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Review Article

Role of Natural Killer Cells in Multiple Sclerosis

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Although the etiology of multiple sclerosis (MS) is not known, the consensus is that Th1 cells sensitized to myelin proteins in the periphery are recruited into the CNS and damage the myelin sheath. Natural killers (NK) are cells that spontaneously lyse tumor target cells and have immunoregulatory activity secreting multiple cytokines and chemokines, as well as interacting with cells of innate and adaptive immune systems. A great discovery in the field is the cloning of several inhibitory and activating receptors. Another important contribution is the discovery that these cells express many seven-transmembrane-spanning domain receptors which aid them in extravasations into injured tissues. Despite all this progress, the role of NK cells in autoimmune diseases including MS is still not quite clear. In this paper, I will summarize recent findings related to the effects of these cells in both MS and the animal model of experimental autoimmune encephalomyelitis (EAE). Hence, I will discuss the effects of drugs used to treat MS/EAE and then explain their effects on NK cells. These include anti-CD25 or daclizumab, interferon-β (IFN-β), natalizumab, glatiramer acetate (GA), and fingolimod (FTY720). Finally, I will explain the contribution of the recently discovered NK17/NK1 cells in MS disease.

1. Introduction

Multiple sclerosis (MS) is an inflammatory disorder that results in demyelination and destruction of neurons. At the onset of disease, the majority of patients have a relapsing-remitting (RR) pattern, which over time can proceed into secondary progressive stage [1]. The experimental autoimmune encephalomyelitis (EAE), a mouse model, provides great insights into MS and particularly the role that autoreactive $CD4^+$ cells play in this disease [2, 3]. The popular view is that these cells are primed in the peripheral organs by interacting with antigen-presenting cells such as dendritic cells (DCs) and then migrate into the CNS where they are reactivated by resident APCs such as the microglia (Figure 1). Reactivation of T cells triggers parenchymal inflammation, which recruits other inflammatory cells into the site of inflammation [1–4]. A hallmark of the immune response in MS is the formation of isolated areas of inflammation called MS lesions. Lesions can appear both in the white matter and in the grey matter of the brain and are often found around the ventricles, in the optic nerve, in the brain stem, and in the spinal cord [5]. These lesions could be the results of direct destruction by the immune cells, or through the release of inflammatory cytokines by $CD4^+$ and other cells. The role of several inflammatory cell types in MS/EAE has been reviewed [5, 6] and will not be further discussed here.

2. Natural Killer (NK) Cells

NK cells perform several important functions; among them is the regulation of the adaptive immune response by secreting cytokines such as IFN-γ [7], shaping the innate immune system by interacting with dendritic cells [8], defending against viral infection [9], and lysing and destroying tumor cells [10]. NK cells express many receptors which can be either inhibitory or activating. The inhibitory receptors exist as monomers and transduce inhibitory signals, whereas most activating receptors lack signaling capabilities but associate with adaptor molecules that have signaling motifs. Each NK cell expresses one or two inhibitory receptors ensuring that under normal conditions NK cells are inhibited upon ligating self-MHC molecules, which guards against autoimmunity.

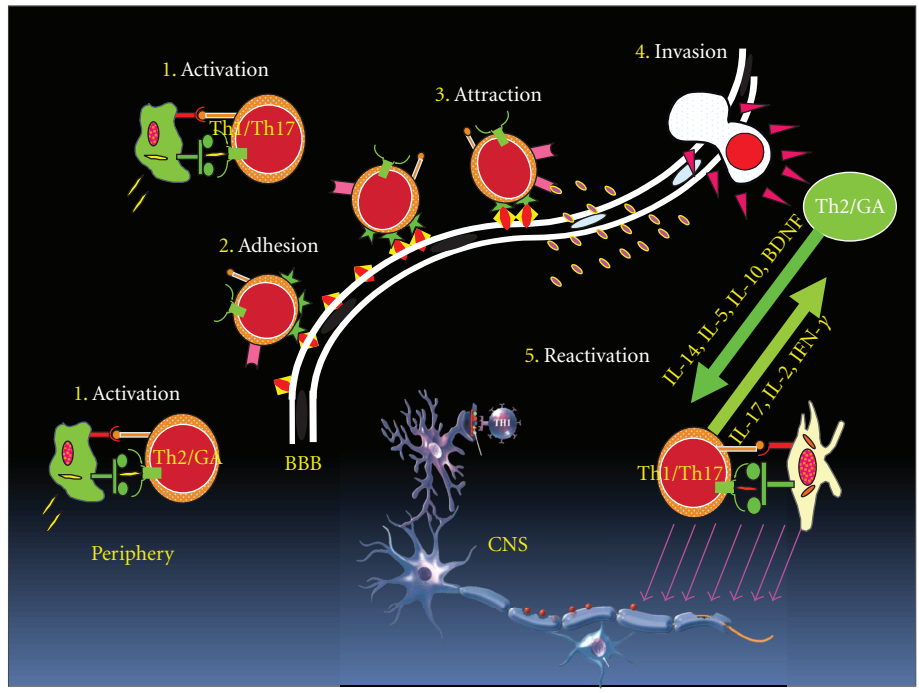

Figure 1: T-cell model of MS immunopathogenesis. Damage to the CNS occurs in sequential steps. Th1 cells are activated in the periphery by antigen-presenting cells (APCs) such as DCs (step 1). After activation, these cells adhere to the endothelial cell layer lining the blood-brain-barrier "BBB" (step 2). Activated cells are attracted towards chemotactic molecules, including the chemokines (step 3). The cells invade the CNS tissues (step 4). These invaders are reactivated in the CNS by either resident APCs like microglia cells, or by migrating DCs (step 5). The figure also explains the interaction of inflammatory T cells (Th1 or Th17) and GA-reactive T cells in the CNS. In this model, the autoreactive Th1 or Th17 cells as well as GA-reactive T cells arrive into the CNS. In the CNS, GA-reactive T cells secrete the beneficiary molecules IL-4, IL-5, IL-10, and BDNF which counteract the effects of the inflammatory molecules IL-2, IL-17, or IFN-γ released by Th1 or Th17 cells. The latter cells may also downregulate the expression of chemokine receptors on the surface of GA-reactive T cells, making them unable to migrate into the desired areas in the brain [16].

