this disease. However, falls in $\beta 2$ -microglobulin parallel those observed for paraproteins and it has been argued that little additional information is gained from measurement of both of these disease markers^[79].

ALLERGY

Specific IgE in food allergy

In general, the magnitude of specific IgE that is reactive against a food allergen correlates with the likelihood but not severity of true IgE-mediated allergy. In keeping with this, values of specific IgE have been defined that have a positive predictive value of 95% for childhood allergy to egg, milk, peanuts, tree nuts or fish^[80]. With lower levels and depending upon the history, diagnosis of food allergy may also require additional testing such as skin prick tests and supervised open food challenge.

In the setting of previously diagnosed food allergy, serial measurement of specific IgE may also be useful in monitoring of allergic status. This is particularly the case in children with allergy to cows milk and egg. Over 90% of children with cows milk allergy will become tolerant by 6 years of age^[81] while 66% of children with egg allergy acquire tolerance after 5 years of follow-up^[82]. Progressive development of tolerance can be predicted by lowered levels of specific IgE, combined with declining reactivity in skin prick testing and may be confirmed by judicious use of open food challenge^[83].

Total IgE

In some circumstances, serial measurement of total IgE may be of use in disease monitoring. For example, in patients with allergic bronchopulmonary aspergillosis, total serum IgE provides a measure of disease status and response to corticosteroids^[84]. However, IgE measurement is not useful in monitoring the depletion of this isotype by anti-IgE (omalizumab) therapy since commonly used assays do not distinguish between free and omalizumab-complexed forms of this antibody^[85].

GASTROENTEROLOGY AND

HEPATOLOGY

Celiac serology

Celiac screening generally involves the measurement of

IgA anti-tissue transglutaminase (tTG) antibodies, often followed by confirmatory testing for IgA anti-endomysial antibodies. Serological testing for these antibodies is highly useful in identifying individuals who may have coeliac disease (and who generally require duodenal biopsy while on a gluten-containing diet to confirm the diagnosis). Antibody testing has been advocated to aid disease monitoring in two circumstances. First, periodic retesting may be performed in an effort to assess compliance with a gluten-free diet in patients with an established diagnosis of coeliac disease. Second, repeated serological screening is commonly undertaken to detect the development of coeliac disease, particularly individuals at higher risk such as patients with type 1 diabetes. Importantly however, sequential serological testing in coeliac disease is compromised by the fact that assays are not standardised^[86].

In the sections that follow, these potential areas of disease monitoring are considered in turn.

Monitoring compliance with a gluten-free diet: Both anti-tTG IgA and anti-endomysial IgA antibodies can normalise on a gluten-free diet and can increase in titre following re-exposure to gluten. However, the value of repeated measurement of these antibodies in the monitoring of patients with coeliac disease remains complex and controversial. Anti-tTG IgA antibody levels were reported to be scarcely reliable in some^[87-89], but not all^[90] studies in detecting compliance with a gluten-free diet, when compared with duodenal biopsy. Despite following such a diet for 2 years with normalisation of anti-tTG IgA antibody levels, villous atrophy had not improved in 25% of asymptomatic adults^[91]. Furthermore, in patients with associated autoimmune disorders such as type 1 diabetes, anti-tTG IgA antibody levels may remain elevated despite dietary compliance and thus may not be useful for this purpose in such patients^[92]. In keeping with this, Armstrong and colleagues have recently concluded that repeat testing of anti-tTG IgA in patients diagnosed with coeliac disease and managed with a gluten-free diet is not indicated to confirm response to treatment. They acknowledge that studies have shown that quantitative results of anti-tTG IgA assays do commonly change in response to the initiation of a gluten-free diet. However, they point out that there is no evidence to suggest that regular, continued monitoring of anti-tTG titres improves outcomes^[93].

The Ontario Health Technology Advisory Committee have also commented on this issue. They concluded that: "Repeat serologic testing for patients diagnosed with celiac disease is reasonable for those patients who remain symptomatic despite strict adherence to a gluten-free diet. In this case, serologic testing for celiac disease should not be repeated more than once a year for each patient"^[94]. Taken together, these considerations argue that repeated testing of anti-tTG IgA antibodies in patients with an established diagnosis of coeliac disease is of highly limited value.

Monitoring high-risk populations for development of coeliac disease: High-risk populations such as indi-

viduals with type 1 diabetes are often monitored periodically using anti-tTG IgA and/or anti-endomysial IgA assays for the development of coeliac disease. To facilitate this process, the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) have recently recommended initial testing for coeliac disease-associated human leukocyte antigen (HLA) alleles (HLA-DQ2/ DQ8) in order to ascertain which of these children warrant such monitoring. However, no firm evidence exists as to how frequently coeliac serology should be re-tested in individuals who have these disease-associated alleles. In the face of this lack of evidence, it was the opinion of the ESPGHAN working group that testing should be undertaken every 2 to 3 years^[95]. To complicate matters further, recent data suggests that positivity for one or both coeliac disease-associated antibodies can spontaneously revert in some individuals, particularly children, who maintain a gluten-containing diet^[96,97].

The National Institute for Clinical Excellence has also commented on the issue of repeated serological testing for coeliac disease. It has concluded that "there is a lack of evidence on the need for repeat testing. Studies are needed to determine whether serological tests should be repeated if the initial results are negative and there is no high clinical suspicion of coeliac disease, and if so, when and how often they should be repeated"^[98]. It is clear that further research is required to clarify the optimum means to achieve such monitoring.

Autoimmune liver disease

Autoimmune hepatitis is characterized by hypergammaglobulinaemia accompanied by either ANA and anti-smooth muscle antibodies (type 1) or anti-liver kidney microsomal-1 antibodies (type 2). Anti-LC1 antibodies may also be detected, more commonly in European patients and in the absence of ANA or SMA^[99]. However, autoantibody titres in adults correlate imprecisely with disease severity, clinical course, and treatment response^[4,100]. By contrast, IgG levels and autoantibody titres have all been correlated with disease activity in children with this disease^[101], providing a rationale for repeated measurement.

Anti-mitochondrial antibodies reactive against the 2-oxoaldehyde dehydrogenase complex - previously named anti-M2 mitochondrial antibodies - are excellent serological markers for the serological diagnosis of primary biliary cirrhosis. However, these antibodies do not correlate with any clinical, histological or laboratory parameters of disease. Antibody titres remain stable over time^[102] and are not influenced by ursodeoxycholate treatment. Furthermore, antibody titres decrease only transiently after liver transplantation^[103,104].

HUMAN IMMUNODEFICIENCY VIRUS INFECTION

CD4 counts in human immunodeficiency virus

Monitoring CD4 cell counts in asymptomatic human im-

munodeficiency virus-1 infected patients to decide when to start antiretroviral therapy is unanimously recommended and is cost-effective. However, no recommendations have yet been formulated regarding the optimal frequency of CD4 monitoring, which most United Kingdom practitioners perform every 3-4 mo^[105]. Although formal guidelines do not exist, a recent study suggests that this frequency of testing may be excessive in those whose CD4 counts are well above the treatment threshold^[106].

DERMATOLOGY

In autoimmune blistering skin disease, diagnosis relies upon histopathological analysis of a skin biopsy together with direct immunofluorescence microscopy to demonstrate tissue-bound autoantibodies and/or C3 in the patient's skin or mucous membranes. Nonetheless, skin antibody testing (commonly by indirect immunofluorescence) provides useful information in the monitoring of these conditions. Increasingly however, as target autoantigens are being identified, ELISAs are being produced which can be used to measure antibody levels over the course of the disease. Target antigens include desmoglein 3 (pemphigus vulgaris), desmoglein 1 (pemphigus foliaceus), envoplakin (paraneoplastic pemphigus), NC16A domain of BP180 (bullous pemphigoid/pemphigus gestationis/mucous membrane pemphigoid) and type VII collagen (epidermolysis bullosa acquisita). In general, a reduction in antibody level can be demonstrated with disease remission, although the correlation is by no means absolute^[107-109].

IMMUNE MONITORING IN CLINICAL IMMUNOLOGY

Serum immunoglobulin measurements

Serum immunoglobulin concentrations (IgG, IgA and IgM) are markedly raised in many autoimmune diseases. However, the magnitude of increase of these proteins is not related to the severity of disease; repeated measurements are therefore not useful^[110].

Trough IgG levels provide a useful marker of adequacy of immunoglobulin replacement therapy in many patients with hypogammaglobulinaemia. Repeated measurement at least quarterly is recommended by the United Kingdom primary immunodeficiency network. (<http://www.ukpin.org.uk/home/standards-CVID.htm>, accessed February 21st, 2013).

CONCLUSION

Immunology laboratories make an enormous contribution to the diagnosis of several disorders. However, the role of immunology testing in disease monitoring has been subject to considerably less scrutiny. In this review, evidence has been evaluated to assess the role of several commonly requested tests in disease monitoring. Some investigations, such as paraprotein quantification or

measurement of anti-GBM antibody titres, provide critical information to inform therapeutic decision-making. However, in many other cases, repeated performance of immunology tests generates data that do not alter disease management or accurately reflect disease status. As ever, liaison between clinical and laboratory staff remains vital to achieve optimal evidence-based selection and interpretation of test results to guide disease management.

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Probiotics in inflammatory bowel disease: Pathophysiological background and clinical applications

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Abstract

Ulcerative colitis and Crohn's disease, collectively termed the inflammatory bowel diseases (IBD), are chronic inflammatory disorders of the gastrointestinal tract. A "dysbiotic" relationship between the commensal gut flora and the intestinal mucosa-associated immune system has been at the core of the pathogenesis of these conditions. Probiotics are "good bacteria" with the ability to benefit the health of the host and their therapeutic application has been studied in IBD. The theoretical basis for such utilization relies upon the ability of probiotic microorganisms to interfere with the dysregulated homeostasis that takes place in IBD and restore the immune-bacterial interaction at the intestinal mucosa. Proposed mechanisms of action include the reconstitution of altered flora composition, enhancement of the integrity of the epithelial barrier, promotion of tolerogenic action by dendritic cells, strengthening of the defensive mechanisms of the innate immunity, and the suppression of pro-inflammatory adaptive immune responses. Despite this abundance of supporting experimental evidence, clinical application of probiotics in IBD has been disappointing. Possible expla-

nations for such discrepancy include the great diversity of microorganisms that fall under the definition of probiotics, the lack of standardization of dosages and administration schemes, the heterogeneity between clinical trials, and the inclusion in the treatment arms of patients with a large variety of clinical phenotypes. Addressing these important issues will be critical for the optimal usage of probiotic-based therapies for patients with IBD.

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Key words: Probiotics; Commensal flora; Mucosa-associated immune system; Inflammatory bowel disease; Clinical trials; Pouchitis; Ulcerative colitis; Crohn's disease

Core tip: Inflammatory bowel diseases are chronic debilitating diseases of the gastrointestinal tract. Current therapies are not effective in a great proportion of patients and are associated with serious adverse effects. The use of probiotics has been tried as an alternative and safe treatment. Herein, we review the pathophysiological basis for such an application and summarize the data from the major clinical trials.

Pagnini C, Delle Fave G, Bamias G. Probiotics in inflammatory bowel disease: Pathophysiological background and clinical applications. *World J Immunol* 2013; 3(3): 31-43 Available from: URL: <http://www.wjgnet.com/2219-2824/full/v3/i3/31.htm>
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INTRODUCTION

In recent years, great progress has been made regarding our understanding of the structure and function of intestinal microbiota, leading to attractive novel perspectives for different pathologic conditions. An increasing number of investigational studies have shed light to the

pivotal role of the physiologic host-bacterial mutualistic relationship for health maintenance, as well as the pathophysiological relevance of the disturbance of such balance for the occurrence of several human diseases^[1]. Research in this field has been directed towards the two main actors of this interplay: the gut microflora and the intestinal mucosal immune system.

The great advancement that has been achieved regarding laboratory techniques for the quantitative and qualitative evaluation of commensal flora has led to the evolution from a culture-based approach to the metagenomic (molecular profile) and metabolomic (metabolic activity) investigation of bacterial species hosted inside the human gut^[2]. Modern analytical methodologies provided a large body of information which, on the one hand, has expanded our comprehension of genera- and species-composition of intestinal microbiota; furthermore, the effects of internal (aging, pathology) and external (drugs, diet) factors on the stability and variation of the flora can be accurately determined^[1]. On the other hand, paradoxically, data interpretation has become a difficult task, in the face of the growing diversity of flora composition and its temporal and spatial distribution and the need to correctly balance similarity with inter- and intra-individual variability. In an attempt to simplify this complexity, a stable core microbiome has been described for healthy individuals and people affected by some pathologic conditions (enterotype)^[3]. Surprisingly, complexity decreases when considering gene expression profile rather than bacterial species definition^[4], indicating that functional analysis of microbiota may provide a more solid framework for future studies.

A comprehensive study of the microbiota is inseparable from the parallel investigation of the host counterpart of the intestinal ecosystem, *i.e.*, the innate and adaptive immunity of the intestinal mucosa. Evidence that the host immune system can shape intestinal gut flora relies on data from animal models. Mice with deficiency of T-bet, a T-box transcription factor family member with important roles in adaptive and innate immunological responses, develop spontaneous colitis that is transmissible to immunological intact mice by fecal transplantation^[5]. To date, molecular insight into the mechanism of bacterial recognition points to a binomial pathway, which originates from pathogen-associated molecular pattern/pattern recognition receptor (PAMP/PRR) interaction and, through the activation of transduction proteins (*i.e.*, MYD88, IRAK), leads in turn to the activation of specific genes and production of proteins (*i.e.*, mucins, defensins) that are able to modulate intestinal microflora^[6]. To further complicate the picture, many external and internal factors may activate molecular modulators with the potential to induce different pathways^[7]. Perturbations affecting microflora and/or innate mucosal immunity are likely to alter the physiological balance between the two compartments. The real impact of such alteration on the development of specific diseases represents a challenge for scientists involved in this field of research.

One major application of research in this field is the opportunity to interfere with the host-bacterial cross-talk. One such way of reinforcing the communication to the benefit of the host, is by means of specific bacterial supplements, namely probiotic bacteria. Although the concept of “good bacteria”, capable of benefiting individual health is about 100-year-old, it is only in the last decades that the implementation of probiotics for therapeutic purposes has become widely tested. This was driven on the one side by the impulse of microbiota research, and on the other by market suggestions. To date, rigorous scientific evidence for probiotic utilization has been accomplished for few clinical conditions, such as treatment of acute diarrhea in children and post-antibiotic diarrhea prevention^[8,9], prevention of *Clostridium difficile* infection^[10], and prevention of atopic eczema associated with cow milk allergy^[11].

When considering probiotic administration for the treatment of a specific condition several confounding factors need to be taken into account. First, a variety of different bacterial species and compounds fall under the “probiotic” label. As such microbial diversity is inevitably translated into functional variability, individual strain properties may render one product applicable to a specific clinical setting but not in a different one. At the same time, not every probiotic microorganism may be suitable for a particular condition. Second, the majority of evidence is being generated in experimental models, including animal mimics of human diseases. Although the important contribution of such methods to the elucidation pathogenetic mechanisms cannot be overemphasized, data are not directly translatable in clinical scenarios. Finally, therapeutic doses and schemes are far from being characterized, even for the same probiotic species.

Investigation of the role of probiotic bacteria in inflammatory bowel diseases (IBD) is a valid exemplification of the skepticism presented above. IBD represents a heterogeneous group of disorders that are characterized by persistent intestinal inflammation of unknown etiology. The major forms of IBD are ulcerative colitis (UC) and Crohn’s disease (CD), which share many clinical, pathological and immunological characteristics, but are also distinguished by unique phenotypic signatures^[12]. Despite a large number of studies that has been published in recent years, solid evidence for the efficacy of probiotics in IBD remains scarce. On the other hand, current research has led to clarification of different molecular pathways that are involved in the action of such bacteria, both during the active phase of inflammation (induction of remission) and as prevention of disease flares (maintenance of remission). Such progress should ideally lead to a critical revision of previous clinical experience with probiotic compounds in IBD and will hopefully lead to the design of translational clinical studies.

In the present review, we critically address the influence of commensal flora in IBD, and the current concept of IBD pathogenesis, in order to evaluate the rational of probiotic use and their putative mechanisms of action

in inflammatory conditions. Moreover, we present and discuss current clinical data of probiotic supplementation in IBD, and we try to delineate the indications for future investigation.

PATHOPHYSIOLOGICAL BACKGROUND

The “dysbiotic” microbiota in IBD

Evidence for a “microbiota influence” in IBD originates from observations in both experimental models and patients. Early studies have outlined a “negative” role of commensal bacteria on IBD onset. First, the majority of animal models of IBD do not develop inflammation when raised on “germ free” conditions; furthermore, re-introduction of bacteria in the gut induces the development of colitis^[13,14]. Second, antibiotic therapy has been associated with amelioration of the severity of clinical and experimental intestinal inflammation^[5,15]. Third, fecal diversion has been proven efficient in ameliorating inflammation in CD patients and reinfusion of fecal content into a previously excluded ileum resulted in reappearance of the disease^[16,17]. Nevertheless, more recent studies have questioned the previous data, putting forward the hypothesis that some commensal species may have protective roles against the development of inflammation. In fact, spontaneous ileitis in SAMP1/YitFc mice develops in a germ-free environment, even though with attenuated severity in comparison to their specific-pathogen-free (SPF) counterparts^[18]. In addition, DSS-colitis is more severe in germ free than in SPF mice^[19].

