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The role of natural killer cells in curbing neuroinflammation

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Abstract

Natural killer (NK) cells are evolutionarily early lymphocytes that lack antigen-specific receptors and, hence, are considered to be part of the innate immune system. The majority of research on NK cells has focused on their ability to lyse “target cells”, generally identified by low or absent MHC Class I expression, such as tumor cells and virus infected cells. However, an alternative role of these leukocytes as regulators of adaptive (and potentially destructive) immune responses, in particular organ-specific autoimmune diseases, has been increasingly recognized. Here we discuss the growing body of evidence that NK cells limit damage in autoimmune demyelinating disease by inhibiting autoreactive T cell responses without harming resident neurons or glia.

Keywords

Multiple sclerosis; experimental autoimmune encephalomyelitis; natural killer cells; immunoregulation

1. Introduction

Over the past few years the majority of studies on immunoregulatory networks have focused on the role of thymic derived and inducible FoxP3⁺ CD4⁺ T cells, IL-10 producing Tr1 cells and TGF- β producing Th3 cells (Baecher-Allan and Hafler, 2006; Carrier et al., 2007; Miyara and Sakaguchi, 2007; Roncarolo et al., 2006). This trend may create the impression that the adaptive arm of the immune system has primary responsibility for monitoring its own activities. However, multiple studies in the literature provide evidence that cells within the innate immune system have the potential to inhibit autoreactive CD4⁺ T cells from mediating autoimmune disease and foreign antigen reactive CD4⁺ T cells from inflicting collateral damage to healthy tissues. In addition to so called “suppressor” myeloid cells (Nagaraj and Gabrilovich, 2007; Serafini et al., 2006) and resting or “homeostatic” CD205⁺ dendritic cells (Hawiger et al., 2001), NK cells are emerging as key participants in the immunomodulatory circuitry.

Natural killer (NK) cells are evolutionarily primitive lymphocytes that possess cytotoxic properties, classically directed against transformed and virus infected cells. Unlike T and B cells, NK cells are not antigen specific. Their cytotoxicity is determined by the collective signaling of an array of inhibitory and stimulatory receptors expressed on their surface (Borrego et al., 2006; Kirwan and Burshtyn, 2007). NK cell inhibitory receptors, commonly referred to as killer inhibitory receptors or KIRs, interact with classical and non-classical MHC Class I molecules. Hence, the presence of self MHC Class I molecules on healthy cells sends an

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inactivating signal to NK cells. Examples of KIRs include the Ly49 family of molecules (that bind classical MHC Class I) and NGK2A (that binds Qa-1, a non-classical MHC Class I molecule expressed on activated T cells, B cells and dendritic cells). Conversely, NK cell cytotoxicity is generally elicited by a distinct set of inducible molecules that have a weak homology with MHC Class I and bind NK stimulatory receptors. For example, retinoic acid early inducible gene (RAE-1) encoded proteins, distantly related to MHC Class I, are ligands for the murine NK cell stimulatory receptor, NGK2D (Diefenbach et al., 2000; Smyth et al., 2005). In many instances, RAE-1 expression correlates with susceptibility to NK cell mediated cytotoxicity (Backstrom et al., 2003). RAE-1 was originally isolated from tumor cell lines (Nomura et al., 1994); it is also expressed on virus infected cells (Backstrom et al., 2007).

Once activated, NK cells mediate cytotoxicity via contact dependent pathways involving perforin/ granzyme, Fas/ Fas-ligand and TRAIL/ TRAIL ligand interactions (Screpanti et al., 2005; Takeda et al., 2001; Warren and Smyth, 1999). They also produce proinflammatory cytokines, such as IFN γ , and chemokines such as CCL5 (Biron et al., 1999). A premise of this review article is that, while NK cells originally evolved as a defense against microbes and neoplasms, their cytotoxic properties were later subverted to eliminate potentially harmful T cell responses. Here we will limit our discussion to the putative regulatory role of NK cells in EAE and MS.

