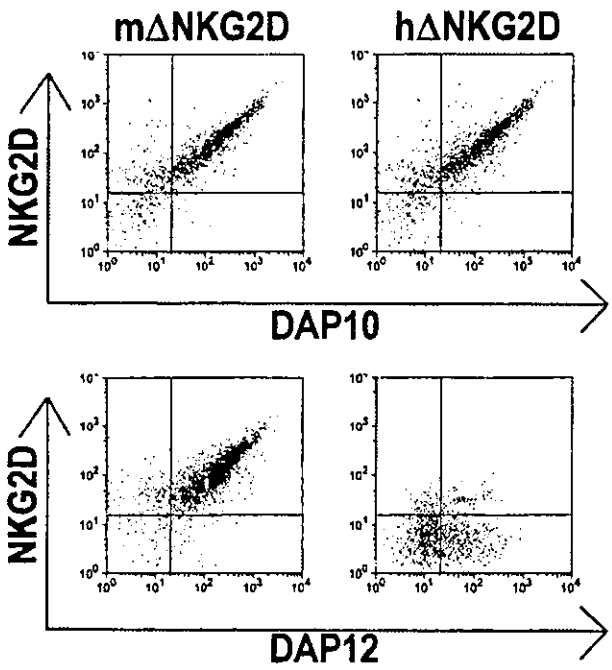

FIGURE 4. Mouse NKG2D TM and EC domains are sufficient for DAP12 association. Ba/F3 reporter cells stably expressing Myc-DAP10 or Flag-DAP12 were infected with retroviruses with the indicated NKG2D constructs, as shown in Fig. 2*b*, in a vector containing an IRES-GFP element. Samples were stained with the relevant anti-NKG2D and anti-epitope tag mAbs and analyzed by flow cytometry. Results shown are representative of at least three independent experiments.

based on prior reports (12, 16) (Fig. 3). In accordance with prior findings (12, 30), mNKG2D-S efficiently paired with DAP12. By contrast, although the human short NKG2D associated with DAP10, it was completely unable to pair with DAP12. Human and mouse NKG2D-L were able to pair interchangeably with either human or mouse DAP10, without species preference (data not shown). Similarly, mNKG2D-S associated equally with mouse or human DAP12, whereas human short NKG2D failed to assemble with either mouse or human DAP12. Collectively, these results demonstrate a fundamental difference between the human and mouse NKG2D proteins, rather than species-specific differences in the conserved adapter proteins.

Mouse NKG2D cytoplasmic domain is not required for DAP12 association

In mouse NKG2D, the 13 amino acid N-terminal cytoplasmic tail present in mNKG2D-L, absent in mNKG2D-S, abrogates DAP12 binding (12). Similar to mouse NKG2D, the first 14 amino acids of human NKG2D preceding the second methionine in the cytoplasmic domain is a highly charged region (isoelectric point pH 12). As our artificial human short NKG2D also lacks this potentially inhibitory sequence, and is still unable to pair with DAP12, other structural elements must explain the difference between human and mouse NKG2D with respect to association with DAP12. By comparison of mouse and human NKG2D (Fig. 2), one explanation lies in a 13 amino acid stretch present in mouse, but not human NKG2D, in the cytoplasmic juxtamembrane region. This unique murine cytoplasmic juxtamembrane sequence (mCJS), designated in Fig. 2*a*, might provide a positive signal for DAP12 association, as this sequence is present in mouse but not human NKG2D. To address this possibility, we created two N-terminal truncations of mouse NKG2D: one that contained the mCJS and another that only contained the TM and EC domains (mΔNKG2D). Similarly, we created a truncated human NKG2D, which contained only the TM and EC domains (hΔNKG2D) (Fig. 2*b*). Retroviruses encoding mΔNKG2D and hΔNKG2D were used to infect Myc-DAP10 or Flag-DAP12 reporter BaF/3 cells, as previously described.

Ab staining for the appropriate epitope tags and NKG2D revealed that mΔNKG2D was capable of pairing with both DAP10 and DAP12 (Fig. 4). This result suggests that the mCJS region is not required for DAP12 association and that the mouse NKG2D TM and EC domains are sufficient for DAP12 association. The same experiment with a truncated mouse NKG2D still containing the mCJS did not significantly improve DAP12 association, suggesting that this sequence plays no major role in DAP12 association (data not shown). Like human short NKG2D, hΔNKG2D was still able to pair with DAP10, but was incapable of pairing with DAP12. This excluded the possibility that the inability of human NKG2D to pair with DAP12 was due to an inhibitory sequence present in the cytoplasmic region. Furthermore, these results suggest that the difference between mouse and human NKG2D, with respect to DAP12 association, must lie within the TM and/or EC domains.