The development of novel techniques for microbiota evaluation has opened the way for the identification and characterization of a “dysbiotic state” between commensal flora and gut-associated mucosal immune system in IBD patients compared with normal individuals. Although studies so far failed to identify a single etiologic microorganism for the pathogenesis of disease, many studies reported a reduced diversity of intraluminal and mucosa-adherent microbiota in IBD patients^[20]. In general, a reduction of *Firmicutes* have been observed in patients with IBD, with consequent relative increase of *Enterobacteriaceae*^[21,22]. Controversial data are available regarding differences between UC and CD patients, as well as between inflamed and uninfamed areas from individual patients^[20,23-25]. Alteration of the interspecies balance may have detrimental effects on the homeostasis and development of inflammatory disease by two different reasons. First, the reduction of bacterial diversity may favor the overgrowth of “enteropathogens” with pro-inflammatory properties. Along that line, several studies have reported increased concentration of mucosa-adherent-invasive *E. coli* (AIEC) strains with specific virulence factors in IBD patients^[26-28]. Such bacteria were isolated in 36% of ileal specimens of CD patients, compared with 6% of normal control^[28], and have the ability to stimulate the production of pro-inflammatory cytokines from macrophages both *in vitro* and *in vivo*^[29,30]. Conflicting data are also available for the pro-inflammatory role

of *Mycobacterium avium sub. paratuberculosis*^[31], pathogenic *Yersinia*^[32,33] and *Listeria monocytogenes*^[34,35]. Alternatively, dysbiosis may be signified by the reduction of “protective” bacterial species. Several members of *Firmicutes* are strong producers of short chain fatty acids metabolites (acetate and butyrate) with anti-inflammatory properties and important trophic function for colonic mucosa^[36]. In this regard, of particular relevance are *Clostridial* cluster IV and XIV, which are relatively less abundant in IBD patients compared with normal controls^[23]. Moreover, *Bacteroides fragilis* and *Faecalibacterium prausnitzii* are bacteria with anti-inflammatory properties that are reduced in IBD patients^[37,38].

Several probiotic bacteria supplements have been shown to affect microbiota composition and, in particular, to increase diversity. *Lactobacillus casei* and *L. plantarum* have been shown to increase *Lactobacillus* diversity in mouse colon^[39]. *L. rhamnosus subsp. GG* (LGG) administered to mothers increases Bifidobacterial diversity in 5-day-old newborns^[40]. Several studies indicate that the administration of the multiple probiotic compound VSL#3, a high dose (450 billions/g) supplement of 8 different bacterial species (4 *Lactobacilli*, 3 *Bifidobacteria* and *S. thermophilus*), affects human and mouse gut flora composition, incrementing diversity^[41-43]. Still a matter of debate is the question of the real role of the dysbiosis in the IBD pathogenesis. Recent support to the hypothesis that microflora alteration is not just a consequence but may have a causative role in the development of inflammatory disease in wild type mice by fecal content transplantation from T-bet-deficient mice^[5], and by the promising results of microbiota fecal transplantation in humans^[44]. Thus, both gut flora composition and mucosal immune response appears to be equally involved and interconnected in homeostasis maintenance and in the onset of inflammation.

Restoring the barrier defect: Stimulating innate immunity and IEC activity

In the last years, the general conception of IBD pathogenesis has been drastically changed. Indeed, focus has been shifted from an over-reactive immune response, mainly driven by activated T cells^[45], to a primarily immunodeficient condition whereby the chronic inflammatory reaction is a paradoxical consequence of a defective immune response, in particular of the innate compartment^[46]. This leads to an insufficient handling of the bacterial burden at the intestinal mucosa and, eventually, to the onset of pathological chronic inflammation^[47]. In this scheme of events, a compromise of the barrier function of intestinal epithelial cells may be the initiating event. Defective barrier function at the mucosal level can be identified at two separate levels: intestinal permeability and anti-bacterial molecule production^[48,49]. Several studies have outlined increased intestinal permeability as an early event in IBD^[50]. Older studies have reported increased permeability in patients with CD and their first

degree relatives, suggesting a possible primary etiological role of this alteration for disease occurrence^[51]. Derangement of tight junctions, with up-regulation of claudin 2 and down-regulation and redistribution of claudins 5 and 8, has been described in patients with mild to moderate CD^[52]. In the spontaneous ileitic SAMP/YitFc murine strain, the primary defect leading to the onset of chronic intestinal inflammation appears to be related to an epithelial permeability increment^[53].

Additional levels of homeostatic control include the active secretion of mucins and defensins by epithelial cells. Pathologic mucin production and assembly has been described in human IBD^[54], and such alterations has been demonstrated to be associated with colitis in experimental models^[55,56]. Alterations in α - and β -defensins production, by Paneth cells and enterocytes, has been reported in IBD^[57,58]. The possible role of flawed defensin production in the pathogenesis of CD has been further confirmed by the detection of such defects in patients with polymorphisms of the CD-associated genes nucleotide oligomerization domain 2 (NOD2) and autophagy related protein 16-like 1 (ATG16L1)^[59,60]. Moreover, importance of epithelial production of NF- κ B mediated production of cytokines (IL-1b, IL-8, TNF) for mucosal homeostasis maintenance has been demonstrated^[61,62].

Probiotics have been shown to positively stimulate IEC activity and intestinal permeability. LGG effectively improved intestinal permeability in rats with ethanol-induced colitis, by increased gastric production of Muc6 and PGE2^[63]. *L. acidophilus* upregulated muc2 and IL-8, IL-1b and TNF α in IEC, thus inhibiting the attachment of *E. coli* O157:H7^[64]. VSL#3 increased mucins genes expression and secretion in IEC^[65], and improved intestinal permeability in IL-10 deficient mice^[66]. VSL#3 and *L. fermentum* improved intestinal permeability by upregulation of human beta defensin 2 by NF- κ B and MAPKs related pathways^[67]. VSL#3 effectively prevented ileal inflammation in SAMP1/YitFc mice by NF- κ B activation in epithelial cells and consequent production of TNF α and restoring of intestinal permeability; notably, the beneficial effect of probiotics was completely abrogated by the concomitant administration of anti-TNF antibodies^[68]. In a further study, the direct effect of epithelial TNF α on amelioration of epithelial barrier permeability in this experimental model, via the modulation of tight junctions proteins, occludin and claudin, was confirmed^[69].

Dendritic cells

Intestinal dendritic cells (DC) in the lamina propria of the mucosal layer represent a unique population of innate immune cells whose characteristics are shaped by cell-cell and host-bacteria interactions^[70]. Among the specific function of those cells are luminal antigen sampling, T-cell stimulation and differentiation, microbial uptake, pathogen defense, and, notably, the modulation of tolerance toward non pathologic stimuli, such as commensal bacteria, by means of IL10/IL12 balance at mucosal side^[71]. The possible relevance of DCs alterations in IBD

is supported by experimental data in which mice with deregulated DC-related pathways (*i.e.*, A20, β -catenin and phosphotylinositol-3-kinase) develop either spontaneous colitis or increased susceptibility to experimental colitis^[72,73]. Furthermore, in IBD patients, lamina propria DCs and macrophages produce higher amount of pro-inflammatory cytokines compared with normal controls^[74].

For their relevance in the mucosal homeostasis maintenance, and for their crucial “bridging” role between innate and adaptive immunity activation, DCs has been indicated as potential targets of probiotic bacteria in IBD. In fact, *L. salivarius* Ls33 and *L. rhamnosus* Lr32 promoted tolerogenic action of DC *in vitro*, and administration of probiotic-incubated DC ameliorated trinitro-benzene-sulfonic acid induced colitis in mice^[75]. A fermentation product of *B. breve* has been shown the potential to modulate DC function *in vitro* by selective activation of MAPK, GSK3 and PI3K^[76]. Five commensal/probiotic bacteria incubated with immature DC induced a distinct cytokines profile and a tolerogenic phenotype through the selection of hyporesponsive T cells^[77]. The probiotic mixture VSL#3 increased IL-10 in intestinal and blood DC's from healthy volunteers, and inhibited Th-1 cells and IL-12 production^[78].

Immunomodulation of the acquired immune response

IBD has been considered for a long time as a disease primarily caused by dysregulation of acquired immune responses, with over-reactive effector pathways of the Th1 (CD) or Th2 (UC) type. This view was sustained by the observed increase of cytokines of the Th1 pathways (TNF, IFN γ) in CD patients and overexpression of IL-5 and IL-13 in colonic specimens of UC patients^[79]. Furthermore, the importance of activated lymphocytes has been confirmed by the development of animal models of IBD with specific up-regulation of Th1 derived cytokines (IL-10 KO, TNF Δ ARE mice)^[80,81], and by the observation that inflammatory disease in experimental models can be induced to SCID mice by the adoptive transfer of specific subpopulations of lymphocytes^[82]. The immune imbalance may be due either to defective regulatory pathways (T-regs, IL-10 and TGF β) and/or the increment of pro-inflammatory Th1 and/or Th2 derived cytokines. In recent years, this schematic paradigm has been consistently disputed: cytokines of both Th1 and Th2 derivation has been observed both in CD and UC patients^[46] and efficacy of anti-TNF antibodies in UC confirmed that Th1 cytokines has an important role for inflammation even in UC and not only in CD^[83]. At present the classical pathogenic scheme has been replaced by a model in which different mediators (cytokines) interplay in the induction, development and maintenance of inflammatory disease (in CD and UC)^[46], thus overcoming the schematic division of the past. Despite the overwhelming recent evidence supporting a primary role of innate immunity in the pathogenic cascade leading to IBD development, the dramatic efficacy of anti-TNF antibodies, the fact that blockage of adaptive immune responses

remains the main target of therapy (*i.e.*, corticosteroids, immunosuppressants), the promising data from leukocytopheresis^[84], bone marrow and mesenchymal stem cells transplantation^[85], testify that acquired immune response over-activation is a important factor for inflammation onset and maintenance in IBD. In addition, the recent discovery of the relevance of IL23/IL17 pathways, supported by the observation of increased concentration of Th17 lymphocytes (producing IL17 upon stimulation of IL23) in the inflamed mucosa of CD patients^[86,87], and the effective amelioration of experimental inflammation by selective blocking of IL23/IL17 pathways^[88,89], has given new impulse to the investigation of the role of the adaptive compartment in IBD pathogenesis. Accordingly, monoclonal antibody targeting of the p40 subunit of IL12/IL23 (Ustekinumab) is currently under investigation as potential therapy in CD^[90]. Commensal bacteria have an important role in the physiologic maturation of the adaptive compartment of the immune system, since germ-free mice have low number of CD4 T cells producing IL17, IFN γ , TNF α and IL-10^[91].

Several probiotic bacteria have a direct inhibitory effect on the pro-inflammatory cytokines production and a concomitant stimulatory function on T-reg cells and modulatory cytokines. The probiotic mixture VSL#3 was shown to induce IL-10 dependent T-reg cells in an animal model of recurrent chemical-induced colitis^[92], and to increase tissue levels of IL-10 and reduce pro-inflammatory cytokines, nitric oxide synthesis and melaproteinase activity in the inflamed pouch^[93]. In a microarray analysis, LGG altered the expression of several genes related to immune response and inflammation in human duodenal mucosa^[94]. In the IL-10 KO model of colitis, the probiotic bacteria *L. plantarum*, *B. infantis* and *L. salivarius* prevented and/or ameliorated inflammation by down-regulation of pro-inflammatory Th1 derived cytokines^[95,96]. Three strains of Lactobacilli reduced Th2 response and activated T-reg frequency in health mice in a strain dependent way^[97]. In a pediatric study, rectal enemas with *L. reuteri* ameliorated inflammation by augmenting mucosal expression of IL-10 and reducing of IL1 β , TNF α and IL8^[98]. *Bifidobacteria* and *S. thermophilus* stimulated the production of TGF β and the development of T-reg cells in PBMC from humans^[99]. Finally, in a mouse model of allergic asthma, *L. gasseri* suppressed the Th-17 pro-inflammatory response^[100].

CLINICAL APPLICATION

Despite the large amount of experimental data and the pathophysiological rationale for their use in IBD, solid clinical evidence for the applicability of probiotic supplementation in these conditions is still lacking. Existing data are convincing for maintenance of remission in pouchitis, promising for maintenance of remission in UC, but disappointing for CD (Table 1). In fact, a recent workshop of an expert committee expressed a grade A (supported by strong positive studies) recommendation for probi-

otic use for prevention and maintenance of remission of pouchitis (VSL#3) and for remission maintenance in UC (VSL#3 and *E. coli* Nissle 1917), a grade B (positive-controlled studies but some negative studies) for inducing remission in UC (VSL#3 and *E. coli* Nissle 1917), and a grade C (some positive studies but clearly inadequate amount of work to support the outcome) for induction of remission of pouchitis (VSL #3) and in CD (*E. coli* Nissle 1917, *S. boulardii*, LGG)^[101]. Interestingly, in the same expert workshop held three years before, all IBD indications scored a C grade of recommendation, with the sole exception of pouchitis prevention and maintenance of remission (A grade)^[102].

One of the major limitations for probiotic utilization is that considerable variability exists between studies in regards to probiotic species used, dosage and duration of therapy, as well as variability of the underlying inflammatory disease and concomitant medication usage. Indeed, IBD comprises a continuum spectrum of disease, which far exceeds the simplistic subdivision in the two main clinical entities of UC and CD. In the two far ends of the spectrum are patients with very mild and limited rectal disease and patients with extended and devastating complications such as perianal and systemic involvement. Thus, it is not surprising that the best results for probiotics have been accomplished in experimental models of IBD, where the inflammatory condition is the consequence of alteration of single specific pathways. Besides the appropriate design of clinical trials which should include clear end-points, satisfactory statistical power, well-selected probiotic formulations given in adequate dose and scheme^[103,104], the key for the future clinical investigation of probiotics application in IBD is the comprehension of the complexity of the single conditions and the appropriate selection of patients with similar characteristics in terms of disease activity, extension, phenotype and molecular features.

Pouchitis

Acute and chronic pouchitis are relatively frequent events in patients with Ileal Pouch Anal Anastomosis (IPAA) following colectomy for UC. The application of probiotics for the maintenance of remission after pouchitis represented the earliest and most established clinical indication of probiotics in IBD. The therapeutic administration of probiotics in patients with pouch has been proven effective in the remission maintenance and prevention of pouchitis, and for this indication the probiotic mixture VSL#3 has been included in the European Crohn and Colitis Organization (ECCO) guidelines^[105]. In fact, a recent Cochrane systematic review included two studies of VSL#3 *vs* placebo for remission maintenance of pouchitis after remission induced by antibiotics, and probiotic treated patients had significant lower rate of recurrence after 9 mo^[106] and 1 year^[107]. Accordingly, LGG administration significantly increased the duration time of remission in a three-year follow-up^[108]. The same meta-analysis included two studies analyzing the VSL#3 administration

Table 1 Main published randomized control trial on probiotics administration in patients with inflammatory bowel diseases

Author, year	Disease	Indication	Probiotic	n	Outcome
Gionchetti <i>et al</i> ^[106] 2000	IPAA	Remission maintenance	VSL#3	20 treated 20 placebo	Superior to placebo
Mimura <i>et al</i> ^[107] 2004	IPAA	Remission maintenance	VSL#3	20 treated 16 placebo	Superior to placebo
Gionchetti <i>et al</i> ^[109] 2003	IPAA	Prevention	VSL#3	20 treated 20 placebo	Superior to placebo
Gosselink <i>et al</i> ^[108] 2004	IPAA	Prevention	LGG	38 treated 35 no treatment	Superior to no treatment
Pronio <i>et al</i> ^[110] 2008	IPAA	Prevention	VSL#3	16 treated 15 no treatment	Superior to no treatment
Kuisma <i>et al</i> ^[111] 2003	IPAA	Pouchitis	LGG	10 treated 10 placebo	No difference
Rembacken <i>et al</i> ^[117] 1999	UC	Inducing and maintenance remission	<i>E. coli</i> Nissle 1917	57 treated 59 mesalamine	Not inferior
Kato <i>et al</i> ^[116] 2009	UC	Inducing remission	Fermented milk (<i>B. breve</i> + <i>B. bifidum</i>)	10 treated 10 placebo	No difference
Tursi <i>et al</i> ^[118] 2004	UC	Inducing remission	VSL#3	30 VSL#3 + balsalazide 30 balsalazide 28 mesalamine	VSL#3 + balsalazide better
Furrie <i>et al</i> ^[115] 2005	UC	Inducing remission	Synbiotic (<i>B. longum</i>)	9 treated 9 placebo	No difference
Matthes <i>et al</i> ^[124] 2006	UC	Inducing remission	<i>E. coli</i> Nissle 1917 (enemas)	20 treated (3 groups different doses) 20 placebo	Superior to placebo
Sood <i>et al</i> ^[126] 2009	UC	Inducing remission	VSL#3	77 treated 70 placebo	Superior to placebo
Miele <i>et al</i> ^[125] 2009	UC (pediatric)	Inducing and maintenance remission	VSL#3	14 treated 15 placebo	Superior to placebo (corticosteroids)
Tursi <i>et al</i> ^[118] 2004	UC	Inducing remission	VSL#3	71 treated 73 placebo	No difference
Kruis <i>et al</i> ^[119] 1997	UC	Remission maintenance	<i>E. coli</i> Nissle 1917	58 treated 60 mesalamine	Not inferior
Ishikawa <i>et al</i> ^[127] 2003	UC	Remission maintenance	Bifidobacteria-fermented milk	11 treated 10 placebo	Superior to placebo
Cui <i>et al</i> ^[128] 2004	UC	Remission maintenance	Bifidobacteria	15 treated 15 placebo	Superior to placebo
Kruis <i>et al</i> ^[120] 2004	UC	Remission maintenance	<i>E. coli</i> Nissle 1917	162 treated 165 mesalamine	Not inferior
Zocco <i>et al</i> ^[121] 2006	UC	Remission maintenance	LGG	65 LGG 62 LGG + mesalamine 60 mesalamine	No difference remission rate LGG longer relapse-free time
Wildt <i>et al</i> ^[122] 2011	UC	Remission maintenance	<i>L. acidophilus</i> LA-5 and <i>B. animalis</i> subsp. <i>Lactis</i> BB-12	20 treated 12 placebo	No difference
Malchow <i>et al</i> ^[136] 1997	CD	Remission maintenance	<i>E. coli</i> Nissle 1917	10 treated 10 placebo	No difference
Guslandi <i>et al</i> ^[135] 2000	CD	Remission maintenance	<i>S. boulardii</i>	16 mesalamine + probiotic 16 mesalamine	No difference
Prantera <i>et al</i> ^[131] 2002	CD	Remission maintenance (post-surgery)	LGG	18 treated 19 placebo	No difference
Schultz <i>et al</i> ^[134] 2004	CD	Inducing and maintenance remission	LGG	4 treated 5 placebo	No difference
Bousvaros <i>et al</i> ^[133] 2005	CD (pediatric)	Remission maintenance	LGG	39 treated 36 placebo	No difference
Marteau <i>et al</i> ^[139] 2006	CD	Remission maintenance	<i>L. johnsonii</i> (LA1)	48 treated 50 placebo	No difference
Van Gossum <i>et al</i> ^[140] 2007	CD	Remission maintenance	<i>L. johnsonii</i> (LA1)	34 treated 36 placebo	No difference
Chermesh <i>et al</i> ^[142] 2007	CD	Remission maintenance	Symbiotic 2000	15 treated 15 placebo	No difference

RCT: Randomized control trial; IPAA: Ileal pouch anal anastomosis; UC: Ulcerative colitis; CD: Crohn's disease; LGG: *L. rhamnosus* subsp. GG; *E. coli*: *Escherichia coli*.

in the prevention of pouchitis onset, finding the probiotic mixture superior to placebo^[109] and to no treatment^[110].