Resting NK cells respond to dangers occurring at sites of injury. Here, they change their adhesion molecules and start upregulating chemokine receptors that aid them in extravasating into injurious tissues. In the blood circulation, human NK cells can be classified into two major subsets: those that express CD56 but not CD16 (known as $CD56^{bright}CD16^{-}$) and those that express CD16 and low CD56 (known as $CD56^{dim}CD16^{+}$) [7, 10]. The former subset comprises about 10% of total NK cells in the blood, whereas the latter subset comprises about 90%. Other differences were noted between these subsets of NK cells which can be either functional or phenotypic differences. For example, $CD56^{bright}CD16^{-}$ cells are more regulatory (they secrete IFN-γ and other cytokines) and less cytolytic than $CD56^{dim}CD16^{+}$ cells which are highly cytolytic but secrete cytokines with less intensity than the former cells. Phenotypically, $CD56^{dim}$ cells are CD94/NKG2^{+}, killer inhibitory receptor $(KIR)^{+}$, natural cytotoxicity $(NC)^{+}$, and perforin^{+}, whereas $CD56^{bright}$ cells are CD94/NKG2^{+}, $KIR^{low\,or\,-}$, NC^{low}, and perforin^{-} [11, 12]. These two subsets also differ in their expression of adhesion molecules [13, 14] or chemokine receptors [15].

Chemokines play essential roles in linking the innate and adaptive immune responses [17] and are crucial in health and diseases [18]. They have low molecular weights and are divided into four subfamilies based on the position of the cystein (C) residue in the amino terminal end of the molecules; these are known as CXC or α, CC or β, C or γ, and CX3C or δ. Chemokines and their receptors are also classified based on their functions with those that are upregulated during inflammation and under pathological conditions are known as inflammatory chemokines or inflammatory chemokine receptors, whereas those that perform housekeeping functions and are involved in the circulation and homing of cells under physiological conditions are known as constitutive chemokines or constitutive chemokine receptors [19, 20]. These molecules also play important roles in NK cell biology maintaining them in the bone marrow, guiding them into the circulation, and aiding their accumulation at sites of injury. The field examining the expression of chemokine receptors on the surface of NK cells and their ability to induce their migration started in my laboratory in early 1990s [21, 22]. In certain circumstances, there have been controversies about what chemokine receptor is expressed on which subset of NK cells. This is due to the fact that

investigators isolated NK cells in different ways, where some used freshly isolated cells and others used activated cells. In other cases, chemokine receptors expression was examined in $CD56^{bright}$ and $CD56^{dim}$. All these parameters contributed to conflicting concepts regarding which chemokine receptor is involved and what chemokine affects NK cells. Nonetheless, there is an overwhelming data describing the effects of chemokines on the *in vitro* chemotaxis of NK cells [23–36], on their *in situ* distribution [37–42], and on their accumulation during virus infections [43–46], or tumor growth sites [43–48], and other inflammatory diseases or sites [49–52]. Figure 2 is a collection of results regarding the expression of chemokines and chemokine receptors on the two most important subsets of NK cells as described by various investigators and reviewed elsewhere [53].

3. Role of NK Cells in MS/EAE

The consensus is that the activity and number of NK cells in autoimmune diseases are reduced [54]. However, the role of NK cells in MS is controversial with one school of thought suggests that NK cells ameliorate the disease, whereas another school indicates that they exacerbate the disease [reviewed in [6, 53]]. For example, Segal suggested that NK cells play a primary role in ameliorating EAE due to their accumulation within target organs, and that depletion of these cells results in aggravating the disease [55]. Depleting NK cells before immunization of sensitive mice with myelin oligodendrocyte glycoprotein (MOG35–55) peptide resulted in clinically more severe relapsing EAE. This disease enhancement was associated with increased T-cell proliferation and production of Th1 cytokines indicating that NK cells inhibit T-cell proliferation triggered by encephaloantigens [56]. NK cells recruitment into the central nervous system (CNS) of CX_3CR1-deficient mice with EAE is notably impaired, corroborated with increasing the severity of the disease [57]. Lünemann et al. reported that $CD56^+$ NK cells activity is reduced in patients with MS [58]. Those authors observed that IFN-γ production by these cells was inhibited, whereas no effect on the cytolytic activity of $CD16^+$ cells was observed. Intriguingly, Matsumoto et al. observed that NK cells are recruited from the spleen into the CNS of EAE rats, and that treatment of animals with anti-3.2.3 antibody or antiasialo GM1, antibodies that deplete NK cells, exacerbated the clinical scores of EAE [59].