The mouse NKG2D TM sequence allows human NKG2D to pair with DAP12

Previous studies with a chimeric protein composed of the DAP10 EC-DAP12 TM-DAP10 cytoplasmic domains suggested a nonpermissive interaction between the TM regions of DAP12 and human NKG2D (27). Furthermore, examination of the protein sequences of human and mouse NKG2D reveals significant sequence divergence within the TM region (Fig. 2*a*, underlined). These observations spurred the question of whether the difference in DAP12 pairing ability between mouse and human NKG2D could be attributed to their TM regions. To test the hypothesis that the mouse NKG2D TM is permissive for DAP12 association whereas the human NKG2D TM is not, we created a chimeric NKG2D construct containing the mTM hEC domain (Fig. 2*b*) and tested its ability to pair with DAP10 and DAP12 in BaF/3 reporter cells, as previously described. We found that replacing the human TM with the mouse TM region allowed the chimeric protein to stabilize expression of human DAP12 on the cell surface of the transfectants (Fig. 5*a*). In other words, the mouse NKG2D TM permitted the chimeric receptor to associate with DAP12. This conclusion was further supported by the ability to coimmunoprecipitate both DAP10 and DAP12 with the chimeric receptor containing the mouse NKG2D TM region (Fig. 5*c*). By contrast, as reported previously, human NKG2D coimmunoprecipitates with DAP10, but not with DAP12 (27, 31). These results suggest a critical difference between the TM domains of human and mouse NKG2D such that human NKG2D is not permissive for DAP12 pairing whereas mouse NKG2D is permissive.

In these experiments, we also tested whether the FQPV motif (Fig. 2*a*, boxed) on the EC membrane-proximal side of the mouse TM was necessary for DAP12 interaction, as this motif is absent from human NKG2D. Our experiments suggest this motif is unnecessary as chimeric mTM hEC proteins with or without this sequence associate equivalently with DAP12 (data not shown).

The human NKG2D cytoplasmic domain when associated with the mouse NKG2D TM region is permissive for DAP12 association

Like the mouse NKG2D 13 amino acid tail, the N-terminal portion of human NKG2D-L contains multiple charged amino acid residues (Fig. 2*a*). Because of this similarity, it is possible that the