For acute pouchitis data are still sparse and conflicting. LGG was not superior to placebo in a randomized con-

trol trial (RCT)^[111], while in an open-label study VSL#3 was effective in patients with mild pouchitis^[112].

UC

Probiotic applications in UC are encouraging, although solid evidence for their use is still unproven. Two Cochrane systematic reviews analyzed the utilization of probiotics in induction of remission and in remission maintenance, respectively^[113,114]. The first included four studies: two small trials compared a symbiotic preparation with *B. longum* ($n = 9$ patients in treatment group *vs* $n = 9$ controls)^[115] and fermented milk supplemented with *B. breve* and *B. bifidum* ($n = 10$ treated *vs* $n = 10$ controls) to placebo in addition to standard therapy^[116]; in one trial supplementation of *E. coli* Nissle 1917 was equal to mesalamine ($n = 57$ probiotics *vs* $n = 59$ mesalamine group)^[117], and in one study patients treated with balsalazide plus VSL#3 ($n = 30$) had increased remission compared with patients receiving only balsalazide ($n = 30$) or mesalamine ($n = 28$)^[118]. The authors concluded that there is no evidence that probiotics are superior to placebo or mesalamine in inducing remission in UC^[113]. In the meta-analysis for remission maintenance, four studies were included. Two multicenter studies compared the administration of *E. coli* Nissle 1917 to mesalamine for 3 ($n = 58$ probiotic and $n = 60$ mesalamine) and 12 mo ($n = 162$ probiotic and $n = 165$ mesalamine), finding no difference in recurrence rate^[119,120], and one study compared LGG ($n = 65$), LGG+ mesalamine ($n = 62$) and mesalamine alone ($n = 60$) at 12 mo, finding no difference in remission rate but a longer disease free time in LGG treated group^[121]. One small trial compared administration of a probiotic mixture of *L. acidophilus* strain LA-5 and *B. animalis subsp. Lactis* strain BB-12 ($n = 20$) to placebo ($n = 12$), finding no difference^[122]. The authors of the meta-analysis conclude that there is no evidence that probiotics are superior to mesalamine or placebo for maintenance of remission in UC patients^[114]. Another recent meta-analysis analyzed 13 RCT of probiotics utilization in UC, both in induction of remission ($n = 7$) and in remission maintenance ($n = 8$, two studies analyzed both the outcomes)^[123]. For induction of remission, three supplemental studies were analyzed in addition to the ones already included in Cochrane's review, in which *E. coli* Nissle (only in abstract form^[124]) and VSL#3, in a pediatric^[125] and adult group of UC patients^[126], were tested. Overall, in a total of 399 patients (219 probiotics *vs* 180 standard therapy/placebo) the authors did not report significant difference in inducing remission between probiotic treated and placebo/standard therapy treated patients. In the maintenance of remission, eight clinical trials were included, five of which were previously excluded by the Cochrane meta-analysis due to lack of remission of the patients at the beginning of the trial^[117,125,127], short follow-up^[128], or because it was not a RCT^[129]. A total of 709 patients were globally analyzed (390 probiotics *vs* 319 standard therapy/placebo), and there was a statistically significant difference in favor of probiotic group for re-

mission rate (recurrence rate = 0.69, 95%CI: 0.47-1.01, $P < 0.05$). It is of note that the results of this meta-analysis should be interpreted with caution for the presence of significant heterogeneity across the studies.

CD

A Cochrane meta-analysis analyzed seven RCT (five full papers and two abstracts) for application of probiotic for maintenance of remission in CD patients, with a total of 160 patients^[130]. In two studies the remission was surgically-induced^[131,132], and in five medically-induced^[133-137]. Probiotics tested were LGG^[131,133,134,137], VSL#3^[132], *E. coli* Nissle 1917^[136], and *S. Boulardii*^[135]. Authors conclude that probiotics were not superior to placebo or aminosalicylates. Another recent meta-analysis examined probiotics in the post-operative prophylaxis, including five studies, none of which showed effectiveness of probiotics *vs.* placebo for the prevention of recurrence^[138]. Probiotics tested were *L. johnsonii*^[139,140], LGG^[131], VSL#3 in a study available only in abstract form^[141], and the preparation Symbiotic 2000^[142]. Only one small RCT tested the efficacy of probiotic in inducing remission in CD^[134], with no difference *vs* placebo. The disappointing results of human studies are confirmed in experimental models: in SAMP/YitFc spontaneous model of CD, administration of VSL#3 was effective only for prevention of disease, and not in established disease, and the preventive effect was obtained only with a 50-times higher dose comparing to the one effective in experimental colitis^[68]. Those data underscore that probably the application of probiotics in CD needs to be further improved in terms of dose and timing of administration.

CONCLUSION

The recent expansion of research on gut microbiota opened the way to the application of probiotic therapy for IBD. Probiotic bacteria may be of value in IBD, since consistent experimental work has unraveled potential molecular targets in the pathogenesis of intestinal inflammation. In IBD patients, altered bacterial composition, defects in the barrier function, impairment in innate and adaptive immune response, may lead to a deregulated chronic inflammatory condition. Probiotics are likely to act through different mechanism affecting all the aforementioned defects and restore alterations at several levels, thus promoting gut health and homeostasis. Nonetheless, clinical data are still inconclusive, in particular in CD. It is desirable that the progress in the research in IBD pathogenesis and in host-bacteria interaction will drive to a tailored approach, including appropriate selection of patients with specific features of disease, utilization of bacterial species with proven efficacy, and with the characterization of effective therapeutic schemes.

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Decoy receptor 3: Its role as biomarker for chronic inflammatory diseases

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Abstract

Members of the tumor-necrosis factor- α (TNF- α) and TNF- α receptor (TNFR) superfamilies of proteins (TNFSF and TNFRSF, respectively) play important roles in the function of the immune system. Decoy receptor 3 (DcR3, TNFRSF6b) is a decoy receptor that binds to three TNFSF ligands, FasL, LIGHT and TL1A. Association to these ligands competes with the corresponding functional receptors and blocks downstream signaling, leading to immunomodulatory effects, including the prevention of apoptosis. DcR3 lacks a transmembrane region and exists only as a secreted protein, which is detectable in biological fluids. Recent studies have shown that DcR3 is upregulated and may be pathogenetically implicated in several and diverse chronic inflammatory diseases. The strongest associations have been described for rheumatological diseases, mainly systemic lupus erythematosus and rheumatoid arthritis, inflammatory bowel disease, and serious infectious conditions, including systemic inflammatory response syndrome. In the majority of these conditions, DcR3 mRNA and protein expression is elevated both at the target tissues as well as in the systemic circulation. DcR3 concentration in the serum is untraceable in the majority of healthy individuals but can be detected in patients with various inflammatory diseases. In most

such cases, soluble DcR3 correlates with disease severity, as patients with severe forms of disease have significantly higher levels than patients with milder or no activity. In addition, effective anti-inflammatory treatment leads to the disappearance of soluble DcR3 from the circulation. Taken together, current evidence suggests that serum DcR3 may become a useful biomarker for chronic inflammatory disorders, as it is up-regulated in response to inflammatory stimuli, and may serve both as a prognostic marker for disease severity and as a surrogate indicator of response to treatment.

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Key words: Decoy receptor 3; Tumor necrosis factor receptor superfamily of proteins; Chronic inflammation; Infection; Disease activity; Biomarker

Core tip: Members of the tumor-necrosis factor- α (TNF- α) and TNF- α receptor superfamilies play important roles in the function of the immune system. Decoy receptor 3 (DcR3) is a decoy receptor that exists only as soluble protein and has the ability to bind to FasL, LIGHT and TL1A. Recent studies showed that DcR3 is upregulated and may be pathogenetically implicated in systemic lupus erythematosus, rheumatoid arthritis, inflammatory bowel disease and infection. DcR3 may become a useful biomarker for chronic inflammatory disorders, as it is upregulated in response to inflammatory stimuli, and may serve both as a prognostic factor for disease severity and as an indicator of response to treatment.

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INTRODUCTION

Decoy receptor 3 (DcR3, alternative names TR6 or M68) is a member of the tumor necrosis factor receptor superfamily (TNFRSF), officially designated TNFRSF6b^[1]. It forms part of a distinct subset within the family of TNFR-like proteins, namely the decoy receptor family, which also includes DcR1, DcR2 and osteoprotegerin^[2]. DcR3 shares a very similar protein sequence identity with osteoprotegerin in that they both lack a transmembrane region; hence, they exist solely in a secreted form as soluble receptors^[3].

It should be mentioned that the original publications for DcR3/TR6/M68 reported high expression of this protein in a variety of neoplastic tissues. This, in combination to its anti-apoptotic function that is described below led to a large body of research regarding the importance of this cytokine in carcinogenesis. The present review will focus exclusively to the recently appreciated role of DcR3 as an immunological-mediator and inflammatory biomarker. In particular, the association with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), inflammatory bowel disease (IBD) and infection will be presented in detail.

REGULATION OF DcR3 EXPRESSION

The *tnfrsf6b* gene that encodes for the human DcR3 protein is located at chromosome 20 (20q13.3). The translational product is a protein containing 300 amino acids^[4]. Western blot analysis of samples from transgenic mice for the full-length human DcR3 estimated a molecular size of 33 kDa and evidence for a glycosylated protein^[4]. DcR3 is not found in mouse or rat genomes; on the other hand, sequences homologous to the *tnfrsf6b* gene have been described in a variety of species such as chicken, frog, chimpanzee or cattle^[3,5].

DcR3 mRNA is detected in variable amounts in healthy adult tissues, including spleen, colon, lung, stomach, spinal cord, lymph node, and trachea^[1,4,6]. In contrast, its expression is weak in the thymus and undetectable in peripheral blood lymphocytes^[1]. In addition, immunoreactivity against DcR3 protein was usually weak or undetectable in normal tissues, with the exception of the luminal portions of crypt epithelium^[4]. At the cellular level, DcR3 transcripts were present, although weakly, in most hematopoietic cell lines and induced upon activation in Jurkat T leukemia cells. Finally, DcR3 mRNA were constitutively and strongly expressed in the endothelial cell line, HUVEC^[6].

The regulation of DcR3 expression is not fully elucidated and reported results may differ between different cell-types or between different experimental systems (Figure 1). *In vitro* studies in intestinal epithelial cells show elevated expression of DcR3 mRNA after stimulation with lipopolysaccharide or tumor-necrosis factor- α (TNF- α)^[7,8]. LPS-induced DcR3 upregulation in IECs is mediated through the activation of extracellular signal-

regulated kinase 1 and 2 and c-Jun NH2-terminal protein kinase, as well as of the transcription factor NF- κ B. In human monocytes and myeloid-derived dendritic cells, DcR3 secretion was induced *via* Toll-like receptors -2 and -4^[9]. This was dependent upon the p42/p44 mitogen-activated protein kinases, Src-like protein tyrosine kinases, and phosphatidylinositol 3-kinase. Finally, in keratinocytes, DcR3 mRNA expression is downregulated after exposure to UVB irradiation^[10,11].

FUNCTION OF DcR3

The soluble DcR3 protein is capable of binding to three ligands of the tumor necrosis factor superfamily (TNFSF), in particular, Fas ligand (FasL/CD95L/TNFSF6)^[1], lymphotoxin-like inducible protein that competes with glycoprotein D for herpesvirus entry on T cells (LIGHT)^[6] and TNF-like molecule 1A (TL1A)^[12]. All TNFSF members are type II transmembrane proteins. They are initially expressed as membrane-bound molecules but subsequently may be cleaved off from the membrane and released in the peripheral circulation. It should be noted that, both membrane-bound and soluble forms of these ligands are functional^[13]. The three ligands that recognize DcR3 are also capable of binding to additional TNFRSF members that act as the functional (as opposed to decoy) receptors. In particular, FasL binds to Fas^[1], LIGHT to lymphotoxin-beta receptor and to the herpesvirus entry mediator (HVEM)^[14], and TL1A to Death Receptor 3 (DR3)^[12]. DcR3 competes for ligand binding, restricting therefore functional signaling mediated by Fas/FasL, LIGHT/HVEM, and TL1A/DR3 interactions. As a result, the functional importance of DcR3 has been associated with two pathways, primarily: first as an anti-apoptotic protein and second as an immunomodulatory molecule^[15]. It should be noted that the lack of DcR3 expression in the mouse is associated with difficulties in the study of its function, as it prohibits mechanistic studies in knockout strains or pharmacological neutralization of the molecule.

Several studies have clearly shown that DcR3 inhibits apoptosis. This is accomplished through its ability to prevent functional association between TL1A/DR3, FasL/Fas, and LIGHT/HVEM, which all lead to programmed cell death^[1,6,12]. Although DcR3-mediated regulation of cell death has been primarily emphasized for neoplastic conditions, it also has relevance to the pathogenesis of immunological responses as well. Indeed, inhibition of apoptosis of leukocytes may lead to perpetuation of chronic tissue injury^[16]. Such a mechanism is of great importance, as effector lymphocytes are a critical cellular component of most chronic inflammatory conditions^[17].

In addition to inhibiting apoptosis, DcR3 exerts additional immunomodulatory functions as well. Many of these are also mediated through blockade of interactions between its ligands (FasL, LIGHT, TL1A) and their respective functional receptors (Fas, HVEM, DR3), as immunostimulatory signaling is also generated through the latter.

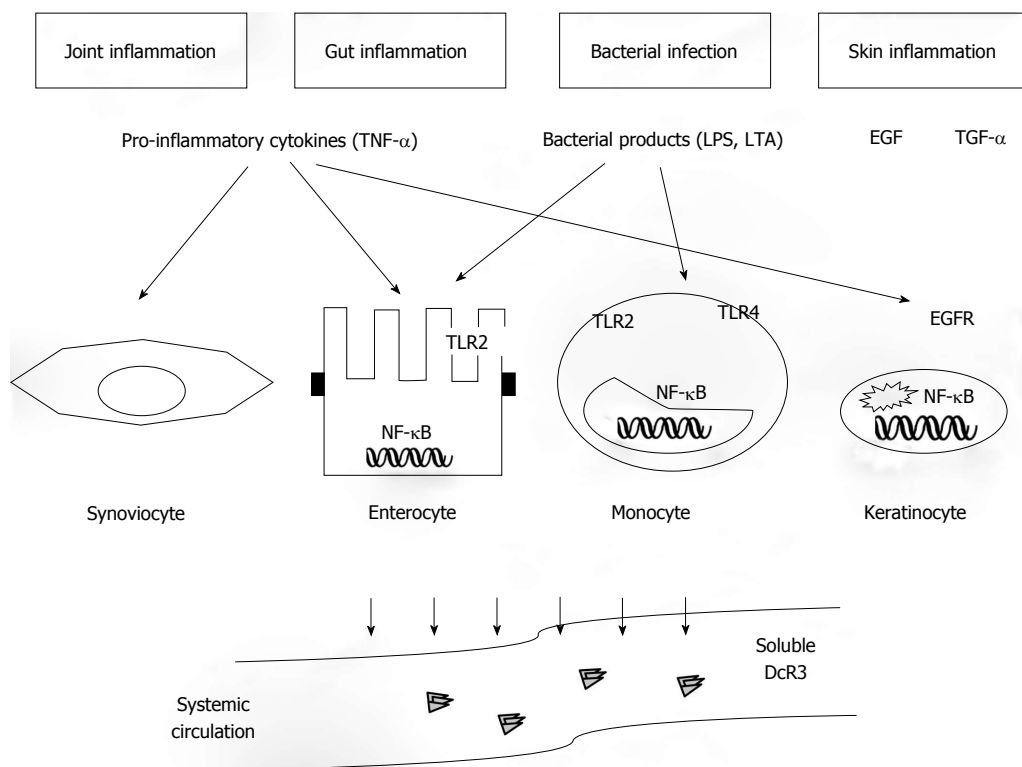


Figure 1 Upregulation of Decoy receptor 3 in chronic inflammation. Decoy receptor 3 (DcR3) exists solely as a secreted protein, which becomes detectable at the peripheral blood of patients with a variety of inflammatory conditions. The most stable associations have been described for gut and joint inflammation as well as for infectious diseases. DcR3 upregulation is mediated via diverse inflammatory stimuli, including pro-inflammatory cytokines [most prominently tumor necrosis factor- α (TNF- α)], bacterial-derived factors such as lipopolysaccharide (LPS) or lipoteichoic acid (LTA), and growth factors including epidermal growth factor and transforming growth factor- α , which interact with their respective receptors. Regulation is highly dependent upon the target cell population, although there is considerable overlap between the various targets. In monocytes and small intestinal epithelial cells LTA and LPS interact with TLR-2 and -4, whereas TNF α is a potent stimulator of DcR3 expression in various cell populations, including epithelial cells and keratinocytes. Nonetheless, a common final signalling pathway for DcR3 induction appears to be the activation of the transcription factor NF- κ B. The latter is a final step that leads to the transcriptional activation of the *tnfrsf6b* gene and release of the DcR3 protein into the systemic circulation. EGF: Epidermal growth factor; TGF- α : Transforming growth factor- α ; TLR: Toll like receptor; NF- κ B: Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1; EGFR: Epidermal growth factor receptor.

