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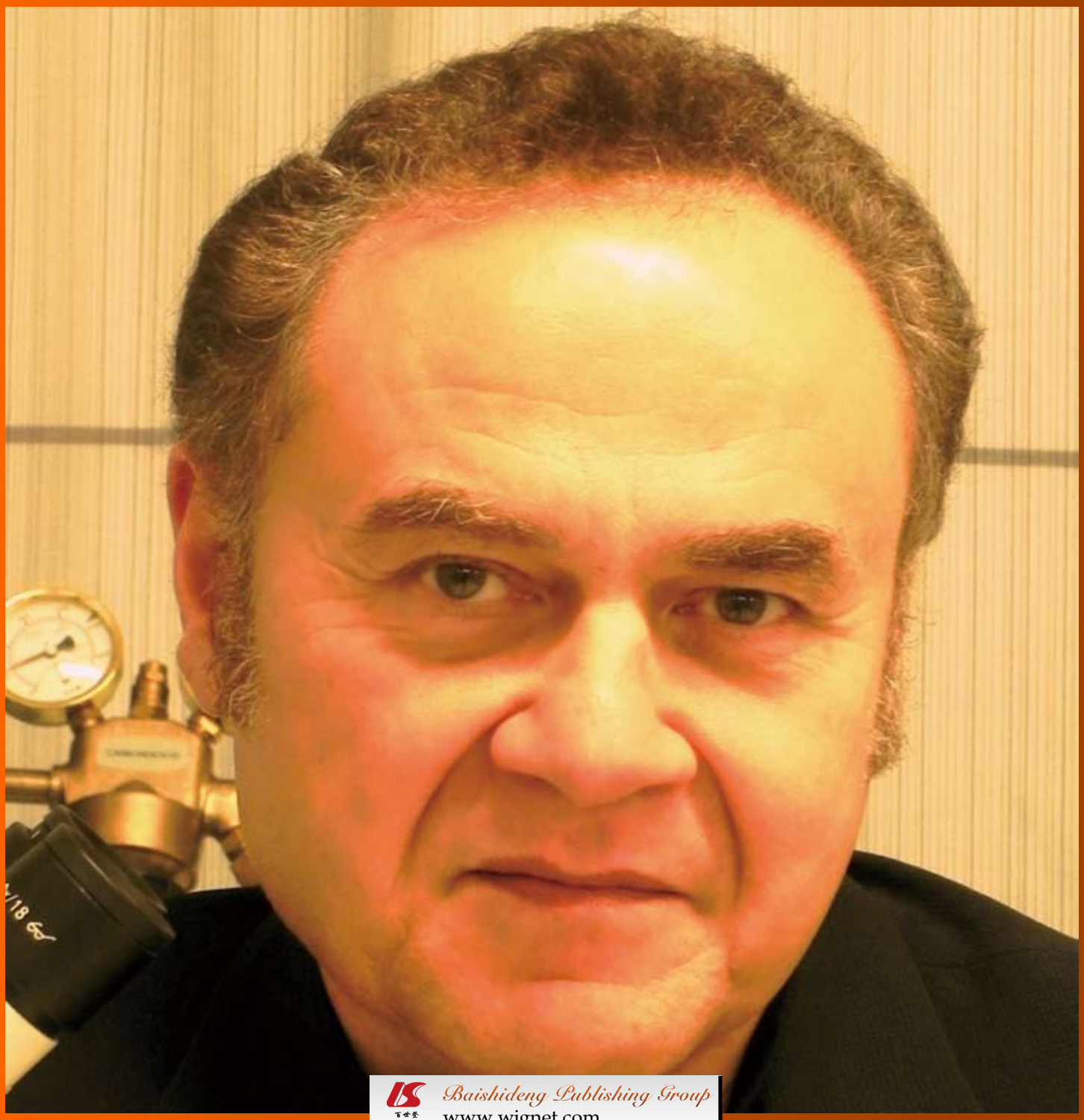

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EDITORIAL

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

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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.

Key words: Sézary syndrome; Mycosis fungoides; Natural killer receptors; KIR3DL2; Cutaneous T-cell lymphomas

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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].