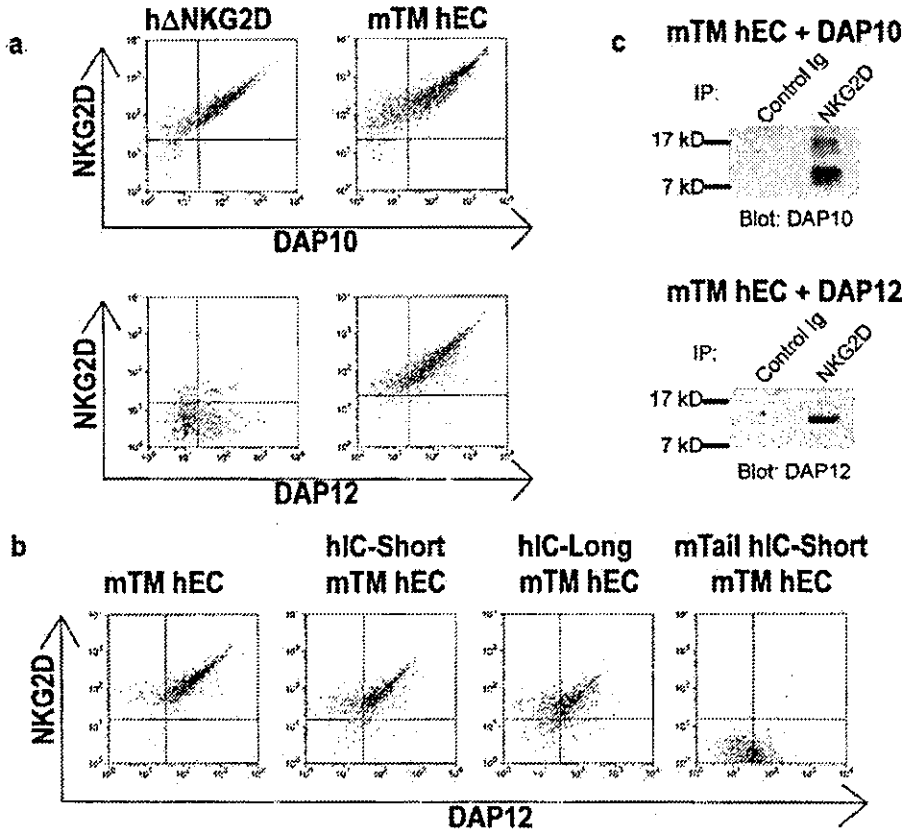

FIGURE 5. The mouse NKG2D TM region conveys DAP12 specificity to human NKG2D. *a* and *b*, Ba/F3 reporter cells stably expressing Myc-DAP10 or Flag-DAP12 were infected with retroviruses with the indicated NKG2D constructs, as shown in Fig. 2*b*, in a vector containing an IRES-GFP element. Samples were stained with the relevant anti-NKG2D and anti-epitope tag mAbs and analyzed by flow cytometry. *c*, mTM hEC chimeric NKG2D receptor coimmunoprecipitates with DAP10 and DAP12. Transfected mTM hEC reporter cells (*a*) were lysed in Brij-Nonidet P-40 lysis buffer. The receptor complexes were immunoprecipitated from lysates with anti-human NKG2D or isotype-matched control mAb. Samples were analyzed by SDS-PAGE and transferred to Immobilon P membrane and probed with goat anti-DAP10 antisera N-17 or anti-DAP12 mAb DX37, followed by HRP-conjugated donkey anti-goat IgG or goat anti-mouse IgG, respectively, and visualized with chemiluminescent substrate. As described previously (16), the heterogeneous migration pattern of DAP10 is likely due to *O*-linked glycosylation of its EC domain.

human tail behaves like the mouse tail and may also be repulsive to DAP12 association. Thus, in addition to having a nonpermissive TM region in human NKG2D, the repulsive segment present in mNKG2D-L cytoplasmic domain might have been conserved in humans to prevent potential association with DAP12. To answer the question of whether the long cytoplasmic domains of human NKG2D or the artificial "short" hNKG2D are permissive for DAP12 binding, we created NKG2D chimeras from the mTM-hEC construct adding back the human "short" intracellular domain (hIC-"Short" mTM hEC), the human long intracellular domain (hIC-Long mTM hEC), or the human "short" intracellular domain with the mouse N-terminal 13-aa tail (mTail hIC-"Short" mTM hEC), as portrayed graphically in Fig. 2*B*. As expected, all of these chimeric constructs paired with DAP10 (data not shown).

Using retroviral infection of the Flag-DAP12 reporter BaF/3 cells, we found that the entire human intracellular domain, in both the artificial short and natural long isoforms, failed to prevent DAP12 association when the TM region of mNKG2D is present in the chimeric receptors (Fig. 5*b*). In contrast, addition of the mouse tail to the human short intracellular domain abrogated DAP12 association. These results demonstrate that the N-terminal tail sequence of mouse NKG2D is necessary to prevent association of DAP12 with the mNKG2D-L isoform. This repulsion cannot be due to charge alone because the N-terminal segments of both mouse and human NKG2D are rich in acidic and basic amino acid residues. From an evolutionary perspective, mouse NKG2D may have evolved a repulsive tail and alternative splicing of NKG2D as mechanisms to regulate DAP12 adapter signaling. In contrast, as the TM of human NKG2D is not permissive for association with DAP12 it requires no such regulatory region in the cytoplasmic domain and hence this structural feature has not been conserved.

NKG2D TM domains are necessary and sufficient to confer adapter specificity

As the mouse NKG2D truncation (mΔNKG2D) and mTM hEC chimeric NKG2D proteins paired with DAP12, it was possible that the EC portions of human or mouse NKG2D could be necessary for association with DAP12. To address this question and further to ask whether the TM regions of mouse and human NKG2D are sufficient to confer adapter specificity, we created chimeric proteins consisting of the TM domain of either mouse or human NKG2D fused to the EC domain of human CD69 (Fig. 6*a*). CD69 has some structural similarity to NKG2D, as it is also a type II TM-anchored homodimer with an EC region consisting of a single C type-like lectin domain (32–34). However, in contrast to NKG2D, CD69 does not pair with signaling adapters, such as DAP10 or DAP12, and does not possess charged amino acids in its TM region. A truncated form of CD69 consisting of only the TM and EC domains was also constructed as a control (in our study designated as ΔCD69) (Fig. 6*a*).