It was also reported that disrupting the interaction of Qa-$1b^+$ T cells and NKG2A/CD94 NK cells led to the ability of NK cells to lyse autoreactive T cells and consequently ameliorate EAE, suggesting that Qa-1b molecule on T cells is important for protecting these cells from lysis by NK cells [60]. Further, administration of an antibody that blocks NK cells interaction with Qa-1^+-activated CD4 T cells resulted in a complete remission from EAE and increased NK cell lytic activity in the CNS [61]. Also, proteolipid peptide-(PLP-) specific autoreactive T cells isolated from SJL mice are killed *in vitro* by syngeneic NK cells suggesting that one of the mechanisms of the beneficial effects of NK cells is perhaps through lysing the encephalitogenic T cells [62]. These results support earlier findings showing that NK cells suppress pathogenic autoreactive T cells which mediate the inflammation in the CNS. Whereas T cells robustly responded to MBP by secreting IFN-γ, such a response was ablated in the presence of NK cells [63]. Recently, Hao et al. confirmed these results and showed that enrichment of NK cells "aided by CX_3CR1" into the CNS of EAE mice leads to their interaction with microglia suppressing the inflammatory Th17 cells and ameliorating the disease [64].

However, these observations contradict other studies showing that NK cells exacerbate rather than ameliorate EAE. Early studies showed that higher NK cell activity as measured by NK cell lysis of K562 target cells is correlated with a higher risk of developing active lesions in relapsing-remitting MS patients "RRMS" [65]. Localized IL-12 by astrocytes promotes the spontaneous development of NK cells that enhance Th1 activity and cytokine secretion [66]. Also, syngeneic IL-2-activated NK cells lyse dorsal root ganglia neurons of C57BL/6 mice by direct cell-cell contact and perforin-dependent mechanisms, suggesting that NK cells damage the neurons [67]. In addition, increased IL-18 production during the primary injection of the MOG35-55 resulted in increased production of IFN-γ secreted by NK cells, a cytokine that promotes autoreactive Th1 responses, whereas an impaired capacity of NK cells to release IFN-γ was found to be a major mechanism underlying resistance to EAE [68]. These results were supported by others showing that *in vivo* depletion of NK cells with specific antibodies led to diminished EAE clinical disease [69], an activity which could be due to activating the adaptive immune cells such as the autoreactive IFN-γ secreting T cells (Th1 cells). Figure 3 summarizes these results and explains the mechanisms proposed in the literature regarding the two contradictory schools of thought.

Due to the importance of NK cells in MS, regardless whether they are stimulatory or inhibitory, several investigators embarked on exploring their activities in MS and other autoimmune diseases. In most cases, these studies particularly those using drugs that treat MS patients revealed the important roles these cells play in this disease. I will touch in this paper on the most important drugs used to treat MS patients and their effects on NK cells. While this paper was under review, Kaur et al. published a related paper which deals with the role of NK cells in MS, as well as explaining the role of NK cell receptors some of them may bind virally infected cells that could be a hidden cause for MS [70].

4. Treatment with Anti-Tac (Anti-CD25 or Daclizumab)

Daclizumab is a humanized IgG1 monoclonal antibody against CD25 molecule which inhibits early IL-2R signal transduction and blocks T-cell activation and their *in vitro* proliferation [71]. This antibody is approved by the FDA for the prevention of acute transplant rejection. It reduces the accumulation of new cortical lesions of RRMS patients, which in one study was significantly better than in the

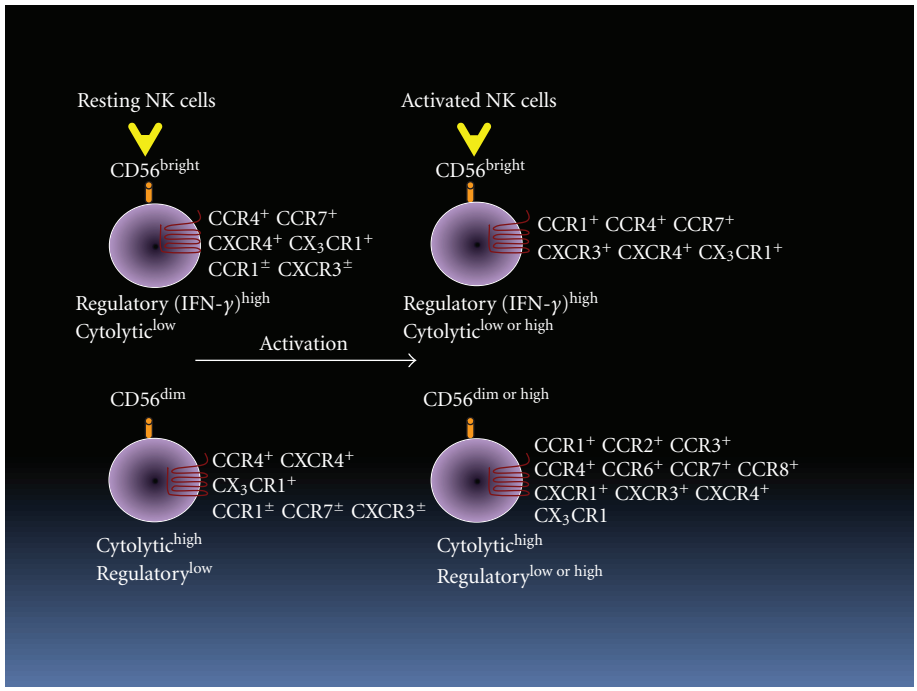

Figure 2: Expression of chemokine receptors on the two major subsets of human NK cells. Resting human NK cells are divided into $CD56^{bright}$ and $CD56^{dim}$ subsets. The former express CCR4, CCR7, CXCR4, and CX_3CR1, and to a lesser extent CCR1 and CXCR3, whereas the latter cells express CCR4, CXCR4, and CX_3CR1, and to a lesser extent CCR1, CCR7, and CXCR3. Upon activation, the $CD56^{bright}$ upregulate the expression of CCR1, CCR4, CCR7, CXCR3, CXCR4, and CX_3CR1, whereas $CD56^{dim}$ cells upregulate the expression of CCR1, CCR2, CCR3, CCR4, CCR6, CCR7, CCR8, CXCR1, CXCR3, CXCR4, and CX_3CR1.