A number of laboratories have found an inverse relationship between the frequency or functional competence of circulating NK cells and clinical or radiological disease activity in patients with multiple sclerosis (MS) (Kastrukoff et al., 2003; Kastrukoff et al., 1998; Oger et al., 1988). In at least two publications the administration of immunomodulatory drugs to MS patients was associated with an expansion of circulating NK cells (Bielekova et al., 2006; Saraste et al., 2007). Furthermore, a substantive body of research demonstrates that conventional (CD3 $^-$ TCR $^-$ NK1.1 $^+$) NK cells limit the severity of experimental autoimmune encephalomyelitis across several different models (Galazka et al., 2006; Huang et al., 2006; Lu et al., 2007; Matsumoto et al., 1998; Xu et al., 2005; Zhang et al., 1997). Collectively this data suggest a significant role of NK cells in reducing neuroinflammation and CNS injury in the context of autoimmune demyelinating disease. Topics that remain to be addressed include: the mechanism and site of action of regulatory NK cells in EAE, factors that influence the migration and biological activities of regulatory NK cells *in vivo*, effects of NK cells on CNS resident neurons and glia, and the feasibility of using NK cells as therapeutic targets and/ or markers of CNS disease activity in MS.

2. Natural killer cells modulate the clinical severity of EAE

In 1997 Zhang and colleagues reported that treatment of MOG₃₅₋₅₅-sensitized C57BL/6 mice with a depleting antibody specific for NK1.1 accelerated the onset and increased the severity of clinical EAE (Zhang et al., 1997). In addition, administration of anti-NK1.1 converted an ordinarily monophasic clinical course into a relapsing one. In parallel experiments, MOG-specific CD4 $^+$ T cell lines induced a more severe form of EAE in syngeneic hosts treated with an anti-NK1.1 antibody than in hosts treated with an isotype matched control antibody. Similar observations were made using C57BL/6 β 2-microglobulin deficient mice that lack NK1.1 $^+$ CD3 $^+$ cells, indicating that depletion of conventional NK cells was responsible for the therapeutic effect of the antibody. (This point is particularly important since it has been speculated that NK T cells play a regulatory role in autoimmune diseases, including EAE and MS (Furlan et al., 2003; Illes et al., 2004)). Conversely, cotransfer of whole splenocytes, but not NK cell depleted splenocytes, ameliorated EAE induced by the injection of myelin-specific T cells into Rag2 $^{-/-}$ hosts.

Shortly thereafter, Matsumoto and colleagues published their finding that injection of MBP-immunized rats with antibodies specific for either NKR-P1 (analogous to NK1.1) or asialo GM1 resulted in aggravation of EAE, reflected by significantly higher maximal clinical scores (and, with anti-asialo GM1, increased mortality rates) (Matsumoto et al., 1998). However, these experiments were not designed to distinguish between effects of the antibodies on NK cells as opposed to NK T cells (with regard to both anti-NKR-P1 and anti-asialo GM1) or peritoneal macrophages and CD8⁺ T cells (with regard to anti-asialo GM1).

While the manuscripts referenced above do not elucidate the mechanism of action of regulatory NK cells in EAE, they provide some clues. Hence, as mentioned earlier, Zhang and colleagues reported that NK cell depletion exacerbates adoptively transferred EAE. Masumoto et al. found that NK cells comprise up to 17 percent of CNS-infiltrating inflammatory cells in Lewis rats at peak disease. Although the study by Zhang, et. al. did not include flow cytometric analysis of CNS mononuclear cells, other researchers have reported that NK cells account for 10-20% of the infiltrate in symptomatic C57BL/6 mice immunized with MOG₃₅₋₅₅ (Huang et al., 2006). Collectively, the observations that significant numbers of NK cells accumulate within the target organ during EAE, and NK depleting antibodies aggravate disease at a point past the priming of encephalitogenic T cells, implicate a role of regulatory NK cells in the effector phase of pathogenesis, possibly within the CNS itself. On the other hand, lymph node cells from anti-NK1.1 treated, MOG₃₅₋₅₅ immunized C57BL/6 mice mount enhanced MOG-specific proliferation and IFN γ recall responses, suggesting that NK cells ordinarily suppress the priming, differentiation and/ or expansion of encephalitogenic T cells in the periphery (Zhang et al., 1997). Of course, it is possible that the immunomodulatory activities of NK cells are important during both induction and effector stages. In fact, reigning theories on their mechanism of action (namely that they drive activation induced cell death and directly lyse encephalitogenic cells) are compatible with such a scenario.