We tested the CD69 chimeras in the DAP10 or DAP12 reporter cells, as previously described, and found that the TM domain of NKG2D was necessary and sufficient to convey adapter specificity to the chimeric proteins (Fig. 6*b*). Although the control protein ΔCD69 did not pair with either adapter, the human TM NKG2D-CD69 protein was able to pair with DAP10, but not DAP12. In contrast, the mouse TM NKG2D-CD69 chimeric protein was able to associate equivalently with both DAP10 and DAP12.

We further wished to define requirements for DAP12 association. Closer examination of the mouse TM region revealed a second basic residue present in the mouse but not human TM region. We tested whether this second arginine residue (Fig. 2*a*, boxed)

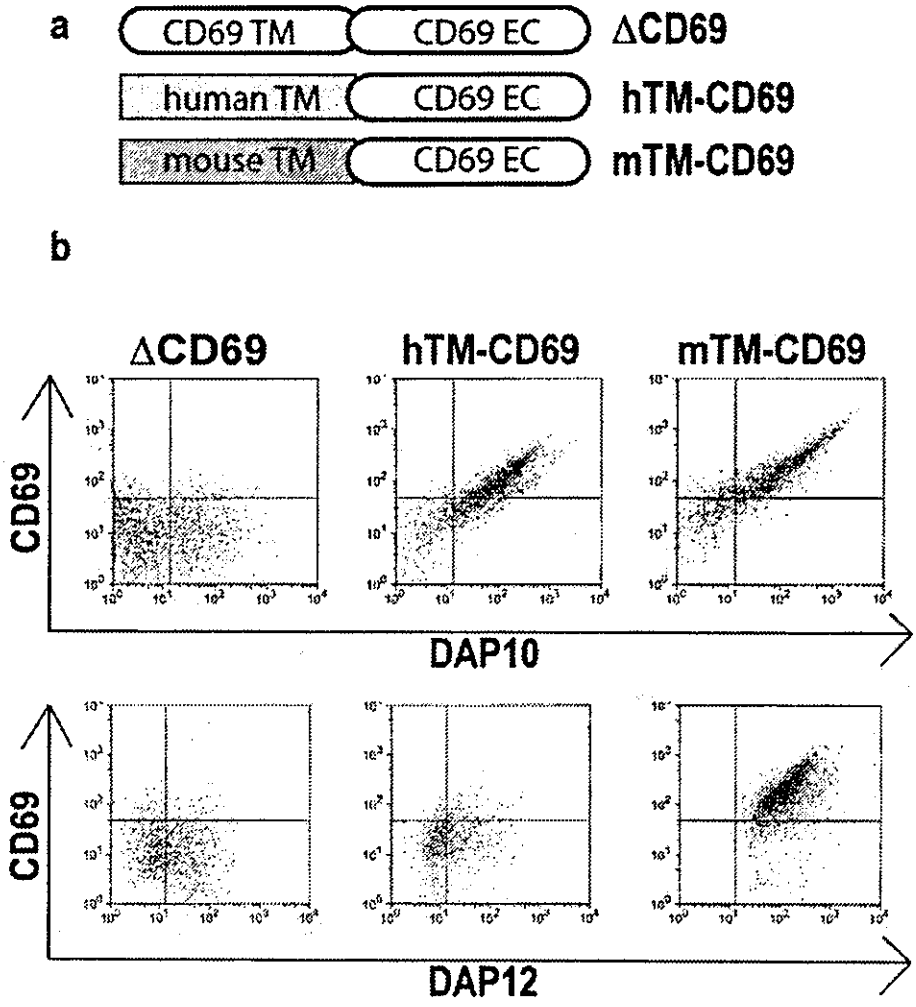

FIGURE 6. NKG2D TM regions are necessary and sufficient to confer DAP10 and DAP12 specificity. *a*, Schematic representation is shown of chimeric receptors containing the EC domain of human CD69 and the TM region of human or mouse NKG2D (as described in *Materials and Methods*). *b*, Ba/F3 reporter cells stably expressing Myc-DAP10 or Flag-DAP12 were infected with retroviruses with the indicated NKG2D TM-CD69 EC constructs in a vector containing an IRES-GFP element. Samples were stained with anti-human CD69 mAb and anti-epitope tag mAbs and analyzed by flow cytometry. Results shown are representative of at least three independent experiments.