As these interactions usually lead to pro-inflammatory responses, an anti-inflammatory role has been attributed to DcR3. For example, DcR3 can ameliorate T-cell responses to alloantigens by neutralizing LIGHT-HVEM association^[18]. Similarly, DcR3 is capable of attenuating T-cell activation and downregulating the secretion of proinflammatory cytokines mediated by TL1A/DR3 signaling^[12]. There is recent evidence that DcR3 also has non-decoy, direct immunomodulatory properties. DcR3 induces polarization of antigen-presenting cells towards a Th2 immunophenotype with concomitant downregulation of their activation status, as indicated by reduced expression of HLA-DR and CD80/B7.1^[19]. Along the same line, DcR3 was shown to divert macrophages towards an M2/immunosuppressive phenotype^[20]. Furthermore, DcR3 facilitates adhesion of circulating monocytes to the endothelium by upregulating the expression of intercellular adhesion molecule-1, vascular cell adhesion molecule 1 and interleukin (IL)-8 through NF- κ B-mediated mechanisms^[21]. This may lead to enhanced homing of cells to inflammatory foci.

Collectively, these data indicate that DcR3 exerts pleiotropic roles during inflammatory disorders (Figure 2). DcR3 is usually undetectable in the sera of healthy in-

dividuals but becomes detectable and/or highly elevated during a number of diseases characterized by chronic inflammation. Although the association between elevated serum DcR3 concentration and chronic inflammatory disorders is indisputable, several critical questions remain unanswered. First, it is often unclear whether the elevated DcR3 protein exerts protective or pathogenic roles during specific immunological conditions, given the vast array of functions that have been attributed to this molecule. Second, the pathogenetic relevance of such systemic elevations of DcR3 may not be easily recognized, as this may be a secondary phenomenon related to the presence of inflammation and not a causative factor. Moreover, the specific disease state may be of critical importance, with DcR3 exerting pro- or anti-inflammatory effects in different settings. In the remaining of this article the strongest associations of DcR3 with inflammatory disorders will be reviewed (Table 1).

Rheumatologic diseases

Increased secretion of DcR3 has been reported in several rheumatologic conditions. A strong association has been described for SLE. Indeed, serum DcR3 is significantly

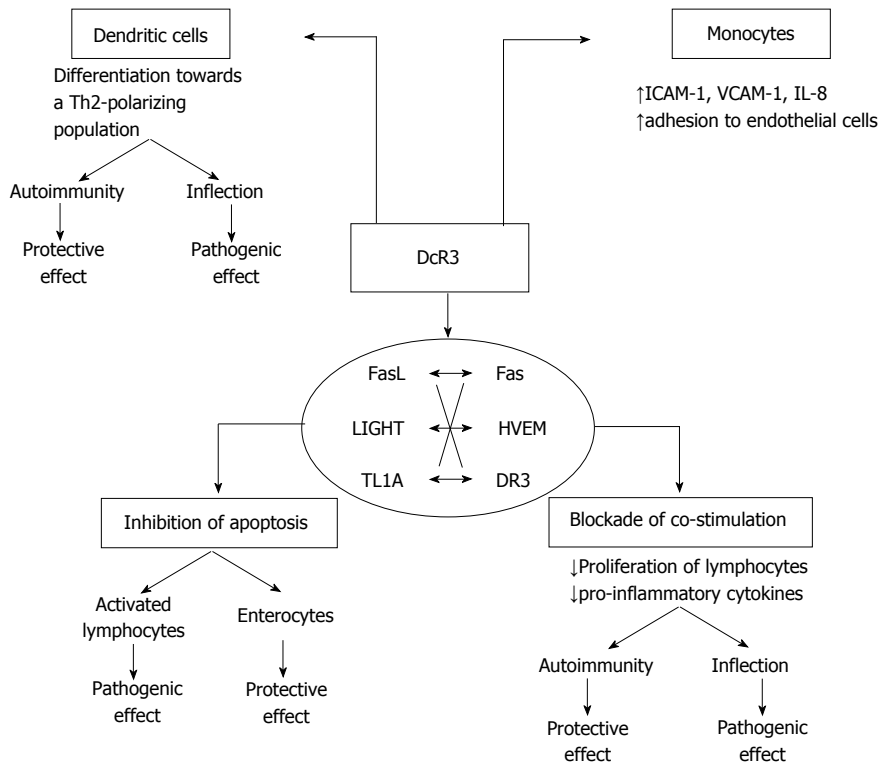


Figure 2 Dual immunomodulatory function of Decoy receptor 3. Decoy receptor 3 (DcR3) displays several properties that are of pathogenetic relevance during inflammatory reactions. The final effect may be either protective or pathogenic depending on several parameters, such as the specific condition (autoimmunity vs infection) and the cell type (lymphocyte, monocyte, epithelial cell). The majority of these effects are associated to the function of DcR3 as a decoy receptor, which inhibits signalling via the tumor necrosis factor superfamily ligands FasL, lymphotoxin-like inducible protein that competes with glycoprotein D for herpesvirus entry on T cells (LIGHT), and tumor necrosis factor-like molecule 1A (TL1A), through competition for ligand binding with the respective functional receptors Fas, herpesvirus entry mediator (HVEM), and DR3. DcR3 inhibits apoptosis induced by ligand/receptor interactions, which may have either deleterious or beneficial effects depending upon the specific cellular component. In particular, blockade of apoptosis in activated effector lymphocytes would lead to perpetuation of chronic inflammatory injury. On the other hand, it may induce the survival of intestinal epithelial cells in inflammatory bowel disease, leading to tissue protection. DcR3 may also interfere with effector immunological responses in several ways. It blocks LIGHT/HVEM- or TL1A/DR3-mediated co-stimulation of activated lymphocytes, leading to impaired proliferation and defective cytokine production. Such an effect may have direct anti-inflammatory function during autoimmune diseases. On the opposite, in the event of an infection such defects in effector lymphocyte activation may lead to impaired clearance of the microorganism and enhanced severity of the infection. The same duality takes place in relation to the effect of DcR3 on dendritic cell differentiation towards a Th2-polarizing antigen-presenting-cell subtype, which is a non-decoy function, as it occurs independently of binding to FasL, LIGHT or TL1A. Such Th2 polarization may dampen Th1-type pro-inflammatory responses in autoimmunity, but may lead to defective anti-microbial function during infections. An inverse correlation may occur for the effects of DcR3 on monocytes that include upregulation of the adhesion molecules intercellular adhesion molecule-1 and vascular cell adhesion protein-1 and enhanced adherence to endothelial cells. This may offer an advantage during infection via the recruitment of inflammatory cells; at the same time, however, it may be deleterious during autoimmune inflammation as it may lead to the constant homing of monocytes to the inflammatory foci and perpetuation of tissue injury. ICAM-1: Intercellular adhesion molecule-1; VCAM-1: Vascular cell adhesion protein-1.

elevated in patients with SLE as compared with healthy controls^[22,23]. In one study, serum DcR3 levels were elevated in 43% of 90 SLE patients and only 2.4% of 123 healthy individuals^[23]. More importantly, patients with active disease (SLE disease activity index ≥ 10) had higher concentrations than patients in remission (SLE disease activity index < 10), indicating a positive correlation between sDcR3 concentration and disease activity^[22]. This is further supported by the fact that, when compared with DcR3-negative SLE patients, a larger percentage of DcR3-positive patients showed abnormally high serum IgE levels, which serves as a surrogate marker of ongoing Th2 immune responses^[23]. The potential involvement of DcR3 in the pathogenesis of SLE is supported by studies in mice expressing a human DcR3 transgene under the control of an actin promoter^[24]. These mice develop a SLE-like syndrome sharing characteristic features of the human condition, including autoantibodies against

double-stranded DNA, glomerular nephritis with IgG and C3 deposition, skin lesions, SLE-like hepatopathy and pancytopenia. This clinical similarity was associated with parallel immunological disturbances as well. Indeed, DcR3 transgenic mice demonstrated dominant Th2 associated features such as enhanced IL-4 expression and elevated serum IgE levels. Of particular interest is the fact that penetrance of this murine syndrome was, like SLE, sex-associated, as it was described in 60% of females *vs* in only 20% of males^[24]. Subsequent studies from the same group showed that the murine clinical and immunological SLE-like syndrome was transmissible to irradiated C57BL/6 female mice upon transplantation of T-cell-depleted bone marrow from DcR3 Tg mice^[23]. These findings showed that DcR3 secreted from cells of hematopoietic origin induces a SLE-like syndrome in mice. Although the exact pathogenetic role of DcR3 in SLE remains to be determined, at least two mechanisms have

Table 1 Expression profile of decoy receptor 3 in chronic inflammatory diseases and potential as a biomarker

Disease	Expression of DcR3	Utility as biomarker
Systemic lupus erythematosus ^[22,23]	Serum	Higher levels in active disease than in remission
Rheumatoid arthritis ^[26-29,32]	Serum Synovial fluid Fibroblast-like synoviocytes Inflamed synovium	Correlates with disease stage "Undetectable DcR3/low TL1A" serotype may predict lack of atheromatic plaque formation/progression in RA patients
Systemic sclerosis ^[34]	Serum	Higher levels in diffuse cutaneous systemic sclerosis than in limited cutaneous disease Correlates with markers of right ventricular function Correlates with inflammatory markers (CRP, ESR)
Osteoarthritis ^[27,28,35]	Serum Chondrocytes	
Ulcerative colitis ^[37,38]	Inflamed colon Lamina propria T cells	Higher levels in active disease than in remission Decrease in serum levels in response to treatment
Crohn's disease ^[7,39]	Serum Inflamed colon Inflamed ileum Small Intestinal epithelium Mononuclear and endothelial cells next to mucosal ulcers	Higher levels in active disease than in remission Decrease in serum levels in response to treatment
Appendicitis ^[8]	Serum	
Sepsis ^[9,43]	Epithelium of inflamed appendix Serum Monocytes Myeloid-derived dendritic cells	DcR3 levels may discriminate sepsis <i>vs</i> SIRS Correlates with APACHE II score
Tuberculosis ^[42]	Serum	High serum levels predictive of development of active disease in patients with latent tuberculosis High serum levels associated with poor prognosis High serum levels predictive of multiple organ failure and 28-d mortality
ARDS ^[44]	Serum	
Atopic dermatitis ^[48]	Serum	
Psoriasis ^[49,50]	Psoriatic lesions Keratinocytes Macrophages Perivascular areas	
Liver disease ^[45]	Regenerative and dysplastic nodules in cirrhosis	
Multiple sclerosis ^[51]	Cerebrospinal fluid	
Chronic peritoneal dialysis ^[46]	Serum	High serum levels predictive of peritonitis
Silicosis ^[53]	Peripheral blood mononuclear cells	

DcR3: Decoy receptor 3; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; SIRS: Systemic inflammatory response syndrome; APACHE: Acute physiology and chronic health evaluation; ARDS: Acute respiratory distress syndrome.

been proposed. Firstly, DcR3 may inhibit FasL-mediated apoptosis in activated T cells. In turn, this may result in impaired activation-induced cell death of T cells, leading to their enhanced survival and intensification of effector pathways. Along that line, missense mutations have been recently identified in adult and juvenile patients with SLE, which resulted in altered binding kinetics to FasL and significantly increased lymphocyte proliferation^[25]. Furthermore, in the previous study with DcR3 transgenic mice it was shown that recombinant DcR3 or endogenous DcR3 produced by transgenic T cells protected T cells from activation-induced apoptosis *in vitro*^[24]. Secondly, co-stimulation of T cells with soluble DcR3-Fc increased IL-2 and interferon-gamma production. Furthermore, T Lymphocytes isolated from patients with SLE displayed enhanced reactivity to DcR3. Thus, DcR3 seems to be responsible at least partly for T cell hyper-reactivity in SLE both by preventing apoptosis induced by cell-activation as well as by providing co-stimulatory signals^[22].

DcR3 expression is also elevated in RA. Previous studies have shown that DcR3 protein is more frequently detected in the sera of patients with RA than in healthy controls^[26]. In addition, the mean DcR3 concentration is significantly higher in RA (*vs* healthy controls) and, more significantly, it correlates with severe RA stage^[26]. In another study sDcR3 was found to be significantly higher in patients with RA than in those with osteoarthritis^[27]. Furthermore, DcR3 is also detected in the synovial fluid of patients with inflammatory arthritis, including RA, ankylosing spondylitis and osteoarthritis^[26-28]. In addition, DcR3 mRNA transcripts were detected in fibroblast-like synoviocytes isolated from patients with RA or osteoarthritis^[29]. A recent study further characterized the distribution of DcR3 expression in the inflamed synovium^[28]. It was shown that no significant differences were observed in synovial lining layer between RA, ankylosing spondylitis and osteoarthritis. On the other hand, at the sublining layer, the expression of DcR3 was more upregulated

in RA and AS than in osteoarthritis. Taken together, the increased local production of DcR3 at the inflamed synovium raises the possibility that this protein participates in the pathogenesis of arthritis. This hypothesis is, in fact, supported by recent experimental evidence. Firstly, DcR3 was shown to inhibit FasL-mediated apoptosis of fibroblast-like synoviocytes^[29]. Secondly, expression of DcR3 was highly upregulated by TNF- α , a cytokine with a pivotal role in the pathogenesis of RA. Indeed, in one study, serum DcR3 concentrations strongly correlated with the respective TNF- α values^[27]; furthermore, TNF- α induced the expression of DcR3 in synoviocytes, an effect that was specific for RA, as it was not observed in osteoarthritis^[29]. Thirdly, a protective role for DcR3 in experimental collagen-induced arthritis was recently proposed^[30]. Forced over-expression of DcR3 in mice *via* plasmid transfer resulted in significantly attenuated arthritis severity, combined with downregulated immunological responses. The latter included a smaller size of inguinal lymph nodes, lower numbers of CD19⁺ B cells and interferon (IFN)- γ , IL-4, IL-17A, and Foxp3-positive CD4⁺ T cells and decreased circulating IL-6. Finally, a protective role for DcR3 was also implicated by a recent *in vitro* study, which reported that DcR3 was capable of binding to TL1A expressed on fibroblast-like synoviocytes from RA patients and inhibited their proliferation induced by pro-inflammatory cytokines^[31]. In all, the aforementioned experimental and clinical evidence indicates that DcR3 may be of pathogenetic relevance in RA. A further intriguing possibility arose from a study from our group that examined the predictive value of DcR3 (and its ligand TL1A) for atheromatic plaque formation in patients with RA^[32]. In this study, 45 patients had a baseline measurement of TL1A and DcR3 serum concentration and were prospectively followed up for more than 3 years with ultrasound of carotid and femoral arteries. It was shown that a serum phenotype of "low TL1A and undetectable DcR3" was predictive of significantly fewer newly formed carotid plaques during the next 3.5 years and a preserved atherosclerosis profile in carotid or carotid and/or femoral arteries. This study expands previous work on the role of the TL1A/DR3/DcR3 pathway in atherogenesis^[33], and indicates that serial measurements of these proteins in the serum may serve as biomarkers of low-grade systemic inflammation and predict the future risk for plaque formation in RA patients.

Recently, an association was also described between serum DcR3 and systemic sclerosis (SSc)^[34]. It was found that patients with diffuse cutaneous SSc had significantly higher serum DcR3 concentrations than healthy controls or those with limited cutaneous disease. The authors concluded that, in SSc, soluble DcR3 values may serve as a surrogate biomarker of pulmonary arterial hypertension and systemic inflammation due to its significant correlation with indicators of right ventricular function or of systemic inflammation (CRP, ESR, and IgG), respectively.

Apart from its involvement in the pathogenesis of arthritis, DcR3 may also interfere with bone metabolism,

as suggested by recent studies. DcR3 is expressed in chondrocytes and affects their proliferation *via* Extracellular Signal-Regulated Kinase signaling and Fas-induced apoptosis^[35]. Thus, DcR3-mediated pathways may interfere with cartilage regeneration, whereas DcR3 manipulation may offer therapeutic opportunities for people with osteoarthritis. In addition, DcR3 has also been shown to promote formation of osteoclasts from monocytes, macrophages, and bone stromal marrow cells^[36]. Mechanistic evidence for such a function was shown in DcR3 transgenic mice which displayed significantly lower bone mineral density and total body bone mineral content when compared with controls. Furthermore, local administration of DcR3 resulted in decreased bone volume. These data indicate an effector role for DcR3 in inducing osteoclast formation and bone resorption activity, and suggest that manipulation of DcR3 expression/signaling may be a therapeutic option for restoring bone metabolism and prevent/reverse osteoporosis^[36].