3. Natural killer cells and disease activity in multiple sclerosis

Since the early 1980's multiple papers have appeared in the literature claiming that patients with MS have reduced numbers of circulating NK cells and/ or functionally deficient NK cells (Benczur et al., 1980; Braakman et al., 1986; Hirsch and Johnson, 1985; Kastrukoff et al., 1998; Kreuzfelder et al., 1992; Munschauer et al., 1995; Uchida et al., 1982; Vranes et al., 1989). With regard to the latter, NK cells isolated from MS patients were found to be inefficient at cytotoxic killing (generally measured by chromium release assays with K-562 erythroleukemia cells as targets) and production of IFN γ , either spontaneously or in response to stimulation with IL-2 (Benczur et al., 1980; Braakman et al., 1986; Hirsch and Johnson, 1985; Kastrukoff et al., 1998; Uchida et al., 1982; Vranes et al., 1989). In a recent longitudinal study, NK cell functional activity fell precipitously in concert with the onset of clinical MS relapses but normalized during remissions (Kastrukoff et al., 2003). Other recent studies have found that MS patients with active disease are relatively deficient in particular subsets of NK cells that suppress cytokine production by autologous myelin-specific T cell lines or are highly cytotoxic *ex vivo*, such as CD56⁺CD3⁻CD95⁺ cells and CD56⁺CD3⁻CX₃CR1⁺ cells, respectively (Infante-Duarte et al., 2005; Takahashi et al., 2001).

The collection of manuscripts published thus far on NK cells in MS differ widely in the criteria used to classify NK cells, the assays and protocols used to measure their functions and/ or frequencies, and the patient populations studied (the latter with regard to relapsing remitting or progressive stage, EDSS range, disease duration, and treatment history). In addition, some researchers have reported no differences, either quantitatively and qualitatively, between NK populations in MS patients versus controls (Hauser et al., 1981; Rauch et al., 1985; Rice et al., 1983; Santoli et al., 1981). While those studies are in the minority, it is still difficult to interpret

the heterogeneous group of manuscripts that support a relationship between NK cell defects and MS exacerbations.

Stronger evidence for a role of regulatory NK cells in MS comes from data on the immunological consequences of initiating novel as well as conventional pharmacological treatments in patients with active disease. In a phase II trial of daclizumab (a humanized monoclonal antibody directed against the IL-2 α chain) in relapsing remitting patients, suppression of contrast enhancing lesions on brain MRI was significantly associated with the expansion of circulating CD56^{bright} NK cells and contraction of CD4⁺ and CD8⁺ T cells (Bielekova et al., 2006). Furthermore, NK cells isolated from patients during, but not before, daclizumab therapy exhibited cytotoxicity towards autologous activated T cells without the need for pre-stimulation with IL-2. In a separate manuscript, newly diagnosed relapsing remitting patients experienced an increase in the percentage of CD56^{bright} NK cells among peripheral blood mononuclear cells within 3 months of starting interferon- β treatment (Saraste et al., 2007). While these observations are intriguing, more definitive conclusions await clinical trials with agents that directly and exclusively interfere with NK cell activities.