was necessary for DAP12 association by mutating it to an alanine residue via site-directed mutagenesis. We found that an R→A mouse TM CD69 mutant still paired with DAP12, indicating that this putative second TM basic residue does not contribute to DAP12 association (data not shown).

These results formally demonstrate that the adapter specificity for human and mouse NKG2D lies exclusively in the TM domain, and this domain is necessary and sufficient to allow adapter pairing. A similar phenomenon has been observed for the receptor specificity of the human DAP10 and DAP12 signaling adapters. Chimeras between DAP10 and DAP12 have illustrated that the receptor specificity of these two adaptors also lies in their TM domains (27). Thus, for NKG2D, DAP10, and DAP12 all of the critical and necessary pairing interactions occur within the TM regions.

Overall, there is little conservation of the TM sequence of human and mouse NKG2D. Only a few amino acids have been conserved, conspicuously the arginine residue in the center of the TM. This extensive diversity in the sequence of the mouse and human NKG2D TM region precludes easy predictions about the critical residues in mouse NKG2D that permit or in human NKG2D prevent association with DAP12.

Discussion

Our results indicate a fundamental structural difference between mouse and human NKG2D. Although mouse NKG2D is capable of associating with both DAP10 and DAP12 signaling adapters, human NKG2D is only able to partner with DAP10. We show that this species difference can be mapped to the TM regions of mouse and human NKG2D. As DAP10 signals through a p85 phosphatidylinositol-3 kinase-mediated AKT pathway (16, 17), and as DAP12 signals through a Syk/ZAP70-mediated ITAM pathway (19), the ability of NKG2D to pair with various combinations of adapters represents its ability to initiate discreet signaling effects in mice, but not humans. Moreover, direct examination of NKG2D-dependent NK cell activation in DAP12-deficient humans suffering from the Nasu-Hakola disorder demonstrated that the NKG2D receptor functions normally with respect to its ability to induce cytolytic activity in the complete absence of DAP12.

Our findings are consistent with the studies of Billadeau et al. (21), which demonstrate that a chimeric protein with the cytoplasmic domain of human DAP10 was capable of triggering NK cell-mediated cytotoxicity. However, in these experiments the chimeric DAP10 was introduced into human NK cells expressing a functional DAP12 protein, leaving open the possibility of indirect interactions between the cytoplasmic domains of the chimeric DAP10 and endogenous DAP12 proteins. Our findings provide conclusive evidence for a DAP12-independent role of DAP10 in human NK cell NKG2D-mediated cytotoxicity.

Activated mouse NK cells expressing mNKG2D-S can thus signal through DAP10 and DAP12, whereas resting mouse NK cells and all human NK cells express long NKG2D and only signal

through DAP10. In many cases, DAP10 alone suffices to initiate cytotoxicity. This is evident from the fact that human NKG2D, which only uses DAP10, stimulates cytotoxicity independent of Syk kinases (21), as well as the evidence that NKG2D triggers cytotoxicity in mouse NK cells lacking DAP12 or Syk family kinases (20). In mice, DAP12 is also independently capable of initiating cytotoxicity through NKG2D-S, as demonstrated by experiments in DAP10-deficient mice (30). Furthermore, mouse NKG2D-S initiated DAP12 signaling contributes to proliferative responses and IFN-γ production (12). A role for DAP10 in IFN-γ production has been suggested using human NK cells and recombinant human NKG2D ligands (17, 31, 35).

It is not completely surprising that the mouse NKG2D TM region can associate with both DAP10 and DAP12 as these two adaptor proteins share significant homology. DAP10 and DAP12 share 20% sequence homology (16) and moreover their TM domains are very homologous in both sequence (45% homology) and structure (27). In the genomes of both mice and humans, the *DAP10* and *DAP12* genes are adjacent to one another, but in opposite transcriptional orientation (36), a circumstance that likely arose through gene duplication.