IBD

The association of DcR3 with chronic inflammatory diseases of the gastrointestinal tract, in particular ulcerative colitis (UC) and Crohn's disease (CD) has been extensively studied. A first study in UC reported increased immunostaining for DcR3 protein (and for other decoy receptors) at inflamed intestinal regions, but not at healthy colon^[37]. T-cells were the major source of DcR3 expression. Similar upregulation of DcR3 was demonstrated in CD^[7]. In particular, DcR3 mRNA transcripts were increased in the small intestinal epithelial compartment of areas affected by CD as compared to non-inflamed controls. Furthermore, Western-blot analysis detected elevated expression of DcR3 protein in areas with active CD. Immunohistochemical analysis revealed increased localization of DcR3 to follicle-associated epithelial cells in areas with CD-related inflammation. Additional staining was observed in mononuclear and endothelial cells in the vicinity of ulcerative lesions^[7]. It should be noted that elevated expression of DcR3 was also reported in the epithelium of acutely inflamed appendices, suggesting a stimulatory effect of mucosal inflammation to the local expression of DcR3^[8]. Recent studies from our group have confirmed the elevated mucosal expression of DcR3 mRNA in areas with CD-or UC-related inflammation in comparison to either healthy controls or non-inflamed areas from IBD^[38,39].

The aforementioned studies provided information regarding the functional importance of DcR3 in IBD. The central pathophysiological hypothesis is that mucosal DcR3 interferes with FasL-mediated apoptosis of lamina propria T cells. This would be pathogenic as it would lead to increased survival of effector lymphocytes and perpetuation of pro-inflammatory responses. Interestingly, the mucosal expression of DcR3 significantly correlated with that of TL1A and Fas-L, indicating that DcR3 is upregulated in response to an initial increase of its ligands and, by blocking functional signaling through DR3 and Fas,

exerts anti-apoptotic function^[39]. At the same time, however, DcR3 also protected small-intestinal epithelial cells from apoptosis, an effect that would lead to beneficial effects. Taken together these studies may imply that DcR3 exerts dichotomous functions at the intestinal mucosa depending on the particular conditions and the specific cellular localization.

The potential of DcR3 as a biomarker for disease activity in IBD was also tested. The concentration of DcR3 in the serum was found significantly elevated in patients with active IBD (UC or CD)^[7,38,39]. This was evident in comparison with either IBD-patients in remission or healthy individuals. More importantly, when pre- and post-treatment values in the same patient were measured, we found that effective anti-inflammatory treatment resulted in almost complete disappearance of sDcR3 from the systemic circulation. This decrease paralleled a similar reduction in CRP concentration^[38]. Interestingly, in the study by Funke *et al.*^[7], TNF- α was shown to be a potent inducer of DcR3 expression. Accordingly, blockade of TNF- α with neutralizing monoclonal antibodies resulted in significant downregulation of the systemic expression of DcR3 in both RA and UC^[26,38,39]. The systemic expression of DcR3 may also be affected by the presence of IBD-associated polymorphisms in the *tnfrsf6b* gene. In recent studies such polymorphisms were associated with early-onset IBD. Interestingly, these polymorphisms resulted not only with higher mucosal expression of DcR3 mRNA but also with elevated circulating levels of this protein^[40].

Taken together, these studies indicate that DcR3 is expressed at the intestinal mucosa, becomes highly upregulated in active inflammatory lesions and is shed to the systemic circulation during active IBD. Larger studies will be needed to further clarify its potential for use as a sensitive biomarker of active disease and whether serial measurements may have a predictive value for response to treatment.

INFECTIOUS DISEASES

The role of DcR3 in infection has been studied in depth and its potential as a biomarker during septic conditions has been intensively sought. One of the first studies reported significantly elevated serum concentrations of patients with various infectious diseases when compared with healthy controls^[9]. Further supporting an association between infectious disease and DcR3, it was shown that the major DcR3 producers were monocytes and myeloid-derived dendritic cells which released DcR3 upon stimulation with bacterial products from both gram-positive and gram-negative microorganisms *via* Toll-like receptor -2 and -4 signaling^[9]. This elevated DcR3 expression following infection may have negative results for the outcome of bacterial disease as it was shown by studies in DcR3 transgenic mice, which demonstrated a defect in developing a Th1-type response^[41]. In particular, splenocytes from transgenic mice displayed upregulation of IL-4 and IL-10 and down-regulation of IFN- γ , IL-12,

and TNF- α upon stimulation *in vitro* by influenza hemagglutinin peptide. More importantly, these mice showed increased susceptibility to infection with *Listeria monocytogenes*, accompanied with reduced expression of IFN- γ . Therefore, a Th2-type bias of immune responses in the face of elevated expression of DcR3 may lead to defective clearance of microorganisms that are eliminated *via* Th1-based cell-mediated immunity. Findings from a recent study in tuberculosis support this hypothesis^[42]. In this work it was shown that high DcR3 serum levels in conjunction with high prostaglandin-2 and low lipoxin levels, predicted with adequate sensitivity the development of active tuberculosis in patients with the latent form of the disease. Interestingly, high serum DcR3 expression correlated with a poorer prognosis. Thus, DcR3 may serve as an indicator of inadequate confinement of latent tuberculosis either through suppression of Th1 responses or through regulation of apoptotic pathways^[42].

Two recent studies looked at the validity of DcR3 serum concentration as a prognostic marker for disease outcome in septic conditions. In the first study, DcR3 levels were found to accurately distinguish sepsis from systemic inflammatory response syndrome (SIRS)^[43]. Receiver-operating characteristic curves analysis was conducted and a cut-off value of DcR3 concentration was proposed which predicted sepsis from SIRS with 96% sensitivity and 82.6% specificity. DcR3 concentration in the peripheral blood was also correlated positively with the APACHE II score; one of the most commonly used indexes for the quantification of sepsis severity. These results were complementary to findings from another study that reported DcR3 as a valuable predictor of adverse outcome in patients with acute respiratory distress syndrome (ARDS)^[44]. Indeed, from a variety of biomarkers tested, only DcR3 serum concentration discriminated those patients with multiple organ failure and mortality within 28-d from survivors. The predictive value of DcR3 was evident from the first week of ARDS. The interpretation of these results may be two-fold and indicate that elevation of DcR3 may be an epiphenomenon resulting from increased secretion of this protein by inflammatory cell-populations. Alternative DcR3 may also be pathogenetically implicated *via* its known immunomodulatory effects which include apoptosis of dendritic cells, suppression of HLA-DR surface expression and diversion of the Th1/Th2 balance towards Th2 responses.

OTHER DISEASES

Although not as well-studied as the conditions mentioned in the previous sections, DcR3 tissue and systemic expression has been reported for several other non-neoplastic conditions. In chronic liver disease, DcR3 was expressed mainly in biliary epithelial cells and infiltrating lymphocytes^[45]. The main DcR3 localization was observed in regenerative and dysplastic nodules of cirrhotic livers. The elevated expression of DcR3 in chronic liver disease, insinuates a role for this molecule in the chronic hepatitis-cirrhosis-hepatocellular carcinoma sequence,

possibly *via* the modulation of apoptosis of hepatocytes^[45]. In the renal system dual roles has been attributed to DcR3 according to the specific clinical scenario. A prospective cohort study in patients receiving chronic peritoneal dialysis demonstrated that elevated circulating DcR3 levels are associated with increased risk for subsequent peritonitis, and may serve as an independent predictor for this, often fatal, complication^[46]. On the other hand, DcR3 expression may have beneficiary effects in IgA nephropathy. In an animal model of this disease DcR3 plasmid administration and subsequent DcR3 protein expression, prevented the clinical onset of nephropathy^[47]. DcR3 was found to act on multiple steps of the disease process, mainly *via* the systemic modulation of T cell activation/proliferation but also *via* the prevention of mononuclear leukocyte infiltration and apoptosis in the kidney. In dermatology, it was reported that serum DcR3 is elevated in patients with atopic dermatitis^[48]. In addition, DcR3 was over-expressed in skin lesions from patients with psoriasis^[49]. Cells staining positive for DcR3 protein expression were among those that are critically involved in the pathogenesis of chronic skin inflammation, such as keratinocytes, macrophages in deep dermis and cells at the perivascular area^[49]. Recently it was reported that up-regulation of DcR3 in keratinocytes is driven by epidermal growth factor receptor, further supporting a potential pathogenetic importance in psoriasis^[50]. DcR3 was also elevated in cerebrospinal fluid samples from patients with multiple sclerosis and other inflammatory neurological diseases in comparison with patients with herniated discs^[51]. This finding prompted an investigation of the therapeutic potential of DcR3 in mice with experimental autoimmune encephalomyelitis by administering DcR3 intrathecally^[52]. This intervention improved significantly the neurological symptoms and ameliorated inflammation in the spinal cord. These findings were replicated in an adoptive transfer study that showed that splenocytes from DcR3-treated mice retained the ability to influence positively the course of the disease. These effects were attributed to the modification of effector T-cell responses, with a shift towards elevated numbers of Th2 and decreased Th1 and Th17 cells^[52]. Finally, DcR3 was studied also in silicosis. It was reported that the DcR3 gene mRNA was significantly overexpressed in the peripheral blood mononuclear cells of patients with silicosis in comparison to healthy controls^[53,54]. The authors suggested that a fraction of CD4⁺ T lymphocytes expresses low levels of membrane Fas and secretes significantly higher levels of DcR3 along with soluble Fas and spliced shorter variants, resulting in considerable resistance to anti-Fas autoantibody-induced apoptosis.

CONCLUSION

The importance of DcR3 as an immunological biomarker has been emphasized in recent years. Indeed, its application appears to be advantageous due to several reasons. First, DcR3 exists only as a secreted protein, therefore, fluctuations of its expression are easily detected and

measurable in the peripheral blood. Second, in healthy conditions, its expression is minimal in most tissues as it is usually absent or very low at the systemic circulation. Detecting, therefore, elevated amounts of DcR3 protein in the serum is usually indicative of an ongoing inflammatory response, when neoplasms have been excluded. Third, soluble DcR3 concentration appears to respond better to the presence of inflammation than the levels of the corresponding ligands. Fourth, DcR3 levels in the systemic circulation are usually associated with disease severity. As mentioned above, patients with active/severe forms of SLE, RA, IBD, SSc, infections and ARDS have significantly higher levels of serum DcR3 than patients with milder forms of the disease. Therefore, elevated DcR3 levels may serve as an adverse prognostic factor. Finally, anti-inflammatory treatment, including biological therapies leads to a definitive decrease in soluble DcR3. This indicates that DcR3 may be useful as marker of response to treatment. In all, DcR3 holds great promise to become a useful biomarker for the estimation of disease activity and follow-up of treatment responses in patients with various inflammatory diseases.

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Role of *IL28B* genotype in older hepatitis C virus-infected patients

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Abstract

The average age of hepatitis C virus (HCV)-infected individuals is becoming increasingly higher in Japan and steps should be taken to treat older individuals infected with HCV. Until an interferon-free regimen becomes available, peginterferon plus ribavirin will play a critical role in the treatment. The perception that older HCV-infected patients may be at higher risk than younger patients for adverse events from peginterferon plus ribavirin treatment but may obtain less clinical benefit from it may be based on the underrepresentation of older patients in clinical trials. A recent genome-wide association study revealed that interleukin-28B (*IL28B*) genotype closely correlates with the treatment response against HCV. The relationship of *IL28B* genotype with the treatment response in older HCV-infected patients is also unknown. In this review, we focused on the treatment response in older patients infected with HCV and the effects of *IL28B* genotype. *IL28B* major genotype is a useful predictor of sustained virological response in the interferon-including treatment of older patients infected with HCV. It also seems useful for avoiding adverse events, although the mechanisms of

the effects of *IL28B* genotype on the treatment outcome are still poorly understood and are currently under investigation. Further studies will be needed.

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Key words: Hepatitis C virus; Interferon lambda 3; Interleukin-28B; Older patients; Telaprevir

Core tip: The exact mechanisms of the effects of interleukin-28B (*IL28B*) genotype on the treatment response in chronic hepatitis C patients are unclear. However, *IL28B* genotype is useful for the continued successful treatment of older patients infected with hepatitis C virus (HCV) and avoiding adverse events. Until the eventual availability of interferon-free regimens, it is important to determine *IL28B* genotype before treating HCV-infected individuals, especially in the screening of older patients.

Kanda T, Nakamoto S, Wu S, Yokosuka O. Role of *IL28B* genotype in older hepatitis C virus-infected patients. *World J Immunol* 2013; 3(3): 54-61 Available from: URL: <http://www.wjgnet.com/2219-2824/full/v3/i3/54.htm> DOI: <http://dx.doi.org/10.5411/wji.v3.i3.54>

INTRODUCTION

Hepatitis C virus (HCV) infection is a cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma in the United States, Western countries and Japan^[1-4]. Most cases of hepatocellular carcinoma are associated with chronic hepatitis B virus or HCV infections^[5]. HCV variants are classified into at least 6 genotypes (representing the 6 genetic groups defined by phylogenetic analysis). It is well known that the treatment response differs among the different HCV genotypes^[6]. Approximately 50% and

Table 1 Association between sustained virological response rates, age and gender in chronic hepatitis C patients treated with standard of care

Ref.	G	Number of patients	Naive	Age (yr, mean $\pm$ SD)/gender (male)	Formula	SVR rates
McHutchison <i>et al</i> ^[17]	1	1016	Yes	47.5 $\pm$ 8.1/59.7%	Low-dose PegIFN alpha-2b plus RBV	38.00%
	1	1019	Yes	47.5 $\pm$ 7.8/60.2%	Standard-dose PegIFN alpha-2b plus RBV	39.80%
	1	1035	Yes	47.6 $\pm$ 8.2/59.2%	PegIFN alpha-2a plus RBV	40.90%
Jeffers <i>et al</i> ^[18]	1	78 (Black)	Yes	46.3 $\pm$ 0.7/72%	PegIFN alpha-2a plus RBV	26.00%
	1	28 (White)	Yes	44.7 $\pm$ 1.4/61%	PegIFN alpha-2a plus RBV	39.00%
Bruno <i>et al</i> ^[19]	1	163	Yes	49.9 $\pm$ 11.1/62%	PegIFN alpha-2b plus RBV	41.10%
	1	148	Yes	49.5 $\pm$ 11.1/62%	IFN alpha-2b plus RBV	29.30%
Conjeevaram <i>et al</i> ^[20]	1	196 (African-Americans)	Yes	49.0 $\pm$ (45.0, 52.5)/64.8%	PegIFN alpha-2a plus RBV	28.00%
	1	205 (Caucasian-Americans)	Yes	48.0 $\pm$ (43.0, 52.0)/65.8%	PegIFN alpha-2a plus RBV	52.00%
Miyauchi <i>et al</i> ^[21]	1	383	-	55.0 $\pm$ 10.9/62%	PegIFN alpha-2b plus RBV	61.80%
Kanda <i>et al</i> ^[22]	1	127	Yes	56.1 $\pm$ 10.7/48.8%	PegIFN alpha-2a plus RBV	56.60%
	1	69	No	59.0 $\pm$ 10.1/49.2%	PegIFN alpha-2a plus RBV	39.10%

G: Genotype; IFN: Interferon; SVR: Sustained virological response; RBV: Ribavirin.

80% achieve a sustained virological response, defined as undetectable HCV RNA at week 24 after stopping the treatment, following the current standard of care combination therapy with peginterferon plus ribavirin for 48 wk in HCV genotype 1 and 24 wk in HCV genotype 2^[6].

In Japan, hepatocellular carcinoma is one of the major malignancies and patients with liver cirrhosis are encouraged to undergo examinations using a combination of real-time ultrasonography and alpha-fetoprotein measurement at regular intervals for an early detection of hepatocellular carcinoma^[7]. The prognosis of elderly hepatocellular carcinoma patients was found to be similar to that for younger cases^[8]. In Japan, as many people live to an advanced age, there are also many elderly hepatocellular carcinoma patients. Therefore, we need to treat elderly patients infected with HCV in order to decrease the mortality and incidence of HCV-related liver diseases^[9,10].

The recent recognition that host genetic polymorphisms near interleukin-28B (IL28B) (*IL28B* genotype), such as a single nucleotide polymorphism (SNP) (rs8099917 or rs12979860), predict the treatment-induced and spontaneous HCV eradications remains an important discovery^[11-15]. IL28B encodes interferon lambda 3 (IFNL3), a cytokine distantly related to type I interferons and IL10^[16]. IL28B, interleukin-28A and interleukin-29 are three closely related cytokine genes that form a cytokine gene cluster on chromosome 19q13. This review focuses on HCV and host factor IL28B genotype in the treatment response of elderly patients with chronic hepatitis C and describes our experiences in treating such patients with chronic hepatitis C.

MOST HCV-INFECTED PATIENTS IN JAPAN ARE OLDER

Treatment response to standard of care in HCV genotype 1-infected patients is shown in Table 1^[17-22]. The reported patients from the United States and European countries

were, on average, younger than those from Japan^[8,21-23]. This suggests that there might be different time spread and roots of transmission of HCV among these countries. In the United States, HCV genotype 1a-infected individuals appeared around 1960, at least 30 years later than the widespread introduction of HCV genotype 1b in the Japanese population^[24]. The spread of HCV genotype 1b in Japan then started to decrease around 1995, whereas HCV genotype 1a in the United States is still growing exponentially^[24]. Japan has high mortality rates from hepatocellular carcinoma, counting about 30000 deaths annually^[25]. According to hepatitis virus carrier rates among first-time blood donors, the peak frequency of antibody to HCV was demonstrated by the 1931-1935 birth cohort, who are 78-82 years old in 2013^[8]. So, to prevent death from hepatocellular carcinoma in Japan, we should treat relatively older patients infected with HCV. We and others previously reported that standard of care was also effective in chronic hepatitis C patients aged > 65 years^[21,26].

SUSTAINED VIROLOGICAL RESPONSE RATES FOLLOWING STANDARD OF CARE IN OLDER HCV-INFECTED PATIENTS

First, we retrospectively analyzed 196 chronic hepatitis C patients aged ≥ 65 years and treated with standard of care in our and affiliated hospitals^[21,22,27,28] (Table 2). This work was carried out in accordance with the Declaration of Helsinki of the World Medical Association. The study protocol was approved by the Ethics Committee of Chiba University School of Medicine.