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 ($CD4^+CD7^- \geqslant 30\%$) or CD26 ($CD4^+CD26^- \geqslant 40\%$) are found in about half of the SS patients[7-9]. However, although it is possible to show using Vß-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 -ß 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-trangenic 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-Vß and TCRß-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, occuring 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 a3-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 cytolysis. 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 CD3ξ or FceRIγ, 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 CD3ξ 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 cytolysis 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-seropositve patients[41]. This raises the question whether NK reprogramming may underlie the transformation of chronically stimulated T-cells.

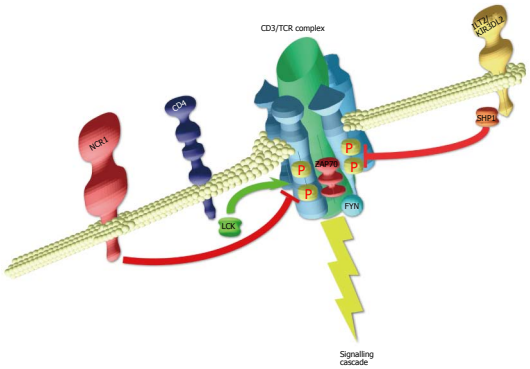

Figure 1 Tuning CD3/T cell receptor signalling threshold by natural killer receptors on Sézary tumoral cells. Ig-like transcript 2 (ILT2) or KIR3DL2 receptors on Sézary cells lower the threshold of T-cell receptor activation through the activation of the SHP1 phosphatase. Natural cytotoxicity receptors (NCR)1 (NKp46) receptors associate with CD3z-g chains and, when in close proximity, the NCR1 prevents the phosphorylation of CD3z 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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ORIGINAL ARTICLE

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 μg or 100 μg 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 μg and D8 100 μg 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 μg 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 μg 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 μg 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 μg 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 consequentially may support a therapeutic potential of anti-eo-2 neutralizing mAb in multiple sclerosis.

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 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 μg 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 μg/mouse myelin-oligodendrocyte glycoprotein peptide (MOG_{35-55}, synthesized by Sigma-Aldrich) in 100 μL PBS. The peptide was emulsified in an equal volume of Complete Freund's Adjuvant (CFA, from DIFCO) containing 500 μg *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 $NaHCO_3$, pH 9.5) overnight at 4 ℃. 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 H_2SO_4. Absorbance at 450 nm was measured using a Termo 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 ℃ 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 D8 100 μg treated groups *vs* 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

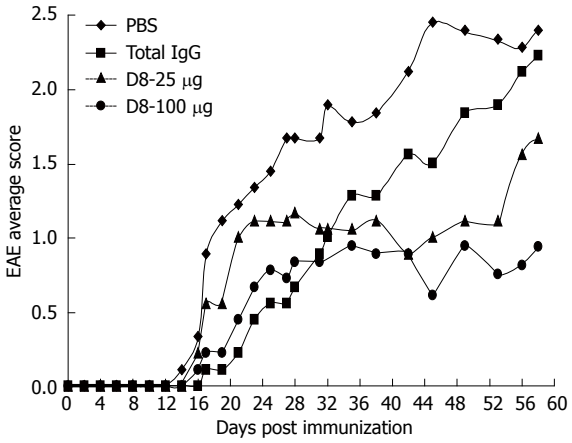

Figure 1 Anti-eo-2 neutralizing mAb treatment effect on progressive experimental autoimmune encephalomyelitis clinical course. Progressive experimental autoimmune encephalomyelitis (EAE) was induced in female C57BL/6 mice by immunization with two following subcutaneous injections of MOG_{35-55} peptide, 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).