Our results illustrate that the TM domain of the immunoreceptor NKG2D provides signaling adapter specificity and accounts for the species difference. Although the human NKG2D TM associates only with DAP10, the mouse NKG2D TM associates with both DAP10 and DAP12. A comparison of NKG2D TM domain sequences across species demonstrates a close relationship between mouse and rat TM sequences (Fig. 2*c*). Furthermore, the only reported sequence of rat NKG2D lacks an N-terminal "tail" region and is thus structurally similar to mNKG2D-S. Based on the sequence similarity of mouse and rat TM regions and the absence on a potentially inhibitory tail sequence in rat NKG2D that could block DAP12 association, rat NKG2D likely pairs with DAP12. In contrast, other species such as macaque, cow, and pig, display TM sequences more homologous to human NKG2D and may solely signal through the DAP10 adapter protein, although this requires experimental verification.

Although many genes are highly conserved in sequence, expression and function between mice and humans, *NKG2D* represents a gene that has undergone evolutionary divergence between the two species. Although it acts as an innate immune receptor in both mice and humans, NKG2D demonstrates distinct signaling mechanisms in the two species and none of the human and mouse NKG2D ligands are highly conserved in primary sequence. For example, mice do not possess structural homologues of the human *MICA* and *MICB* genes that are present in the human MHC (37). Furthermore, although the human ULBP genes clearly are functional orthologs of the mouse *RAE-1*, *H60* and *MULT-1* genes they have diverged significantly and only demonstrate 15–20% sequence similarity (14). Expression of NKG2D also differs in humans and mice. Whereas all human $CD8^+$ T cells constitutively express NKG2D (4, 5), in mice only activated $CD8^+$ T cells possess NKG2D. Nonetheless, there is evidence that NKG2D is important in immune defense in mice and humans. Viruses and tumors have evolved immune evasion mechanisms to counter the effects of NKG2D (38–41). Thus, perhaps an evolutionary pressure from the host to fight rapidly evolving viral infections and tumors helps explain some of the divergence and distinctions of NKG2D between mice and humans.

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The regulatory role of natural killer cells in multiple sclerosis

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Summary

Multiple sclerosis is a chronic demyelinating disease of presumed autoimmune pathogenesis. The patients with multiple sclerosis typically shows alternating relapse and remission in the early stage of illness. We previously found that in the majority of multiple sclerosis patients in a state of remission, natural killer (NK) cells contain unusually high frequencies of the cells expressing CD95 (Fas) on their surface (>36.0%). Here we report that in such 'CD95$^+$ NK-high' patients, NK cells may actively suppress potentially pathogenic autoimmune T cells that can mediate the inflammatory responses in the CNS. Using peripheral blood mononuclear cells (PBMCs) derived from 'CD95$^+$ NK-high' or 'CD95$^+$ NK-low' multiple sclerosis in a state of remission, we studied the effect of NK cell depletion on the memory T cell response to myelin basic protein (MBP), a major target antigen of multiple sclerosis. When we stimulated PBMCs of the 'CD95$^+$ NK-high' multiple sclerosis after depleting CD56$^+$ NK cells, a significant proportion of CD4$^+$ T cells (1/2000 to 1/200) responded rapidly to MBP by secreting interferon (IFN)-γ, whereas such a rapid T cell response to MBP could not be detected in the presence of NK cells. Nor did we detect the memory response to MBP in the 'CD95$^+$ NK-low' multiple sclerosis patients in remission or healthy subjects, regardless of whether NK cells were depleted or not. Depletion of cells expressing CD16, another NK cell marker, also caused IFN-γ secretion from MBP-reactive CD4$^+$ T cells in the PBMCs from 'CD95$^+$ NK-high' multiple sclerosis. Moreover, we showed that NK cells from 'CD95$^+$ NK-high' multiple sclerosis could inhibit the antigen-driven secretion of IFN-γ by autologous MBP-specific T cell clones *in vitro*. These results indicate that NK cells may regulate activation of autoimmune memory T cells in an antigen non-specific fashion to maintain the clinical remission in 'CD95$^+$ NK-high' multiple sclerosis patients.