4. Fractalkine controls NK cell migration to the CNS during EAE

NK cells have been detected in MS plaques and EAE CNS mononuclear infiltrates (Matsumoto et al., 1998; Traugott and Raine, 1984). Studies with genetically engineered mice indicate that their accumulation in the CNS is not a random event, but the consequence of a CX3CL1 (fractalkine) dependent pathway. Huang and colleagues recently reported that mice deficient in CX3CR1 (the fractalkine receptor) mount normal proliferative and cytokine recall responses and generate encephalitogenic T cells comparable to wildtype mice following sensitization against MOG₃₅₋₅₅ in CFA but, nevertheless, experience a relatively severe form of EAE (Huang et al., 2006). Unlike their CX3CR1^{+/-} littermates, CX3CR1^{-/-} mice exhibited a high incidence of CNS hemorrhages, a high mortality rate and they failed to recover function following peak disease. Analysis of spinal cord mononuclear cells from these mice revealed a selective absence of NK1.1⁺CD3⁻ cells (which compose 10-20% of CNS infiltrates in wildtype CX3CR1^{+/+} or heterozygous CX3CR1^{+/-} mice). This finding lead the authors to speculate that the exacerbated disease in CX3CR1^{-/-} mice was due to a deficiency of regulatory NK cells in the target organ. In support of their hypothesis, the majority of CNS infiltrating NK cells in CX3CR1^{+/-} mice expressed CX3CR1 and treatment of MOG-sensitized CX3CR1^{+/-} mice with an anti-NK1.1 depleting antibody resulted in EAE of comparable severity to the CX3CR1^{-/-} cohort. Soluble CX3CL1 was increased in the CNS during EAE and protein extracts from CNS tissues were chemotactic for wildtype NK cells.

The authors included additional experiments to reinforce their contention that a defect in conventional NK cells, rather than NK-T cells, is responsible for the phenotype of CX3CR1^{-/-} mice. Hence, MOG immunized CX3CR1^{-/-} $\times$ CD1d^{-/-} double knock-out mice (that lack NK-T cells) resemble CX3CR1^{-/-} mice with regard to both the clinical course and histological features of EAE, whereas CD1d^{-/-} single knock out mice resemble wildtype controls. In addition, the authors further investigated the extent of the NK cell defect in CX3CR1^{-/-} mice and its bearing on inflammation outside of the CNS. Interestingly, NK cells accumulated at a normal frequency in the spleen and liver of CX3CR1^{-/-} mice infected with cytomegalovirus, suggesting that CX3CR1/ CX3CL1 interactions are of particular importance for NK cell recruitment and/ or survival in the CNS.

A number of experiments that would have solidified the authors' conclusions were omitted. For example, based on their hypothesis, depletion of NK cells from CX3CR1^{-/-}, as opposed CX3CR1^{+/-}, mice should have no effect on EAE severity. However, they did not perform an experiment to test that prediction. Furthermore, they did not include an experiment

demonstrating that reconstitution of CX3CR1^{-/-} mice with wildtype NK cells reduces the severity of EAE to the level of CX3CR1^{+/-} controls. Nonetheless, the manuscript is compelling, particularly in light of a recent report that MS patients are deficient in circulating CX3CR1⁺ NK cells (Infante-Duarte et al., 2005). The latter study found that the frequency of CX3CR1⁺ NK cells in MS patients actually rises in association with radiological disease activity, adding further complexity to their role in MS pathogenesis.

5. Mechanisms of action of “regulatory” NK cells

NK cells could, theoretically, suppress T cell responses by killing antigen presenting cells or by secreting immunosuppressive cytokines such as IL-10 (Gilbertson et al., 1986; Mehrotra et al., 1998). They could also release chemokines that induce the accumulation of other regulatory leukocytes, such as FoxP3⁺ cells. However, the majority of data on regulatory NK cells in EAE and MS suggest that they directly trigger the death of encephalitogenic T cells, most likely by contact dependent interactions or by release of cytokines over short distances.

Hence, PLP-specific encephalitogenic T cell lines derived from the lymph nodes of immunized SJL mice undergo an increased rate of cell death upon co-culture with NK cell-enriched, syngeneic splenocytes (Xu et al., 2005). Similarly, coculture of bone marrow NK cells from naïve DA rats with syngeneic MBP-reactive T cells inhibits myelin-specific T cell proliferation and IFN γ production (Smeltz et al., 1999). Furthermore, NK cells isolated from peripheral blood mononuclear cells of human donors kill activated autologous T cells following prestimulation with IL-2 (Bielekova et al., 2006).