In the 196 patients treated with standard of care, sustained virological response was achieved in 46.9% (92/196). Sustained virological response was achieved in 43.9% (72/164) of HCV genotype 1 compared with 62.5% (20/32) of HCV genotype 2 patients ($P = 0.082$).

Table 2 Clinical characteristics of hepatitis C patients aged ≥ 65 years and treated with standard of care

	Total (<i>n</i> = 196)	Male (<i>n</i> = 104)	Female (<i>n</i> = 92)	<i>P</i> value ¹
Age (yr)	67.8 $\pm$ 2.5	68.1 $\pm$ 2.6	67.4 $\pm$ 2.4	
Gender (male/female)	104/92	104/0	0/92	
HCV viral load (high/low/unknown)	185/7/4	6/2/1996	1/2/1989	0.169
HCV genotypes 1/2	164/32	Nov-93	71/21	0.033
Treatment-naïve (yes/no/unknown)	108/87/1	55/48/1	52/39/1	0.704
ALT (IU/L)	61.3 $\pm$ 50.7	70.1 $\pm$ 57.1	51.4 $\pm$ 40.3	0.010
White blood cells (/ μ L)	4940 $\pm$ 1490	5160 $\pm$ 1670	4690 $\pm$ 1210	0.027
Neutrophils (/ μ L)	2660 $\pm$ 1080	2790 $\pm$ 1270	2530 $\pm$ 830	0.096
Hemoglobin (g/dL)	13.7 $\pm$ 1.5	14.1 $\pm$ 1.6	13.2 $\pm$ 1.3	0.000
Platelets ($\times 10^4$ / μ L)	15.1 $\pm$ 4.7	15.0 $\pm$ 4.8	15.4 $\pm$ 4.6	0.553
Peg-IFN α - 2a / Peg-IFN α - 2b	86/110	41/63	45/47	0.233

Values are expressed as mean $\pm$ SD. ¹*P* value, comparison between male and female groups. HCV: Hepatitis C virus; ALT: Alanine aminotransferase; Peg-IFN: Peginterferon.

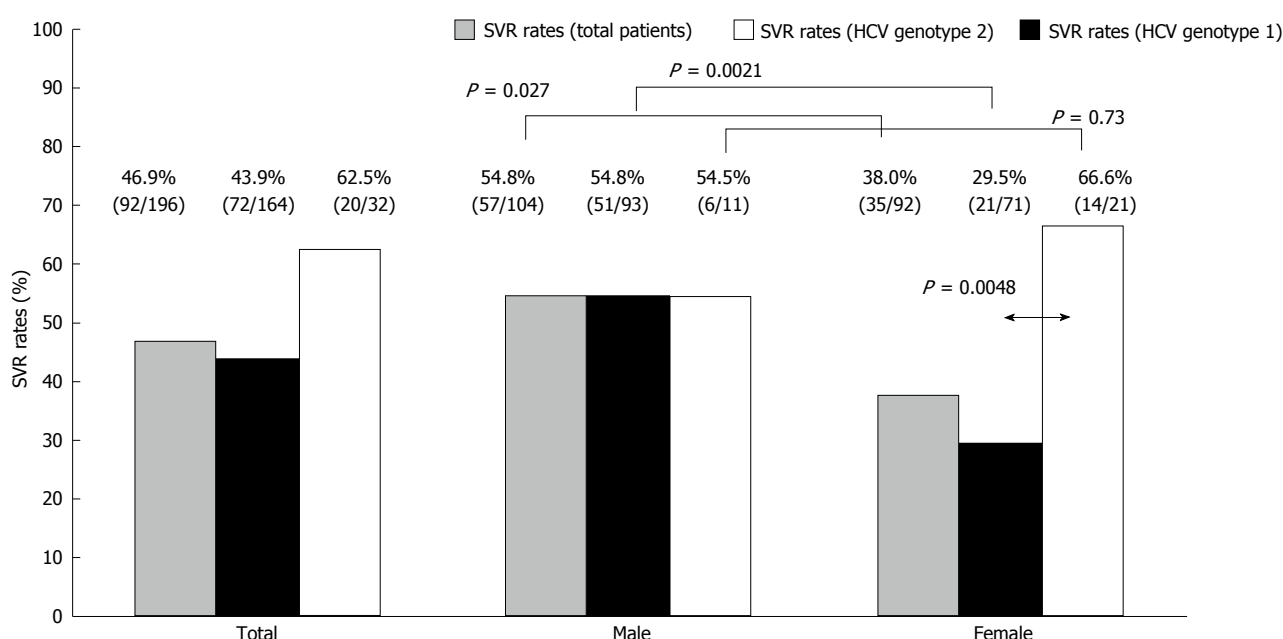


Figure 1 Sustained virological response rates in hepatitis virus genotypes 1/2 patients aged ≥ 65 years and treated with standard of care. Hepatitis C virus variants are classified into at least 6 genotypes (representing the 6 genetic groups defined by phylogenetic analysis). It is well known that the treatment response differs among the different hepatitis C virus genotypes^[6]. SVR: Sustained virological response; HCV: Hepatitis C virus.

In 110 patients treated with peginterferon alpha-2b plus ribavirin, sustained virological response was achieved in 49.4% (44/89) of HCV genotype 1 compared with 66.6% (14/21) of HCV genotype 2 patients. In 86 patients treated with peginterferon alpha-2a plus ribavirin, sustained virological response was achieved in 37.3% (28/75) of HCV genotype 1 compared with 54.5% (6/11) of HCV genotype 2 patients. There were no differences in efficacies or adverse events between the two peginterferons.

In male and female patients aged ≥ 65 years and treated with standard of care, sustained virological response was achieved in 54.8% and 38.0%, respectively (Figure 1). Our results also supported previous studies^[8,26]. Gender differences were more remarkable in HCV genotype 1 than in HCV genotype 2 patients (*P* = 0.0021). In older female patients, the treatment response in HCV genotype 2 was better than that of the others (Figure 1).

ADVERSE EVENTS FOLLOWING STANDARD OF CARE IN OLDER HCV-INFECTED PATIENTS

Interferon plus ribavirin combination therapy for chronic hepatitis C produces a number of well-described side-effects that are dominated by fatigue, influenza-like symptoms, hematological abnormalities and neuropsychiatric symptoms^[29,30]. We defined “severe adverse event” if treatment had to be stopped. In chronic hepatitis C patients aged ≥ 65 years, we identified severe adverse events in 14.7% (29/196) (Table 3). In male and female patients, severe adverse events occurred in 14.4% (15/104) and 15.2% (14/92), respectively. Fried *et al*^[31] reported that severe adverse events were observed in 7% when the mean age of patients treated with standard of care was $42.8 \pm$

Table 3 Severe adverse events in hepatitis C patients aged ≥ 65 years and treated with standard of care *n* (%)

Severe adverse events	No. of patient discontinuities
Occurrence of malignancies (Hepatocellular carcinoma)	5 (13.5)
Severe fatigue	5 (13.5)
Severe pulmonary symptoms (bloody phlegm, cough, or interstitial pneumonitis)	5 (13.5)
Severe anemia	5 (13.5)
Psychiatric disorders	3 (8.1)
Severe skin lesion	3 (8.1)
Neurological disorders	3 (8.1)
Severe thrombocytopenia or neutropenia	3 (8.1)
Severe denutrition	2 (5.4)
Upper gastrointestinal bleeding	1 (2.7)
Severe infection (tuberculosis)	1 (2.7)
Unknown	1 (2.7)

10.1 years. McHutchison *et al.*^[17] reported that severe adverse events were observed in 9.6%-13% when the mean age of patients treated with standard of care was 47 years. So, our results suggested that older HCV-infected patients treated with standard of care might be more susceptible to severe adverse events. Among them, occurrence of hepatocellular carcinoma, severe fatigue, severe pulmonary symptoms and severe anemia were observed in 13.5% each, although we did not use growth factors such as erythropoietin^[32-34] in Japan. It should be emphasized that clinicians regularly check for the possible development of hepatocellular carcinoma even in chronic hepatitis C patients under treatment^[10].

***IL28B* GENOTYPE AND TREATMENT RESPONSE TO STANDARD OF CARE**

There have been several reports showing a close association between *IL28B* genotype and treatment response to standard of care^[11-14,35-42]. Although the mechanisms for this association are still uncertain, several studies have indicated an association between plasma interferon-gamma-inducible protein-10 (IP-10) levels and *IL28B* genotypes^[41], as well as between the expression of hepatic interferon-stimulated genes and *IL28B* genotypes^[38]. We also reported that hepatic signal transducer and activator of transcription 1 -nuclear translocation and *IL28B* genotype could predict treatment outcomes in HCV genotype 1-infected patients^[36]. A (TA) dinucleotide repeat, rs7225881, located in the promoter region, and the transcriptional activity of the promoter increasing gradually in a (TA)_n length-dependent manner, was discovered^[43]. A dinucleotide variant, ss469415590 (-G), is a frame-shift variant that creates a novel gene, designated as IFNL4, encoding the interferon- λ 4 protein (IFNL4), which is moderately similar to IFNL3^[44]. This novel TT/-G polymorphism in the CpG region upstream of *IL28B* is a better predictor of HCV clearance than rs12979860. Induction of *IL28B* and interferon-gamma-inducible protein-10 (IP-10) mRNA of peripheral blood mononuclear

cells (PBMCs) relies on TT/-G polymorphism^[45]. Further studies will be needed to clarify the significance of *IL28B*-related SNPs. In any case, *IL28B* genotypes (rs8099917 and rs12979860) are predictive factors of sustained virological response in standard of care for HCV^[146,47]. Ge *et al.*^[11] reported that the major genotype in rs12979860 leads to 80% sustained virological response with standard of care, but a minor genotype in rs12979860 leads to only 30% sustained virological response. It was reported that the major genotype in rs8099917 leads to 75.3% sustained virological response, but a minor genotype in rs8099917 leads to only 24.7% sustained virological response in HCV genotype 1 patients, and also that major and minor genotypes at rs8099917, respectively, lead to 83.0% and 17.0% sustained virological response in HCV genotype 2 patients^[47].

SUSTAINED VIROLOGICAL RESPONSE RATES BY STANDARD OF CARE AND *IL28B* GENOTYPE IN OLDER HCV-INFECTED PATIENTS

We retrospectively analyzed 41 chronic hepatitis C patients aged ≥ 65 years and treated with standard of care in our hospital (Table 4). This work was carried out in accordance with the Declaration of Helsinki of the World Medical Association. The study protocol was approved by the Ethics Committee of Chiba University School of Medicine. Our results showed that *IL28B* rs8099917 genotype is predictive of early virological response, which is undetectable HCV RNA at week 12 after the commencement of therapy, in standard of care for HCV-infected older patients. As early virological response is a predictive value for sustained virological response in standard of care treatment, *IL28B* genotyping could give us useful information for the prediction of sustained virological response with this treatment.

ROLES OF *IL28B* GENOTYPE IN TRIPLE THERAPY WITH PEGINTERFERON, RIBAVIRIN AND TELAPREVIR

In 2011, telaprevir and boceprevir were approved as direct-acting antivirals against HCV in Japan as well as in the United States and European countries^[48]. Triple therapy with peginterferon, ribavirin, plus telaprevir or boceprevir improved sustained virological response rates greatly, up to 70%-80%, in treatment-naïve individuals and previous treatment relapsers^[39,49-55], but these triple therapies also brought severe adverse events to the patients^[56]. In Japan, only the telaprevir-including regimen was available in clinical practice at this time. We frequently observed dermatological side-effects^[57], drug-drug interactions^[58], anemia^[59] and renal impairment^[60] in chronic hepatitis C patients under triple therapy with telaprevir. Akuta *et al.*^[61] reported that *IL28B* genotype could predict the response

Table 4 Association between *IL28B* rs8099917 and treatment response in hepatitis C patients aged ≥ 65 years and treated with standard of care

	Total (n = 41)	<i>IL28B</i> TT (n = 31)	<i>IL28B</i> TG (n = 10)	¹ P values
Age (yr)	67.7 $\pm$ 2.6	68.0 $\pm$ 2.9	67.0 $\pm$ 1.3	0.141
Gender (male/female)	28/13	22/9	4/6	0.796
HCV viral load, (high/low)	41/0	31/0	10/0	NA
HCV genotype (G1/G2)	41/0	25/6	8/2	0.678
Treatment-naïve (yes/no)	29/12	24/7	5/5	0.208
ALT (IU/L)	56.5 $\pm$ 40.2	54.9 $\pm$ 41.2	61.1 $\pm$ 38.9	0.677
γ -GTP (IU/L)	36.4 $\pm$ 33.3	33.1 $\pm$ 33.1	46.6 $\pm$ 33.4	0.269
White blood cells (/ μ L)	4840 $\pm$ 1300	4730 $\pm$ 1160	5190 $\pm$ 1690	0.337
Hemoglobin (g/dL)	13.5 $\pm$ 1.3	13.6 $\pm$ 1.1	13.3 $\pm$ 1.8	0.528
Platelets (x104/ μ L)	14.9 $\pm$ 5.4	14.4 $\pm$ 5.1	16.5 $\pm$ 6.2	0.289
<i>IL28B</i> rs8099917 (TT/TG)	31/10	31/0	0/10	NA
EVR rates	34.1% (14/41)	45.1% (14/31)	0% (0/10)	0.025
SVR rates	43.9% (18/41)	48.3% (15/31)	30.0% (3/10)	0.514

Values are expressed as mean $\pm$ SD. ¹P value, comparison between *IL28B* TT and *IL28B* TG groups. HCV: Hepatitis C virus; ALT: Alanine aminotransferase; γ -GTP: γ -glutamyl transpeptidase; EVR: Early virological response; SVR: Sustained virological response; NA: Not available.

to peginterferon/ribavirin/telaprevir therapy. The efficacy of triple therapy was high in patients with *IL28B* rs8099917 genotype TT who accomplished sustained virological response (83.8%), but was low in patients with *IL28B* rs8099917 genotype TG or GG who accomplished sustained virological response (27.6%). The efficacy of triple therapy was also high in patients with *IL28B* rs12979860 genotype CC who accomplished sustained virological response (83.8%), but was low in patients with *IL28B* rs12979860 genotype CT or TT who accomplished sustained virological response (32.3%)^[61], although it was reported that *IL28B* genotype had a limited impact on sustained virological response rates with telaprevir-based therapy in treatment-experienced patients^[62].

ROLES OF *IL28B* GENOTYPE IN OLDER PATIENTS TREATED WITH PEGINTERFERON/RIBAVIRIN/TELAPREVR

In our experience, we safely treated a 74 year old female, a previous treatment-relapser who had *IL28B* rs8099917 TT, with peginterferon/ribavirin/telaprevir and obtained a successful result. Although her dose of telaprevir was 1500 mg/d^[63], she did not discontinue the treatment, had no adverse events and finally achieved sustained virological response. Because most Asian individuals are of relatively smaller physical stature compared to American or European individuals, it was reported that 24 wk triple therapy with telaprevir at 1500 mg/d seemed safe and efficacious for elderly patients infected with HCV genotype 1b^[63]. In addition, *IL28B* genotyping seems to be one of the useful screening methods for older patients awaiting treatment with peginterferon/ribavirin/telaprevir^[64]. Furusyo *et al.*^[64] reported that sustained virological response rates for patients aged > 60 with *IL28B* rs8099917 TT genotype (89.4%) was significantly higher than for those with *IL28B* TG/GG genotype (41.2%), and that multivariate analysis extracted *IL28B*TT and rapid virological response, which

was defined as undetectable HCV RNA at week 4 after the commencement of therapy, as independent factors associated with sustained virological response. They observed severe anemia more frequently in patients aged > 60 years than in patients aged ≤ 60 years^[64].

CONCLUSION

In summary, *IL28B* major genotype is a useful predictor of sustained virological response in the interferon-including treatment of older patients infected with HCV. In addition, it seems to play a useful role for avoiding adverse events. The mechanisms of the effects of *IL28B* genotype on the treatment outcome are now under investigation and further studies will be needed. Until the appearance of interferon-free regimens, it is important to determine *IL28B* genotype before treating HCV-infected individuals and this is especially true in the screening of older patients.

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Tumor antigen and MHC expression in glioma cells for immunotherapeutic interventions

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MHC class II in a constitutive manner. Of note, after interferon gamma (IFN- γ) treatment, all seven cell lines expressed MHC class II. The tumor associated antigens (TAA) EGFR and survivin were expressed at high levels in all cell lines; whereas MART-1, RHAMM, WT-1 and IL-13R α were expressed by at least half of the cell lines and HER2/neu, MAGE-1 and tyrosinase were expressed only by few cell lines. However, all cell lines expressed at least two of the candidate antigens included into this analysis.

CONCLUSION: No obvious differences between commercially available and newly-established cell lines were observed. Thus, the latter in low-passages are interesting for (therapy-) screening and immunotherapeutic strategies.

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Key words: Tumor antigens; Glioblastoma multiforme; Major histocompatibility complex molecules; Tumor models; Cell lines

Abstract

AIM: To investigate the expression of tumor-antigens and major histocompatibility complex (MHC)-machinery components in glioblastoma multiforme cell lines flow cytometry staining methods were applied.

METHODS: Ten GBM cell lines (three commercially available: U-87 MG, U-138-MG and GMS-10 as well as seven newly established cell lines from individual patients in low-passages: HROG02, HROG04, HROG05, HROG06, HROG10, HROG13 and HROG17) were analyzed for expression of (I) general and (II) GBM-related tumor antigens as well as of (III) components of the MHC machinery by flow cytometry.