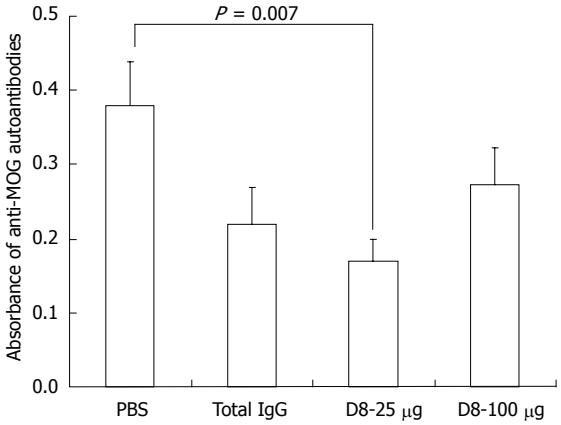

Figure 2 The effect of anti-eo-2 neutralizing mAb treatment on the level of anti-MOG_{35-55} autoantibodies in experimental autoimmune encephalomyelitis sera. Experimental autoimmune encephalomyelitis (EAE) mice daily treated with PBS, total mouse IgG, 25 μg or 100 μg D8, were sacrificed on day 58 and 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} nm, n = 9 in each group).

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, MOG_{35-55} 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 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 = 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 LTB_4 receptor antagonist CP-105,696 selectively inhibited eosinophils recruitment into the spinal cord, without inhibition of

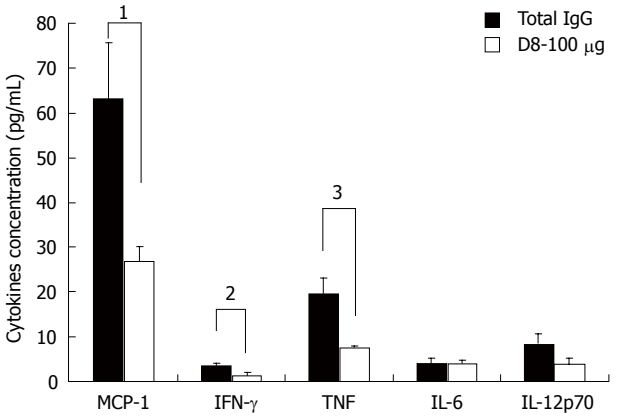

Figure 3 Anti-eo-2 neutralizing mAb treatment effect on pro-inflammatory cytokines profile. Experimental autoimmune encephalomyelitis (EAE) mice sera from total IgG and 100 μg D8 groups were assessed for the presence of interleukin (IL)-6, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, IL-12p70 and 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

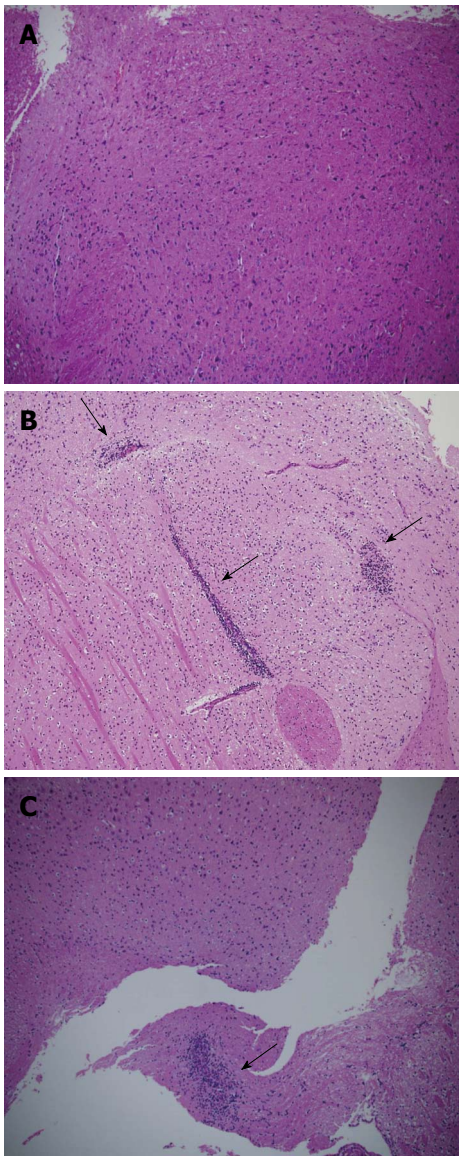

Figure 4 The extent of cellular infiltration in anti-eo-2 mAb treated experimental autoimmune encephalomyelitis mice is low compared with PBS treated experimental autoimmune encephalomyelitis mice and their healthy littermates. A: Hematoxylin and eosin staining of representative brain sections from healthy 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 MOG_{35-55}. 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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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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