Some of the factors controlling the ability of NK cells to lyse activated T cells were revealed in a recent manuscript by Lu and colleagues (Lu et al., 2007). They found that CD4⁺ T cells genetically deficient in the non classical MHC Class I molecule, Qa-1 (which is normally upregulated during T cell activation), undergo an accelerated rate of death when primed in immunocompetent hosts. However, the Qa-1 deficient cells expand and survive as well as wildtype CD4⁺ T cells in perforin deficient or NK-depleted hosts. CD4⁺ T cells that express a mutant form of Qa-1, prohibitive of interactions with the NK inhibitory receptor, NGK2A, exhibited a similar phenotype. Furthermore, transfection of wildtype ovalbumin-specific CD4⁺ T cells with a lentivirus encoding Qa-1 improved their survival upon antigen-driven expansion in syngenic OVA-immunized mice. Collectively this data suggest that upregulation of Qa-1 on CD4⁺ T cells during activation *in vivo* is ordinarily protective against perforin mediated lysis by NGK2A expressing NK cells. With regard to EAE, the authors reported that MOG-specific, T cell receptor transgenic CD4⁺ T cells lose the ability to transfer EAE into Rag2^{-/-} hosts once they are backcrossed to a Qa-1 deficient background. Nevertheless, these cells are able to induce disease in Rag2^{-/-} hosts that are either deficient in perforin or depleted of NK cells. Consistent with these findings, administration of an anti-Qa-1 antibody to MOG-sensitized wildtype mice during the preclinical phase significantly reduced the severity of EAE. However, the therapeutic effect of the anti-Qa-1 antibody was undermined by co-administration of an NK cell depleting antibody. In conclusion, encephalitogenic MOG-specific CD4⁺ T cells, like foreign-reactive T cells, are suppressed by NK cells via a perforin dependent mechanism if they fail to upregulate Qa-1 during *in vivo* priming. Conversely, high or constitutive expression of Qa-1 could, theoretically, favor the expansion and differentiation of autoreactive T cells in patients during relapses of autoimmune disease.

Experiments conducted by Galazka and colleagues raise an alternative mechanism by which NK cells could suppress autoreactive T cells (Galazka et al., 2006). They found that preinjection of mice with heat shock protein 70 (Hsp70)-peptide complexes isolated from inflamed CNS tissue was protective against EAE. Splenic NK cells from the protected mice suppressed myelin-specific T cell proliferation by an IFN γ dependent pathway *in vitro* and inhibited EAE

upon transfer into PLP-immunized mice. Furthermore, PLP-reactive cells from mice preinjected with Hsp70 underwent apoptosis in response to restimulation with PLP *ex vivo*. The authors concluded that the mechanism of Hsp70-induced tolerance to EAE depends on an NK cell mediated increase in IFN γ production leading to enhanced apoptotic death of autoreactive T cells. Their hypothesis is consistent with the findings that activation induced cell death of T lymphocytes is facilitated by IFN γ (Refaeli et al., 2002) and that IFN γ deficient mice are highly susceptible to EAE (Willenborg et al., 1999).

6. NK cells and CNS resident cells

The strategy of exploiting the regulatory properties of NK cells for therapeutic purposes in MS would be sabotaged if NK cells are cytotoxic toward neurons and/or glia. Experimental data on interactions between NK cells and CNS resident cells are both scarce and controversial. The question as to whether NK cells are ordinarily capable of killing oligodendrocytes *in vitro* is particularly contentious. Hence, Satoh and colleagues found that NK cells, isolated from the peripheral blood mononuclear cells of either patients with MS or healthy volunteers, exhibited virtually no cytotoxicity towards primary cultures of bovine oligodendrocytes, even following prestimulation with IL-2 (Satoh et al., 1990; Satoh et al., 1991). In contrast, Morse and colleagues reported that IL-2 activated NK cells readily lyse autologous human primary oligodendrocytes obtained from temporal lobe tissues of individuals undergoing surgery for intractable epilepsy (Morse et al., 2001). More recently, the same laboratory reported that blockade of NK2GD-NK2GD ligand interactions prevents NK cell mediated killing of human oligodendrocytes *ex vivo* (Saikali et al., 2007).