RESULTS: All cell lines expressed MHC class I with seven out of the ten being HLA-A02 positive. Four of the seven primary cell lines additionally expressed

Core tip: Expression of tumor-antigens and major histocompatibility complex (MHC)-machinery components was analyzed in a series of seven novel low-passage glioblastoma multiforme cell lines by flow cytometry in comparison to three commercially available standard lines. MHC class I was always expressed, MHC class II readily after interferon gamma treatment. All cell lines expressed at least two tumor antigens. No differences between newly-established and commercially available cell lines were observed. Since these novel cell lines are available in low-passages upon request, they are interesting tools for future development of immunotherapeutic strategies, associated screenings and the like.

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INTRODUCTION

Glioblastoma multiforme (GBM) is the most common and deadly malignancy of the brain with no current cure. Median survival after standard therapy consisting of resection followed by radio-chemotherapy merely lies at 14.6 mo^[1]. Even more devastating is the fact that most patients relapse and die within the following 18 mo^[2]. Thus, more potent treatment strategies are inevitable.

In the last decade, immunotherapy emerged as a very promising strategy applied in many different varieties. For chimeric antigen receptors and bi-specific T cell engagers, most dendritic cell vaccines, and T cell transfers, the target structures should be known. In general, these can be categorized as tumor associated antigens (TAA), which are overexpressed on tumor tissue (but may also to a lower extent be present on healthy tissue), or tumor specific antigens (TSA), which are exclusively expressed on the malignant tissue^[3]. Further, the distinction between shared or general tumor antigens (*e.g.*, CEA, EGFR, Her2/neu, survivin, mutated TP53) and (entity) specific antigens (*e.g.*, MART and MAGE for melanoma or PSA for prostate cancer) is common^[4].

The most widely used models for (drug and therapy) screenings are cell lines, enabling a multitude of analyses concerning the tumor's genetic and epigenetic composition, biology, phenotype and more. Among these, primary or low passage cultures are most favorable^[5-7], since especially patient individual models have been described to serve most accurately for response and resistance prediction^[8-11]. These patient individual tumor models may be established as *in vitro* (primary cell culture) or *in vivo* (xenograft) models^[12].

In this study, we analyzed the expression of various tumor antigens and in addition that of major histocompatibility complex (MHC) machinery components in a series of patient-derived low-passage glioblastoma cell lines in parallel to commercially available ones. To mimic the *in vivo* situations of absent or weak versus strong inflammation in the tumor bed, expression analyses were performed in the absence and in the presence of interferon gamma (IFN- γ).

MATERIALS AND METHODS

Cell culture

Human GBM cell lines U-87 MG (from cell line service, Eppelheim, Germany), U-138-MG and GMS-10 (both obtained at the DSMZ; Braunschweig, Germany) as well as the newly established cell lines (HROG02, HROG04, HROG05, HROG06, HROG10, HROG13 and HROG17) from our lab^[13] were cultured in DMEM/Ham's F12 supplemented with 10% fetal calf serum

and 2 mmol/L L-glutamine. All analyses with HROG cells were performed at least three times in different but always low passages. Exactly, the following passage numbers apply: HROG02 P23-P29, HROG04 P26-P35, HROG05 P21-P26, HROG06 P16-P25, HROG10 P17-P26, HROG13 P15-P25 and HROG17 P22-P25. The cells were incubated at 37 °C and 5% CO₂. Media and supplements were purchased from PAA (Cölbe, Germany) and all plastic materials were from Greiner bio one (Frickenhausen, Germany).

IFN- γ treatment

For IFN- γ treatment cells were harvested by trypsin treatment and 1.5×10^6 cells were seeded in a T75 culture flask. Cells were allowed to attach overnight and clinical grade IFN- γ (Boeringer Ingelheim, Germany) was added at a final concentration of 200 IU/mL for 48 h. Cells treated in the same manner without IFN- γ addition served as controls.

Flow cytometry

Cells were harvested by incubation with trypsin; the enzymatic reaction was stopped by adding cell culture media. Cells were washed with PBS, counted, and 5×10^5 cells were stained with 1 μ g of the respective antibody or isotype control (Table 1) for surface staining. Cells were washed with PBS and resuspended in final volume of 200 μ L PBS. In case of unlabeled primary antibodies, excess antibody was washed out with PBS and respective secondary antibodies were added and final wash step was performed as above.

Similarly, 5×10^5 cells were fixed with 2% Formafix and then treated with buffer P for 10 min to permeabilize the cell membrane for an intra-cellular staining. Cells were incubated with the antibody and washed with buffer P. After a second 10 min incubation period, the respective secondary antibody was added in buffer P. Cells were washed and resuspended in 2% Formafix at a final volume of 200 μ L.

For the staining with unlabeled primary antibodies, cells handled the same way with no primary antibody served as negative controls. All incubations were performed on ice for 30 min.

All isotype controls were obtained from immunotools (Friesoythe, Germany). The secondary antibodies were purchased from Dako (Glostrup, Denmark) in case of the anti-mouse antibody and from Bethyl (Montgomery, United States) in case of the anti-human antibody.

HLA-A typing

Typing of the HLA-A locus was performed in the department for transfusion medicine at the University medicine in Rostock, Germany.

RESULTS

Expression of tumor antigens

Knowledge about the expression of TAA or even better

Table 1 Antibodies for flow cytometry

Host	Antigen	Clone	Label	Company
Mouse	HLA-ABC	W6/32	FITC	Immunotools
Mouse	HLA-DR, -DP	HL-38	FITC	Immunotools
Mouse	HLA-E	3D12	PE	Bio Legend (London, United Kingdom)
Mouse	HLA-G	87G	APC	BioLegend
Mouse	β 2-microglobulin	2M2	FITC	BioLegend
Mouse	MIC A	2C10	FITC	Santa Cruz (Santa Cruz, United States)
Mouse	MIC B	Jan-47	None	Santa Cruz
Humanized	EGFR	Cetuximab	None	Bristol-Myers Squibb (New York, United States)
Humanized	HER2/neu	Trastuzumab	None	Roche (Basel, Switzerland)
Mouse	ICAM-1	1H4	APC	Immunotools
Rabbit	IL-13R α	Polyclonal	None	AssaybioTech (Sunnyvale, United States)
Mouse	MAGE-1	MA454	None	Thermo Scientific (Waltham, United States)
Mouse	MART-1	M2-7C10	None	Thermo Scientific
Mouse	RHAMM	2D6	None	Leica (Wetzlar, Germany)
Mouse	survivin	3F342	None	Santa Cruz
Mouse	tyrosinase	T311	None	Thermo Scientific
Mouse	WT-1	6F-H2	None	Chemicon international (Temecula, United States)

PE: R-phycoerythrin; APC: Allophycocyanin; FITC: Fluorescein isothiocyanate.

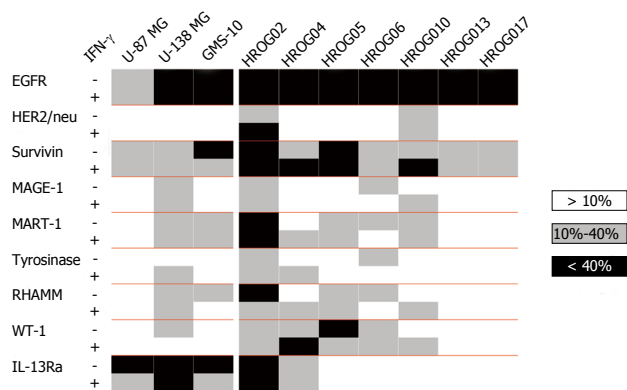


Figure 1 The percentage of cells staining positive for antibodies against tumor associated antigens was assessed by flow cytometry. The results are depicted in the diagram with a white box representing staining in less than 10% of cells, a grey box representing 10%-40% cells positive and a black box in case of more than 40% positive cells. Values with and without the 48 h interferon gamma pretreatment (200 IU/mL) are given. Measurements were performed in three separate experiments and mean values were used to design the blot. IL: Interleukin; IFN: Interferon.

TSA is of interest for a wide range of therapeutic interventions; especially for targeted therapies and immunotherapeutic approaches. Thus, the expression of a variety of antigens was assessed in seven newly established GBM cell lines in comparison to commercially available GBM cell lines (U-87, U-138 and GMS-10). This analysis included TAA shared between different tumor entities (EGFR, HER2/neu and survivin), classical melanoma (MAGE, MART and tyrosinase) and leukemia-associated antigens (RHAMM and WT-1), as well as the glioma specific IL-13R α .

The more general antigens EGFR and survivin were found to be expressed-highly in most cases-in all cell lines (Figure 1). MART-1 and RHAMM were expressed in seven, WT-1 in six cell lines. The glioma specific antigen IL-13R α was expressed in half of the cell lines; however, if positive, than with a high percentage. MAGE-1 and tyrosinase were only expressed in four cell lines and to a low degree. HER2/

neu was expressed rarely (HROG02 and HROG10). In summary, each cell line expressed at least two antigens (EGFR and survivin) and half even seven or more. Of note, the cell line HROG02 expressed all of the assessed antigens.

In addition, we analyzed the influence of IFN- γ on the intensity of antigen expression in these cell lines. Here, the degree of antigen expression was rarely and if only barely affected by IFN- γ treatment (Figure 1).

Expression of MHC class I and II

Next, the expression of MHC class I and II molecules of the GBM cell lines was analyzed. All cell lines expressed high levels (> 50% expression) of the HLA-ABC antigens; though no further up-regulation could be achieved by IFN- γ treatment (Figure 2A). In contrast, only three cell lines (HROG05, HROG06 and U-87 MG) highly expressed HLA-DR, -DP, three (HROG10, HROG13 and HROG17) had intermediate levels and four (HROG02, HROG04, U-138-MG and GMS-10) did not express MHC class II at all. Here, treatment of the cells with IFN- γ resulted in a clear induction of expression in the non-expressing cell lines and an up-regulation in the intermediate ones (Figure 2B).

With regard to unraveling novel T cell epitopes from candidate tumor antigens, the HLA-A02 sub-type is most frequently used as it is the most common one in Caucasian and Asian population. Thus, HLA-A02 expression was assessed by flow cytometric staining. Results were subsequently confirmed by a molecular genetic HLA-A typing (Table 2). Five out of the seven cell lines stained positive for HLA-A02 and were at least heterozygous for *A2. Of the three commercially available cell lines, GMS-10 and U-87 MG were additionally found to express HLA-A02 as judged by flow cytometric staining analysis (data not shown).

Components of the MHC machinery

The cell lines were further characterized with regard to a broader range of MHC machinery components such as

Table 2 HLA-A and A02 expression

	HROG02		HROG04		HROG05		HROG06		HROG10		HROG13		HROG17	
HLA-A typing	*01	*02	*01	*02	*02	-	*01	*03	*02	*23	*02	-	*11	*66
Flow cytometry (HLA-A02)	88% ($\pm 10\%$)		95% ($\pm 1\%$)		83% ($\pm 10\%$)		1% ($\pm 1\%$)		89% ($\pm 4\%$)		92% ($\pm 3\%$)		0% ($\pm 0\%$)	

In the upper row the results of a 2-digits encompassing HLA typing for the loci HLA-A are listed. Information on both alleles is provided; in case of homozygosity the "second" allele is marked by -. In the lower row the mean percentage (of three repetitions) of cells stained positive for HLA-A02 in flow cytometry is given and the SD is provided in brackets.

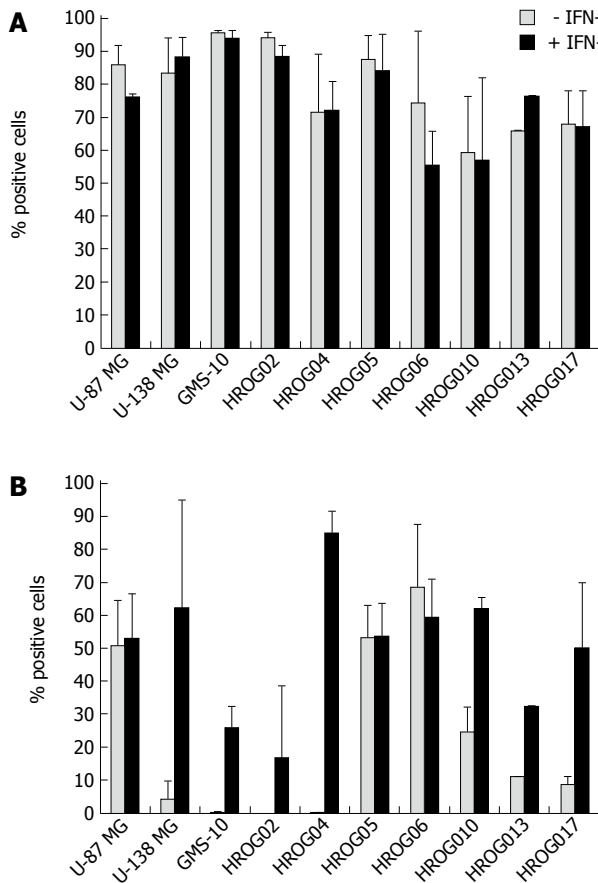


Figure 2 The percentage of cells stained positive for antibodies against (A) HLA-ABC and (B) HLA-DR, -DP as assessed by flow cytometry are depicted in the bar diagram. The black bars represent results for cells treated with 200 IU/ml interferon gamma (IFN- γ) for 48 h prior to staining. The grey bars represent results for cells handled in the same manner but without IFN- γ . Measurements were performed in three separate experiments; values are given as mean $\pm$ SD.

HLA-E and -G, MIC A and B as well as β 2-microglobulin (Figure 3). In line with the results of the HLA-ABC expression analysis, β 2-microglobulin was highly expressed in all cell lines. Further, all cell lines expressed HLA-E; with the exception of HROG13 all cell lines even very high levels ($> 75\%$ expression). HLA-G was expressed by three cell lines (HROG05, HROG06 and HROG17). MIC A was highly expressed by two cell lines (HROG02 and HROG06), two cell lines (HROG05 and HROG17) had intermediate and three (HROG04, HROG10 and HROG13) no MIC A expression. None of the cell lines stained positive for MIC B. In addition, the immune-

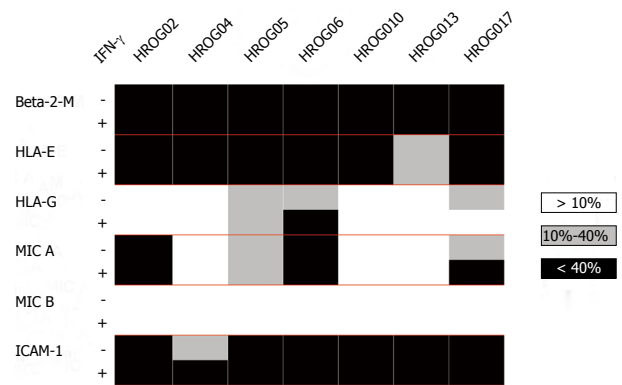


Figure 3 The percentage of cells staining positive for components of the major histocompatibility complex machinery as well as the immune-recognition-enhancing molecule ICAM (CD54) was assessed by flow cytometry. The results are depicted in the diagram with a white box representing staining in less than 10% of cells, a grey box representing 10%-40% cells positive and a black box in case of more than 40% positive cells. Values with and without the 48 h interferon gamma pretreatment (200 IU/mL) are given. IFN: Interferon.

recognition-enhancing molecule ICAM-1 was analyzed (Figure 3). This was highly expressed by all cell lines.

DISCUSSION

Cell lines have served in unraveling cellular- and tumor-related mechanisms as well as in therapy development for many decades. Since no severe differences were observed between the standard cultures and our patient-derived low-passage cell lines with regard to TAA expression, the latter are preferable for such analyses. By using (ultra) low-passage cell lines, culture artifacts can (nearly) be excluded. Long term cultures are reported to acquire a multitude of culture related changes such as increase in doubling time with the number of passages^[14,15], a more methylated MGMT promoter phenotype^[16] and the acquisition of novel mutations and chromosomal aberrations^[17].

Individual patients' tumor models are especially interesting for testing of drug responses and resistance development^[8,9] but are also ideal for establishing and testing of immune-based treatment strategies if autologous or tumor infiltrating lymphocytes (TIL) are available^[18,19]. Together with expert pathological assessment of tumor characteristics and lymphocytic infiltration, low-passage cell lines offer an outstanding possibility to develop and optimize immunotherapeutic approaches. Such approaches seem to at least not be hindered by inflamma-

tion or IFN- γ secreted, *e.g.*, by T cells, since no adverse effects of IFN- γ on TAA expression were observed. However, we did not analyze changes in the presentation of TAA-derived peptides in MHC class I and II molecules.

In case of MHC class II, an induction or up-regulation of the molecules by IFN- γ was frequently observed. This phenomenon is described in the literature^[20,21].

However, the biological background of the high variation in MHC class II expression is not known at present. We suspect that because of the role of MHC class II in T cell activation and regulation this to be a potential novel mechanism of immune evasion. Respective experiments are ongoing in our laboratory.

Immune evasion is one of the major mechanisms employed by tumors to avoid destruction by the patient's immune system. Beside low expression of MHC class I and secretion of immunosuppressive factors like transforming growth factor- β , prostaglandin E2 or Interleukin-6 particularly the upregulation of molecules like Fas and programmed cell death 1 ligand 1 (PD-L1) allow tumor cells (and especially glioblastoma cells) to directly induce T cell anergy or even apoptosis and, as a consequence, to from an immunosuppressive environment^[22]. Especially glioma stem cells are capable of most if not all of these mechanisms^[22].

To sum up our findings, all cell lines expressed multiple TAA; this enables targeting more than one antigen in order to prevent immune escape and resistance development^[23,24]. Additionally, antigen spreading could readily be analyzed.

Finally, the models allow testing the immunological potential of the (selected) antigen (combinations) through *in vitro* T cell stimulation and functional read-outs (for example ELISpot or kill assays). As we have recently shown that the generation of GBM cell lines is feasible and successful to a high percentage even from vitally frozen material^[12], accompanying research on immunotherapies becomes possible.