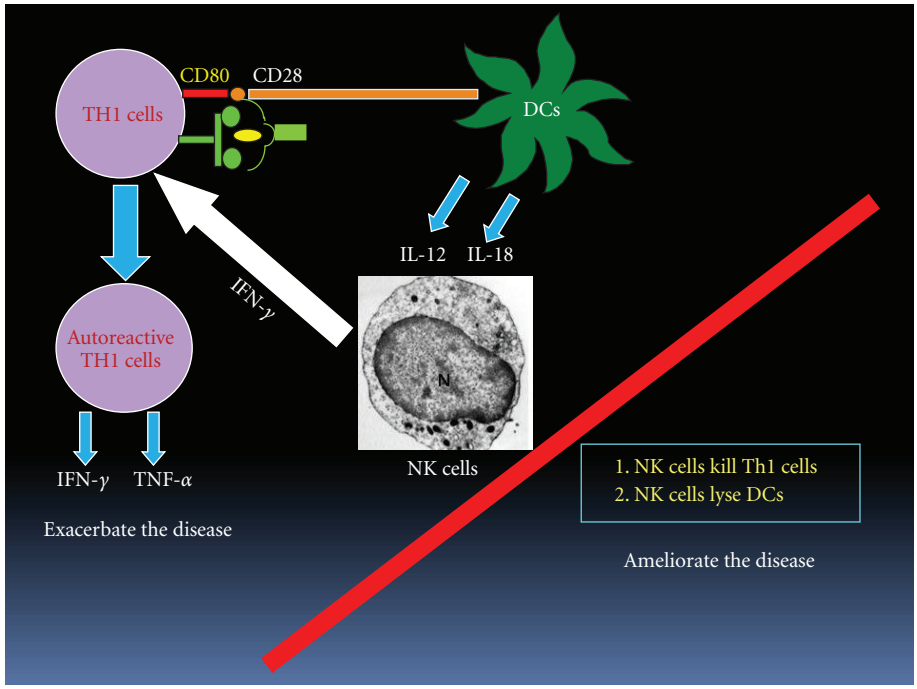

Figure 3: Role of NK cells in MS. Two schools of thought were developed. The first indicates that NK cells help Th1 cells to differentiate into Th1 through the release of IFN-γ. Th1 cells must also receive a signal from APCs that present encephaloantigens which include PLP, MOG, or MBP. These APCs also activate NK cells to release IFN-γ by secreting IL-12 and IL-18. Autoreactive Th1 cells damage the myelin sheath by the release of IFN-γ and TNF-α, among other toxic molecules. The second school indicates that NK cells ameliorate the disease via either directly lysing autoreactive Th1 cells or by lysing APCs, and hence, impeding antigen presentation to these T cells.

vehicle group or in RRMS patients receiving IFN-β1 or glatiramer acetate "GA" [72]. Surprisingly, administration of this antibody into MS patients did not affect *in vivo* T-cell activation but in contrast induced the expansion and activation of CD56^{bright} NK cells, which was corroborated with a positive response in those patients [73]. Further studies showed that daclizumab expanded CD56^{bright} NK cells by about 24-fold compared to IL-2 therapy which expanded them about 7-fold in MS patients. This was corroborated with improvement of the disease as fewer lesions during weeks 8–24 of treatment were observed in the brain of those patients [74].

In this regard, a combination of IL-2 and antibody to IL-2 administered into EAE mice resulted in expansion of NK cells in the spleen, lymph nodes, blood, and CNS. These expanded NK cells did not increase NK cytotoxicity receptors but produced more IFN-γ and MIP-1α/CCL3, which correlated with delayed disease onset and reduced disease severity [75].

5. Treatment with Interferon-β (IFN-β)

IFN-β has been successfully used to treat RRMS patients, where it slows the progression into secondary progressive MS [76]. Although therapy with IFN-β resulted in reduced IFN-γ production and T-cell proliferation [77, 78], other mechanisms of action are still not completely resolved. Early reports showed that RRMS patients responded better to IFN-β treatment when NK cell activity is enhanced in those patients [79]. Also, a significant expansion of CD56^{bright} in IFN-β treated MS patients in the circulation was concomitant with a positive clinical response after one year of therapy [80]. These results were supported by Vandenbark et al. study demonstrating that IFN-β-1a significantly increased the percentages of CD56^{bright} cells and T regulatory "Treg" cells in RRMS treated with this drug for 12 months [81].

Similar treatment resulted in changing the phenotypic profile of NK cells favoring increased expression of NKG2A and decreased expression of killer inhibitory receptors [82]. Treating MS patients with IFN-β alone or in combination with corticosteroids increased the proportion of NK cells during the active stage of cell cycle as determined by the nuclear antigen Ki-67. Although such an expansion did not result in clinical significance, the authors suggested that increased NK cell proliferation may induce their migration into the CNS where they modify the disease outcome [83]. Of note, combining IFN-β with immunosuppressive drugs such as methylprednisolone did not add to the efficacy of this cytokine in treating RRMS patients [84].