There appears to be more of a consensus that central nervous system neurons are relatively resistant to injury by NK cells, particularly in comparison to peripheral neurons. Hence, while murine dorsal root ganglion cells are destroyed upon exposure to syngeneic, IL-2 activated NK cells, CNS-derived hippocampal and ventral horn neurons are unaffected (Backstrom et al., 2003). Protection of CNS neurons from NK cell killing does not appear to be secondary to MHC Class I-KIR interactions since hippocampal neurons from β 2-microglobulin deficient mice are equally resistant to cytotoxicity. An alternative explanation is that CNS neurons fail to express ligands that activate NK cells. Indeed, Backstrom et al. found that hippocampal and ventral horn neurons, by contrast to dorsal root ganglion neurons, express relatively low levels of RAE-1, a ligand for the NK cell activating receptor NGK2D (Backstrom et al., 2003). Furthermore, administration of a blocking antibody against NGK2D protected dorsal root ganglion cells from NK cell killing, indicating that elevated expression of RAE-1 is a critical factor in determining their vulnerability.

According to experiments by Hammarberg et al., NK cells might not simply be innocuous towards neurons, but could actually promote their survival (Hammarberg et al., 2000). This group found that induction of EAE in rats with myelin basic protein dramatically reduced the loss of spinal motor neurons after ventral root avulsion. CNS infiltrates in the protected rats contained T cells and NK cells that expressed high levels of brain-derived neurotrophic factor, neurotrophin-3 and glial cell line-derived neurotrophic factor. However, the relative importance of NK cells and individual neurotrophic factors to neuroprotection was not formally demonstrated.

7. Conclusions, therapeutic implications and future directions

Experiments in the EAE model have clearly demonstrated that endogenous conventional NK cells can suppress neuroinflammation and subsequent damage to CNS tissues in the course of autoimmune demyelinating disease. These findings are made even more compelling by the observation that administration of an immunomodulatory agent resulted in the expansion of a

subset of NK cells in MS patients in association with clinical and radiological improvement. Nonetheless, a number of critical issues remain unresolved.

Despite the demonstration that, under certain conditions, NK cells are cytotoxic towards activated T cells *in vitro* (including encephalitogenic myelin-specific T cells), the mechanism of action of regulatory NK cells in autoimmune disease *in vivo* remains uncertain. It is also unclear whether regulatory functions are a global property of NK cells or, more likely, possessed by a discrete subset. Such a subset might be distinguished a unique cytokine and chemokine repertoire that facilitates its immunomodulatory functions. There is already a suggestion in the literature that regulatory NK cells preferentially express CX3CR1; by extrapolation, they might express a unique panel of adhesion molecules and chemokine receptors that facilitate trafficking and/ or contact-dependent interactions necessary for their function. While CX3CL1 signaling in NK cells appears to be critical for their recruitment to the CNS (at least during EAE), it is thus far unproven that secretion of soluble CX3CL1 by neurons is responsible for NK cell migration across the blood-brain-barrier. Alternatively, it is possible that NK cells are altered by a CX3CL1 dependent event in the periphery, that in turn potentiates their trafficking to, or retention in, the CNS and/ or promotes the acquisition of immunoregulatory traits.

The prospect of exploiting regulatory NK cells for therapeutic purposes, while intriguing, holds a number of caveats. If a cell surface profile of these cells is defined, one could imagine expanding them *in vitro* for autologous infusion. However, while a few of the studies referenced above contained an experiment in which purified NK cells were transferred into mice to prevent EAE, none showed that NK transfers could be used to suppress established disease. Furthermore, potential negative side effects of therapies designed to promote NK cell activity and/ or increase NK cell frequency (such as cytokine release syndromes or cytotoxic injury) have not been rigorously explored. Future experimental therapies will more likely be based on manipulation of cytokine/ chemokine pathways and cell-to-cell interactions involving regulatory NK cells in a manner that will boost their efficiency without stimulating pro-inflammatory NK subsets to damage healthy tissues. Before such Phase I clinical trials are conceived, a great deal more will need to be discovered regarding the biological properties of this interesting leukocyte.

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