A decade ago, Liu and colleagues assessed the expression of the antigens HER-2, gp100 and MAGE-1 in human glioblastoma. They additionally described a correlation between the cytolytic potential of antigen-specific T cells and the level of antigen expression^[25]. A few years later, Zhang *et al.*^[26] performed an antigenic profiling of 20 commercially available GBM cell lines with potential regard to allogenic immunotherapies. In their study, all cell lines were found to express the antigens HER2/neu, IL-13R α , MAGE-1 and survivin and two thirds were positive for tyrosinase.

Recently peptide-pulsed dendritic cells entered a phase I clinical study for patients with recurrent high grade gliomas. The peptides included in the study were derived from the antigens gp100, HER-2, MAGE and WT-1^[27]. With this approach, one out of nine patients had stable disease after vaccination; thus hinting toward efficacy of the strategy but likewise demonstrating the necessity of improvement. The antigens survivin^[28] and

RHAMM^[29] are further being exploited for their potential to cure cancer in vaccination strategies for head and neck cancer as well as chronic B cell lymphoma.

COMMENTS

Background

For development of therapeutic strategies, cell lines are an inevitable tool. With the rise of immunotherapy, precise knowledge on the expression of relevant molecules in the cell models used is appreciated by basic and translational researchers. For the most part, these molecules include components of the major histocompatibility complex (MHC) machinery on the one hand and tumor antigens on the other.

Research frontiers

Development of novel immunotherapeutic approaches is an exploding field of research. Examples here for are chimeric antigen receptors, bi specific T cell engagers, cellular (mostly dendritic cell) vaccines and T cell transfers.

Innovations and breakthroughs

Similar phenotypic characterizations of cell lines from numerous different tumor entities have been performed many times. However, the authors group's focus for several years is development and characterization of low-passage, patient-individual cell lines. These have several major advantages when compared to standard cell lines. Lower passages mean less change in the cell's genome and epigenome. Lymphocytes and sera biobanked together with the cell models allow research to be performed in a complete autologous setting.

Applications

These novel cell lines are interesting tools for drug screening, therapy optimization and even basic and further translational research applications. With the data presented here, they additionally recommend themselves for future development of immunotherapeutic strategies, associated screenings and the like. The cell lines are available in low-passages upon request and thus it is very likely that subsequent studies will broaden the data presented here.

Terminology

Patient-derived, low-passage cell lines: a cell line which has been established only recently from a tumor of a patient and underwent a very limited number of cell divisions.

Peer review

The authors have conducted an investigation of the expression of tumor-antigens and MHC-machinery components in glioblastoma multiforme cell lines. The conception and relevance of this work could be enormous; indeed the authors have analyzed patient specific cell cultures. This is of extreme relevance and actuality due to the more and more recognized limitations of the immortalized cell line model.

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Aberrant expression of CD56 by circulating Sézary syndrome malignant T lymphocytes

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T-cell markers CD2, CD3 or CD4 was detected while a complete down-modulation of CD26 was observed. Despite several lines of treatment, no durable amelioration was observed and patient died after 10 mo of follow-up. Because this CD4⁺ CD56⁺ SS case is the only one reported so far, the functional significance of CD56 expression remained difficult to assess in terms of aggressiveness and prognosis.

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Key words: Cutaneous T cell lymphomas; Sézary syndrome; CD56

Core tip: Sézary syndrome (SS) is an aggressive variant of cutaneous T cell lymphoma characterized by the presence of malignant CD4⁺ memory T cells in the skin, peripheral blood and lymph nodes. We here report a case of SS with an aberrant CD56⁺ immunophenotype. The aberrant expression of this natural killer cell marker by the tumoral cells raises the question of its relevance in terms of function and prognosis.

Abstract

Sézary syndrome (SS) is an aggressive variant of cutaneous T cell lymphoma characterized by the presence of malignant T cells in the skin, peripheral blood and lymph nodes. The tumoral population typically displays a CD3⁺ CD4⁺ CD45RO⁺ memory T cell phenotype. We report a case of SS with an aberrant CD56⁺ immunophenotype. This patient presented with a generalized erythroderma and palpable small axillary lymph nodes. SS (stage IVA) was diagnosed on histological criteria and by the detection of a major T cell clone in skin and blood, an elevated CD4/CD8 T cell ratio and Sézary cells count > 1000/mm³. Beside the Sézary cell marker KIR3DL2, immunostainings revealed that two third of the malignant cells expressed CD56 but no other natural killer (NK) cell marker such as CD16, CD160 or NKp46. This atypical expression was not linked to an activation-dependent process and remained stable during the time course of the disease. No loss of the pan

Thonnart N, Ram-Wolff C, Bagot M, Bensussan A, Marie-Cardine A. Aberrant expression of CD56 by circulating Sézary syndrome malignant T lymphocytes. *World J Immunol* 2013; 3(3): 68-71 Available from: URL: <http://www.wjgnet.com/2219-2824/full/v3/i3/68.htm> DOI: <http://dx.doi.org/10.5411/wji.v3.i3.68>

INTRODUCTION

Cutaneous T cell lymphomas (CTCL) represent a heterogeneous group of diseases characterized by clonal accumulation of lymphocytes with initial restriction to the skin. Mycosis fungoides (MF) is the most common form of CTCL that follows an indolent course in most

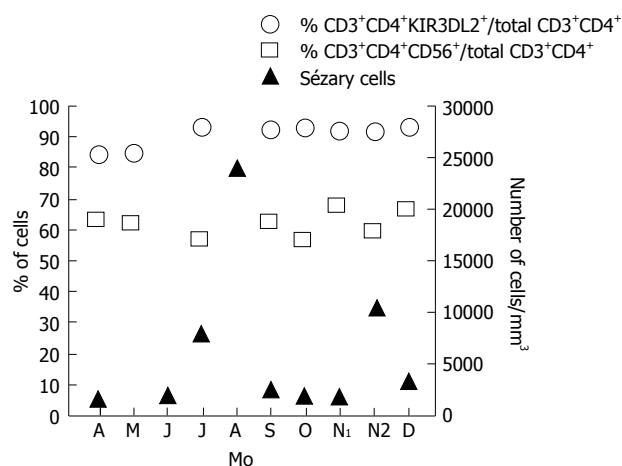


Figure 1 Evolution of the percentage of circulating KIR3DL2⁺ (○) and CD56⁺ (□) CD4⁺ T lymphocytes among the total CD4⁺ T cell population, and of Sézary cell count (▲) from April to December. N1: November/sample 1; N2: November/sample 2.

cases. Sézary syndrome (SS) is an aggressive leukemic and erythrodermic variant of CTCL, characterized by the presence of a clonal T lymphocyte population with atypical cerebriform nuclei (Sézary cells) in the skin, lymph nodes, and peripheral blood. A consensus statement was proposed by the International Society for Cutaneous Lymphoma and the European Organization for Research and Treatment of Cancer for the establishment of SS diagnosis^[1]. The retained criteria are (1) the evidence of a dominant T-cell clone as assessed by polymerase chain reaction or Southern blot; (2) an absolute Sézary cell count of at least 1000/mm³; (3) a CD4/CD8 T cell ratio of 10 or higher; and (4) expanded CD4⁺ T cells with abnormal immunophenotype. Regarding this last point, in most cases, Sézary cells show a CD3⁺ CD4⁺ CD45RO⁺ memory T cell phenotype. However, abnormal expression of cellular markers was reported over the last years. Immunophenotypic analyses of SS patient circulating lymphocytes evidenced a frequent down-modulation of CD2, CD3, CD4 or CD45 surface expression^[2-4]. In addition, a loss of CD7 or CD26 has also been detected and integrated as part of the diagnosis criteria^[5-7]. We further reported that the natural killer (NK) cell receptor KIR3DL2 represents a valuable diagnostic and prognostic marker for SS both in skin and blood^[8-10]. Nevertheless, it became more and more obvious that a single marker by itself is not sufficient to identify the entire tumoral burden of all patients, and that the use of multiple markers better ensure a reliable detection of circulating Sézary cells^[11].

The surface marker CD56 represents a marker of NK cells. Its expression was initially associated to 3 types of cutaneous lymphoma, namely the extranodal NK/T cell lymphoma of the nasal type, the subcutaneous panniculitis-like T cell lymphoma and the blastic NK cell lymphoma^[12]. Until now 8 cases of CD56⁺ MF have been described^[13-17]. We report a patient with SS whose malignant cells are CD4⁺ T cells that expressed CD56.

CASE REPORT

A 55-year-old man with a history of hypercholesterolemia presented in March 2010 with a two-month history of persistent pruriginous and generalized erythroderma. Small axillary lymph nodes were palpable. Skin biopsy revealed marked epidermotropism of atypical T-cells with Pautrier microabscess. Superficial dermis was also infiltrated with atypical lymphocytes consisting of medium to large cells with hyperchromatic and cerebriform nuclei. A blood cell count revealed 15800/mm³ leukocytes, 9000/mm³ neutrophils, 3600/mm³ lymphocytes and an elevated Sézary cells count of 1896/mm³ (Figure 1). Flow cytometry analysis revealed an elevated CD4/CD8 ratio of 41 in the peripheral blood. Molecular biology studies confirmed the presence of a clonal T cell identical in blood and skin biopsy. The level of serum LDH was elevated to 562 UI/L. Thoracic, abdominal and pelvic CT-scans were normal. Sézary syndrome (T4NxM0B2) was diagnosed.

In April 2010 the patient started combined treatment with interferon and bimonthly photopheresis for 3 mo with clinical and biological progression (majoration of infiltration of skin with leonine facies, Sézary cell count: 8241/mm³). In July 2010 bexarotene was started (with continuation of photopheresis) and progression after 2 months of treatment (Sézary cell count: 24211/mm³) was noted. Gemcitabine was introduced in September 2010 (1000 mg/m² at day 1, 8 and 15). Initial amelioration was observed with diminution of Sézary cell count at 2074/mm³ after 2 cycles. Despite four cycles of gemcitabine, the patient progressed clinically and biologically (Sézary cell count: 11004/mm³) and died suddenly in December 2010.

From March to December 2010, elevated levels of tumoral cells within the circulating CD3⁺ CD4⁺ T lymphocyte population were detected, as assessed by KIR3DL2 staining, with a mean percentage of 90.6% ± 4.5% (Figures 1 and 2). In addition, two-third of the malignant cells were found to express CD56 (mean: 61.5 ± 5.3%; Figures 1 and 2). Notably, the percentage of CD3⁺ CD4⁺ KIR3DL2⁺ CD56⁺ lymphocytes remained stable over the course of the disease, regardless of the ongoing treatment. Additional flow cytometric analyses of peripheral blood demonstrated that all neoplastic cells have a CD2⁺ CD3⁺ CD4⁺ CD5⁺ CD7⁺ CD8⁺ CD26⁺ CD27⁺ CD45RO⁺ CCR4⁺ phenotype (Figure 2 and data not shown). Furthermore, none of the specific NK cell markers CD16, NKp46 or CD160 was detected (Figure 2). These cells were also negative for all KIR tested (except for KIR3DL2; data not shown) and did not exhibit an activated NK- or T-cell phenotype as assessed by the lack of expression of NKp44 or CD69 (Figure 2).

DISCUSSION

CD56 is a well-known marker of NK cells that is usually expressed in NK cell tumors such as extranodal NK/T cell lymphoma and aggressive NK cell leukemia. However, atypical cases of CD56⁺ CTCL have been re-

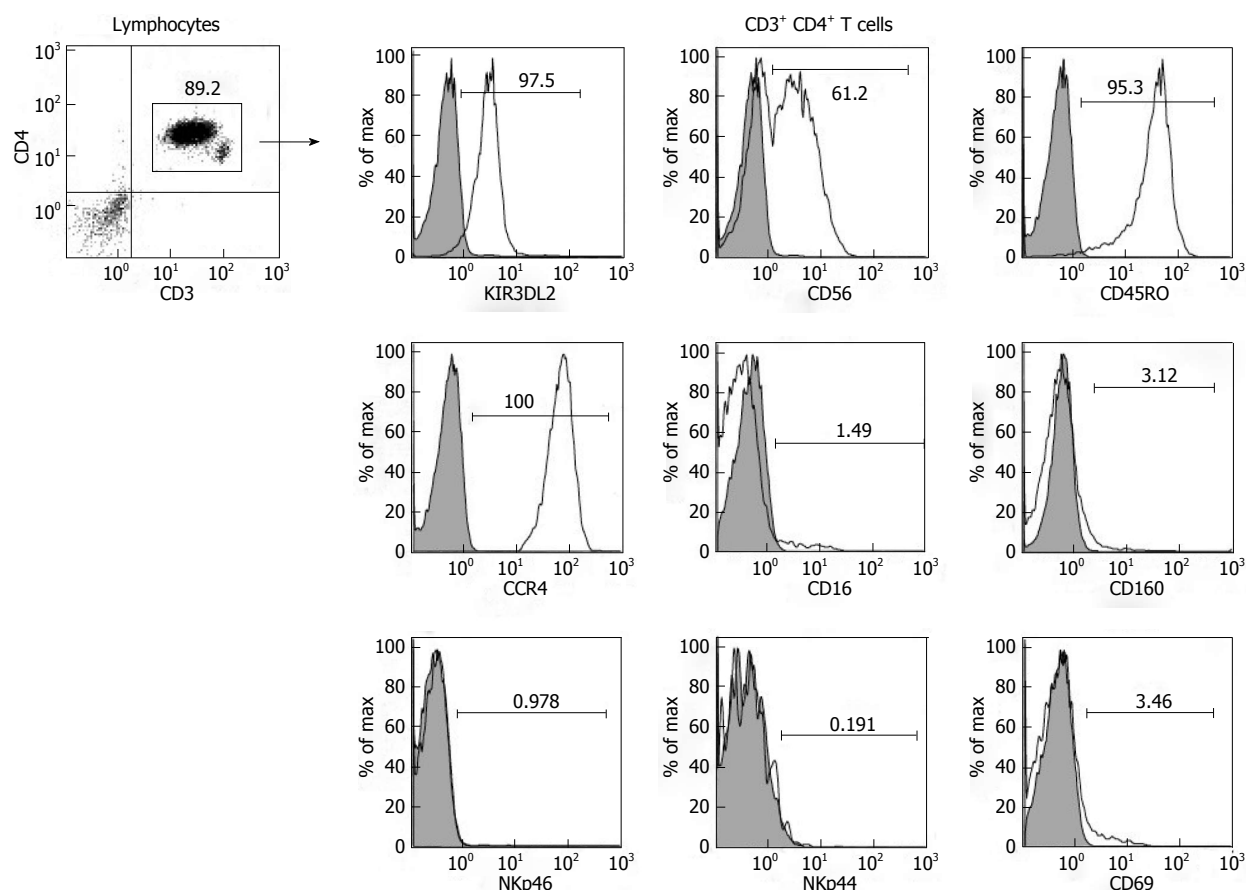


Figure 2 Immunostainings of the CD4⁺ T lymphocyte population. Cells were positive for KIR3DL2, CD56, CD45RO and CCR4, but negative for the NK cell markers CD16, CD160 and NKp46 and for the activation markers NKp44 and CD69. Analyses were performed on a FC500 flow cytometer (Beckman Coulter).

ported over the last years. Among them six MF patients exhibited malignant cells with a CD4⁺ CD8⁺ CD56⁺ (2 cases) or CD4⁺ CD8⁻ CD56⁺ (4 cases) immunophenotype, consistent with their classification as cytotoxic MF^[14,16,17]. All patients were younger than the typical age of disease onset for MF (< 55-60-year-old) and presented with a stage I A or I B disease that followed a slowly progressive course. An atypical CD4⁺ CD8⁺ CD56⁺ phenotype was also described for a 26-year-old Japanese patient who presented with a stage IVA disease, however the appearance of peripheral blood involvement was rather consistent with SS^[13]. Finally an additional case of an 85-year-old patient with clinical MF stage I A, whose neoplastic cells were characterized by a CD4⁺ CD8⁻ CD56⁺ phenotype, was reported^[13]. To our knowledge, we here report the first case of SS patient with a CD4⁺ CD8⁻ CD56⁺ malignant cell phenotype. Besides CD56, our previous studies already allowed the detection of other NK receptors at the surface of Sézary cells. Thus, we showed that circulating malignant Sézary cells might be distinguished from autologous reactive CD4⁺ T cells by the detection of the ILT2/CD85j receptor at their cell surface, this receptor exhibiting an exclusive cytoplasmic location in non-malignant T cells. In this tumoral cell context, ILT2 was found to play an inhibitory co-receptor function, its ligation leading to a down-modulation of the CD3-dependent cell activation process^[18]. Similarly, apart from

KIR3DL2, other KIR receptors such as KIR2DS1/S2 can be detected on Sézary cells. In such case, these activating receptors were found to act as co-stimulatory molecules, their engagement allowing the amplification of the CD3-mediated activation pathway^[19]. More recently, we reported the expression of the NK cell receptor NKp46 by Sézary patient malignant cells. While NKp46 represents a fully autonomous activating receptor in NK cells, it acts as an inhibitory co-receptor in Sézary cells, preventing the delivery of activating signals following engagement of the TCR/CD3 complex^[20]. Remarkably, in most of the cases, the atypical expression of these NK receptors seems to confer a growth advantage to the tumor cells either by promoting an otherwise low proliferation response to activating stimuli (as seen for KIR2DS1/S2) or by delaying or preventing activation-induced cell-death (*e.g.*, NKp46 or KIR3DL2; A.M-C, personal communication). We observed that one third of the malignant cells were negative for CD56, suggesting that these cells either never expressed the receptor or lose expression during tumoral expansion. Notably, such loss of expression has already been observed for numerous markers such as CD3, CD4, CD7 or CD26. The functional relevance of CD56 expression in the context of MF or SS remains to be established. Previous studies did not favor a direct link between this expression and the aggressiveness of the disease, at least in the reported

cases of MF. The identification of more SS patients with a CD4⁺ CD56⁺ malignant cell population will be required to address this issue.

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