6. Treatment with Natalizumab

Natalizumab (anti-α4β1) is IgG4κ monoclonal antibody that does not fix complement or lyse cells via complement-dependent cytotoxicity mode of action. Instead, it inhibits cells that express VLA-4 integrin from migrating across endothelial cell layer that expresses the ligand for this molecule, that is, VCAM-1 [85]. Because it blocks cells that express α4, it was also used for treating Crohn's disease since it prevents cells that express α4β7 from migrating into mucosal sites where endothelial cells express the ligand mucosal addressin cell adhesion molecule-1 "Mad-CAM-1" [86]. Because granulocytes lack the α4β1 molecule, therapy with natalizumab resulted in preventing lymphocytes trafficking into the parenchyma of the CNS and through the mucosal layer of the gut, and consequently, it inhibits recruitment of inflammatory cells towards these sites. Hence, the numbers of CD4$^+$, CD8$^+$, B cells, and plasma cells were significantly reduced in the CNS of MS patients treated with this antibody. After one year of natalizumab therapy, a global decrease in the levels of various inflammatory cytokines and chemokines in the CSF and peripheral blood of MS patients was observed. For example, the cytokines IL-1β, IL-6, and IL-8 as well as the chemokines CCL22, CXCL9, CXCL10, and CXCL11 levels were all reduced in the CSF of these patients [87], suggesting that blocking the migration of inflammatory cells by natalizumab results in reduced inflammatory cytokines and chemokines release favoring ameliorating the MS disease. Unfortunately, this therapy affected two patients who suffered from progressive multifocal leukoencephalopathy (PML), a demyelinating CNS infection associated with a polyoma JC virus [88]. Hence, in addition to the strategy of blocking leukocyte trafficking into sites of injury such as the CNS of MS patients, therapeutic modalities should also focus on finding procedures that deplete autoreactive T cells without affecting other cell types. It should also be noted that the effect of natalizumab on NK cell activity has not yet been defined, but addition of this drug to IFN-β over 2-year period decreased the MS lesions as compared to the effect of IFN-β alone [89], a strategy that may affect NK cell number and/or activity.

7. Treatment with Glatiramer Acetate (GA)

Glatiramer acetate (GA; commercial name Copaxone) is a synthetic compound made up of four amino acids (Glu, Ala, Lys, and Tyr) that are found in myelin. This drug exerts many actions on the immune system [90], which are depicted in Figure 4. It prevents the incidence of experimental autoimmune encephalomyelitis in animals and reduces relapses in patients with MS [91]. This drug is thought to mediate its beneficial effects by induction of GA-specific T helper (Th) 2 cells in MS patients treated with GA, and consequently, IFN-γ, a Th1 cytokine production, was reduced, and the ratio of IL-4/IFN-γ significantly increased, suggesting a shift from Th1 to Th2 after therapy with GA [92]. In a six-year trial, MS patients who received GA had a steady decline in disease relapses with neurological improvement when compared to patients receiving placebo [93]. Moreover, GA inhibits the secretion of IL-12p70 after stimulating DCs with CD40L, resulting in increased IL-4 and decreased IFN-γ release by T cells stimulated with GA-pretreated DCs [94]. Further, GA-specific cells accumulate in the brain secreting Th2 cytokines and the beneficial brain-derived neurotrophic factors [95].

Hestvik et al. [96] isolated several GA-reactive T cells from the CSF and blood of MS patients treated with GA for 3–36 months. The cell lines and clones generated

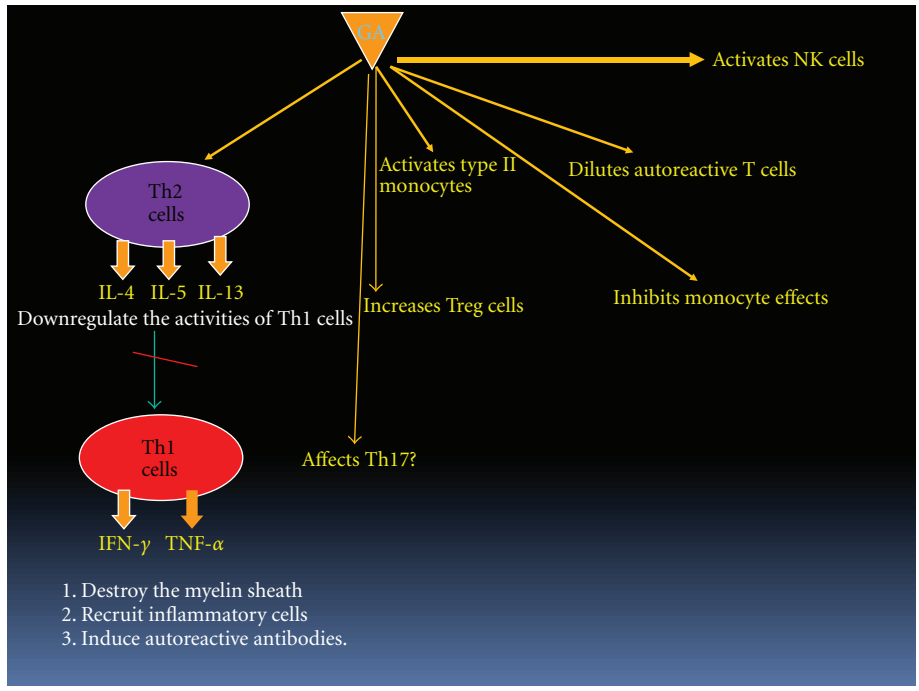

Figure 4: Proposed mechanisms of action (MOA) for GA. GA was reported to be immunomodulatory by shifting the immune system from inflammatory Th1-like to anti-inflammatory Th2-like of response. In addition, various other immunomodulatory activities have been proposed for GA. A red line indicates reversal or inhibition.

showed a bias towards Th2-like cytokine production, which is compatible with the notion that a Th2 shift occurs in the periphery and that these GA-reactive "Th2-like" cells gain access into the CSF [96]. Supporting these findings is the observation that certain of these GA-reactive T-cell clones whether isolated from the peripheral blood or from the CSF of MS patients treated with GA have a limited repertoire of chemokine receptor expression [16]. Only CCR4 and CXCR3 and to lesser extent CCR5 and CCR6 are expressed on nonactivated cells isolated from either blood or CSF of those patients. Surprisingly, these chemokine receptors were downregulated on the surface of these cells upon activation [16]. It was proposed that inflammatory disease activity in spite of GA treatment may be correlated with the loss of expressing chemokine receptors on these cells, leading to reduced migration into sites of injury and the brain parenchyma. The effect of GA may therefore be counteracted by activation, most plausibly by the inflammatory Th1 and Th17 cells. This may explain the delay before any clinical effect of GA treatment is observed in MS patients and could provide additional rational for initial aggressive anti-inflammatory treatment before GA treatment is initiated. In these trials, administration of the immunosuppressive drug mitoxantrone followed by maintenance therapy with GA reduces the mean number of gadolinium-enhancing T1 lesions to almost zero [97–99]. It is plausible that mitoxantrone may suppress Th1/Th17 cells, thereby allowing GA-specific cells to reach the affected areas in the brain. These novel observations may have impact on the way of treating MS patients and in particular those receiving immunomodulatory and immunosuppressive drugs (see Figure 1).

Incubating monocytes *in vitro* with GA renders them less responsive to multiple stimuli including ligands for toll-like receptors (TLRs) and inflammatory cytokines such as IFN-γ and GM-CSF [100]. Also, monocytes from GA-treated MS patients responded with lower efficacy to stimulation with LPS, and they secreted high amounts of the anti-inflammatory cytokine IL-10 and less of the inflammatory cytokine IL-12 [101]. Administration of GA into mice with EAE favors the activation of monocytes type 2 which induce naïve T cells to become Th2, as well as activating Treg cells [102]. Collectively, these results support the idea that GA activates type 2 monocytes corroborated with reduced inflammation in the central nervous system of GA-treated EAE mice or MS patients (see Figure 4).

Dendritic cells play important roles in MS. It was observed that myeloid DCs may accumulate in the CNS during EAE where they present myelin autoantigen to $CD4^+$ T cells that could be skewed into becoming Th17 cells [103]. DC subsets differing in phenotype, function, activation state, and location have been identified [104]. The wide variety of cytokines/growth factors and conditions that have been shown to influence DCs differentiation and activity further highlights the extreme heterogeneity of DC populations, reflecting the diverse phenotype, functions, and ontogeny displayed by these cells [105]. Factors released early after interaction of innate immunity cells with pathogens

represent good candidates as potential natural mediators of differentiation/maturation of DCs *in vivo*. Among them, type I IFN is likely to play a critical role. In fact, type I IFN has been identified as a powerful adjuvant for the development and functional activity of DCs *in vitro* and for IL-15 production in DCs [106]. Of note, DCs endowed with a high migratory and allostimulatory capacity can be rapidly generated by a single-step procedure in the presence of GM-CSF and type I IFNs [107]. However, despite their strong and rapidly induced allostimulatory capacity, IFN-DCs have also been described to produce IL-10 [108] and drive the expansion of T regulatory cells [109].

With respect to the interaction between DCs and NK cells, a number of studies highlighted the importance of such an interaction in the regulation of DC maturation as well as in NK cell activation. The interaction between NK cells and DCs is bidirectional and involves cell-to-cell contact, where DCs activate NK cells by enhancing their proliferation, cytotoxic activity, and IFN-γ production [110]. In turn, activated NK cells provide either maturation signals for DCs or induce their death by direct killing [111]. The mechanisms underlying these opposite effects (maturation versus death) have not yet been clarified. Also how and where such interaction might take place are issues that have not yet been exploited, although it was suggested that the interaction among these cells might take place at inflammatory sites where NK cells and DCs release their contents of inflammatory mediators [8].

It was previously observed that in an animal model of hepatic injury, GA exerted antifibrotic effect corroborated with increased NK cells among many other activities [112]. However, the direct effect of GA on NK cells in MS patients was not known until we embarked on this topic. One of the proposals we entertained is the ability of NK cells to lyse dendritic cells that present antigens to autoreactive T cells (Figure 4). Intriguingly, we observed that GA enhanced the cytolysis of activated human NK cells against autologous and allogeneic human immature and mature monocyte-derived DCs. GA did not modulate the percentage of NK cells expressing NKG2D, NKp30, or NKp44. However, anti-NKp30 or anti-CD86 inhibited GA-enhanced NK cell lysis of immature DCs but not mature DCs, suggesting that the mechanism of GA is different among the two cell types [113]. These results indicate that this drug is capable of activating NK cells to lyse monocyte-derived DCs, a concept that may have vital implications. First, it appears that in addition to switching the system towards Th2, GA may shut down the Th1 axis pathway by ridding the system of monocyte-derived DCs that activate Th1 cells. The fact that NK cells exposed to GA kill both immature and mature DCs ensures that no antigen presentation would be available to Th1 cells. Also, this ensures that during chronic infection where microbes survived the initial attack by the immune system, any stimulation of Th1 cells by monocyte-derived mature DCs would be monitored and controlled by NK cells.

We have translated these findings to mice with EAE, where administration of GA ameliorated the EAE clinical scores [114]. We isolated NK cells from mice that were exposed twice to GA *in vivo* and cultured them with IL-2 for 9 days *in vitro*. Activated NK cells generated by this protocol had high killing of immature and mature DCs that were isolated from either GA-dosed or vehicle-dosed animals [114]. Further, we purified NK cells from the spleens of mice after 16 days of GA therapy and observed that optimal therapy occurred at time points where NK cells had highest killing of DCs [114]. Taking together, these observations indicate that one of the possible mechanisms of GA inhibition of EAE clinical disease may be due to its ability to activate NK cells to lyse DCs. The fact that NK cells exposed to GA kill both immature DCs and mature DCs may lead to the inability of these cells to present antigens to autoreactive T cells. Recent unpublished observations support these findings in MS patients receiving GA, where we monitored the effects of NK cells to lyse DCs in MS patients for a period of one year and observed that dosing with GA enhanced NK cell lysis of autologous DCs (paper submitted).

8. Treatment with Fingolimod (FTY720)

FTY720 "fingolimod; 2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol)" is an immunosuppressive drug derived from myriocin, a fungal metabolite that resembles sphingosine. It proved to be powerful for suppressing allograft rejections during kidney transplantation and is used for treating MS patients [115]. FTY720 inhibits lymphocyte recirculation and induces their sequestration in the secondary lymphoid organs [116]. It was also suggested that in addition to inhibiting the egress of lymphocytes from lymph nodes, FTY720 reduces naïve lymphocytes release from the thymus [117]. Its mechanism of action is related to binding four out five sphingosine-1-phosphate "S1P" receptors, namely, $S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$ and in particular $S1P_1$ and consequently, inducing their internalization and inhibiting S1P activity [118, 119].

IL-2-activated NK cells express $S1P_{1,3,4,5}$ [120], suggesting that S1P may have effects on these cells. Indeed, S1P was observed to be a robust chemoattractant for these activated NK cells [120]. However, administration of FTY720 into kidney transplant patients did not affect the number of NK cells isolated from these patients [121], whereas in an animal transplant model, only high but not low doses of FTY720 prevented NK cell infiltration into allogeneic corneal grafts [122]. NK cells isolated from FTY720-treated mice showed unaltered chemotaxis towards S1P gradient *in vitro* [123]. We also reported that S1P inhibits NK cell lysis of target cells including DCs [124, 125], and that FTY720 as well as the $S1P_1$ antagonist SEW2871 reversed this inhibitory activity [125], suggesting that $S1P_1$ performs an important regulatory function concerning the interaction among NK cells and DCs.

Blocking T-cell infiltration by FTY720 was corroborated with a downregulation of inflammatory genes and vascular adhesion molecules in EAE animals [126, 127]. Late-stage rescue therapy with this drug that started up to 1 month after EAE onset also reversed inflammatory infiltrates and demyelination, as well as normalizing the

disturbances to visual and somatosensory evoked action potentials, suggesting a rapid blockade of ongoing disease by FTY720, and structural restoration of the CNS parenchyma, which are likely due to inhibition of autoimmune T-cell infiltration and direct modulation of neural cells/astrocytes [128]. A phase II clinical trial using FTY720 involving 255 MS patients with RRMS showed a reduction in the median total number of MS lesions [129]. MS patients who participated in FTY720 therapy protocol showed clear reductions in annualized relapse rates and lesion counts compared with the placebo patients during 2-year period [130]. Also MS patients receiving this drug have a relatively increased percentage of $CD56^{dim}$ cells and a reduction in the numbers of circulatory $CD56^{bright}$ NK cells. The authors suggested that the loss of the latter subpopulation may be due to their redistribution into secondary tissues [131]. It was also demonstrated that NK cells as well as monocytes and $CD8^+$ T cells are increased in MS patients receiving FTY720 [132].

9. Discovery of NK17/NK1 Cells

As indicated above, NK cells have been reported to either ameliorate or exacerbate MS disease, but the reasons behind these opposite findings are still a matter of debate. In most of these studies, heterogeneous populations of NK cells have been studied, and no attempt was made to try to dissect whether NK cells might consist of different subpopulation, and that each one of these subsets might exert a separate activity. Although human NK cells have been classified into two subsets based on the intensity of the expression of CD56 molecule, other findings suggest that NK cells might be classified in a different manner. For example, Peritt et al. [133] reported that human NK cells incubated with IL-12-produced Th1-like cytokines such as IFN-γ and were termed NK1 cells, whereas those incubated with IL-4 produced Th2-like cytokines which include IL-5 and IL-13 and were termed NK2 cells. Later work demonstrated that NK cells can be divided into subsets based on the expression of chemokine receptor. In such classification, primary NK cells express CXCR1, CXCR3, and CXCR4 with other subsets expressing CCR1, CCR4, CCR5, CCR6, CCR7, CCR9, CXCR5, and CXCR6 within both $CD56^{bright}$ and $CD56^{dim}$ subsets [134].

A new subset of NK cells was discovered in both human and mouse lining the gut and skin epithelial cell layers [135]. Those isolated from human peyers patches or tonsils express NKp44 and CCR6 molecule, have no cytotoxic granules, do not secrete IFN-γ or IL-17 but secrete IL-22 and CCL20, and are consequently designated as "NK22" cells [136]. Mouse cells lymphoid tissue inducers (LTis) of gut and skin express the transcription factor RORγt and NKp46 receptor molecule [137]. LTi cells that express CD56 among other markers differentiated into cells secreting IL-22 [138]. In tonsil tissues, human NK cells in stage III development express $CD34^+CD117^+2B4^+$ and produced IL-22 and IL-26 but not IL-17 [139]. On the other hand, $NKp46^+$ $NKG2D^+$ $NK1.1^{int}$ $ROR\gamma t^{high}$ NK cells in intestinal lamina propria were found to secrete the Th17 cytokine IL-22 [140]. These findings exposed a new concept suggesting that NK cells may consist of several subsets each with a distinct phenotype and perhaps function. The cells discovered in mucosal tissues secrete IL-22 and express among many markers RORγt and CCR6.

While examining the effects of lysophospholipids on NK cell functions, we discovered that NK cells are not homogenous in terms of their ability to produce inflammatory cytokines and chemokines, but they secrete these molecules with different intensities. Based on these findings, we subdivided heterogeneous NK cells into 4 subcategories according to their release of inflammatory cytokines and chemokines, which are categorized as such (1) low secretors where cells release IL-4, IL-6, IL-12, TNF-α, and MCP-1/CCL2 with low intensity, (2) intermediate secretors where cells secrete IL-1β, IL-10, TGF-β1, and IL-17A with intermediate intensity, (3) high secretors where activated NK cells release IFN-γ and MIP-1α/CCL3, and MIP-1β/CCL4 with high intensity, and (4) very high secretors where the cells secrete MIP-1β/CCL4 with very high intensity [125]. What is striking about these observations is the first demonstration that NK cells secrete the inflammatory molecule IL-17A. Consequently, the major issue was to identify these cells, which was not an ordinary task since as indicated above NK cells may consist of many subsets, and to try to fish a small subset in the peripheral blood among other subsets was difficult to achieve. However, we took advantage of the fact that T cells secreting IL-17 (Th17 cells) express certain chemokine receptors and in particular CCR6 [141–143]. Because NK cells express an array of chemokine receptors including CCR6 [53, 134], the idea was try to stain NK cells with antibody to a specific chemokine receptor as well as intracellularly with antibody to IL-17. Because certain T cells secrete IL-17 as well as IFN-γ (termed Th1/Th17 cells) [141–143], we decided to also stain NK cells for IFN-γ in addition to IL-17. First, we observed that freshly isolated NK cells whether expressing CD56 molecule or not did not produce IL-17. Consequently, we asked the question whether IL-2-activated NK cells might produce this cytokine. We observed that a certain subset within activated $CD56^+$ NK cells produced both IL-17 and IFN-γ, which were consequently termed NK17/NK1 cells based on Th terminology [144]. Surprisingly, we observed that the cells secreting these cytokines were not $CCR6^+$ as greatly anticipated based on Th17 and NK22 studies but instead were $CCR4^+$ [144]. In addition, we examined most other subsets based on chemokine receptor expression and only "sporadically" observed subsets other than $CD56^+CCR4^+$ producing these two cytokines. Why the rules of NK cell generation are different among those found in the gut versus those generated from the blood circulation is an issue not yet clear. What is important is the abundance of NK17/NK1 in the CSF of MS patients. Considering that NK cells are not normally observed in the CSF of normal mice, their presence at high numbers in the CSF of MS patients may indicate that these cells could play vital roles in the pathogenesis of this disease, although this is not yet proven since IL-17 has been shown to be detrimental to autoimmune diseases, but in the case of colitis, it may even be beneficial.

IL-17 is considered an inflammatory molecule because it induced the production of IL-6, TNF, and IL-8/CXCL8 [145]. The latter chemokine recruits neutrophils which induce injury of various tissues. IL-17 also stimulates the production of matrix metalloproteinases 2, 3, 9, and 13 [146] and facilitates the proliferation of endothelial cells [147]. Consequently, IL-17 or cells that secrete it such as Th17 were considered culprits in autoimmune diseases including MS [148], rheumatoid arthritis [149], psoriasis [150], and asthma [151]. In contrast, one study demonstrated that IL-17 may be beneficial for inflammatory colitis disease [152]. In this study, it was observed that Th17 cells may actually inhibit the development of Th1 cells and, consequently, the release of IFN-γ, and that in the absence of Th17/IL-17, Th1 cells can induce strong colitis disease. Based on these observations, it is still too early to suggest whether NK17/NK1 cells may be beneficial or pathological for MS disease.

10. Conclusions

I have reviewed the up and coming field of the role of natural killer cells in multiple sclerosis. Although two concepts were put forward in the literature regarding their effects, with one idea suggesting that NK cells are detrimental and the other beneficial, in this paper a plausible explanation for these contradictory concepts was provided. Further, the effects of approved drugs for treating MS patients have been reviewed. It is not coincidental that the beneficial effects of these drugs (daclizumab, interferon-β, natalizumab, glatiramer acetate, or FTY720) have been corroborated with their ability to increase NK numbers or to enhance their cytolytic activity against target cells. One is left with one conclusion, and that is activation of NK cells in these patients may be an important factor for the beneficial effects of these drugs. The last aspect of this paper dealt with the discovery of NK17/NK1 cells which were abundant in the CSF of MS patients. The role that these cells play in MS is still not resolved. However, one may take advantages of the presence of these cells at inflammatory sites in one of at least two ways: (1) for diagnostic purposes: the presence of NK17/NK1 cells in the CSF of MS patients, or at inflammatory sites in other autoimmune diseases, for example, in the skins of psoriasis patients may be used as a biomarker for the particular disease; (2) for therapeutic purposes: NK17/NK1 if proven to be pathogenic in autoimmune diseases can be targeted for therapy. Taken together, the discovery of NK17/NK1 cells should have an impact on autoimmune diseases and in particular MS. Finally, it seems that future therapy of autoimmune diseases with NK cells is exciting and holds a great promise.

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