Keywords: multiple sclerosis; myelin basic protein; NK cell; NK2; T cell–NK cell interaction

Abbreviations: CBA = cytokine bead array; HLA = human leukocyte antigen; IFN = interferon; IL = interleukin; MBP = myelin basic protein; MS-rel = multiple sclerosis in relapse; MS-rem = multiple sclerosis in remission; NK = natural killer; NK2 = NK type 2; OVA = ovalbumin; PBMCs = peripheral blood mononuclear cells; PI = propidium iodide; PLP = proteolipid protein; TCC = T-cell clone; TNF = tumour necrosis factor

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Introduction

Multiple sclerosis is a chronic neurological disease the pathology of which is characterized by multiple foci of inflammatory demyelinating lesions accompanying a variable degree of axonal changes (Bjartmar and Trapp, 2001). Regarding the pathogenesis of multiple sclerosis, studies have indicated that autoimmune T cells targeting myelin components play a crucial role in mediating the inflammatory process, particularly in the early stages of relapsing–remitting multiple sclerosis (Steinman, 2001). A number of laboratories have studied the properties of potentially pathogenic autoimmune T cell clones (TCC) reactive to myelin antigens such as myelin basic protein (MBP) and proteolipid protein (PLP), which have been derived from the peripheral blood of multiple sclerosis (Ota *et al.*, 1990; Pette *et al.*, 1990; Martin *et al.*, 1991; Ohashi *et al.*, 1995). The large majority of the TCC are CD4$^+$ and produce T helper type 1 (Th1) cytokines

such as interferon (IFN)-γ after recognizing the myelin peptide bound to human leukocyte antigen (HLA)-DR molecules. These results are consistent with the idea that the inflammatory process of multiple sclerosis is triggered by invasion of autoimmune Th1 cells into the CNS, and that exogenous or endogenous factors altering the Th1/Th2 balance may influence the disease activity. The relevance of this postulate is actually supported by clinical observations that Th2-inducing medications, such as copolymer-1, are beneficial for multiple sclerosis (Duda *et al.*, 2000; Neuhaus *et al.*, 2000), and that administration of IFN-γ showed deleterious effects on multiple sclerosis in previous clinical trials (Panitch *et al.*, 1987).

Although there are a number of candidate target antigens for multiple sclerosis, MBP is thought to be a primary target for autoimmune T cells, at least in some patients (Bielekova *et al.*, 2000). It is of note that MBP- or PLP-specific TCC can be established not only from multiple sclerosis, but also from peripheral blood of healthy subjects, which raised the intriguing issue as to how healthy subjects are protected from self-attack by the potentially pathogenic autoimmune Th1 cells. Although much remains to be clarified, studies in the last decade have showed that regulatory cells are involved in prevention of or recovery from autoimmune diseases in rodent (Das *et al.*, 1997; Zhang *et al.*, 1997; Olivares-Villagomez *et al.*, 1998; Sakaguchi *et al.*, 2001). This allows us to speculate that regulatory cells may contribute to protecting healthy subjects from developing autoimmune diseases such as multiple sclerosis, or to prohibiting acute attacks or enhancing the recovery from clinical exacerbations in patients with relapsing–remitting multiple sclerosis.

Whereas regulatory cells constitute various lymphoid populations, substantial evidence supports that natural killer (NK) cells play significant roles in protecting against autoimmune diseases (Zhang *et al.*, 1997; Matsumoto *et al.*, 1998; Smeltz *et al.*, 1999). In fact, it has previously been demonstrated that NK cell depletion augments the severity of a model for multiple sclerosis, experimental autoimmune encephalomyelitis (EAE) (Zhang *et al.*, 1997; Matsumoto *et al.*, 1998), which can be induced by sensitization against CNS myelin component. Given that autoimmune Th1 cells would mediate the pathology of EAE, we propose a possible involvement of NK cells in suppressing autoimmune Th1 cells in multiple sclerosis.

With the hypothesis that NK cells may contribute to maintaining the remission in relapsing–remitting multiple sclerosis, we have previously examined the cytokine production and surface phenotype of NK cells freshly isolated from the peripheral blood mononuclear cells (PBMCs) of multiple sclerosis in remission (MS-rem) or relapse (MS-rel) (Takahashi *et al.*, 2001). The results demonstrate that NK cells in MS-rem (but not MS-rel) are characterized by a remarkable elevation of interleukin (IL)-5 mRNA and a decreased expression of IL-12Rβ2 mRNA, as well as a higher percentage of $CD95^+$ cells among the $CD56^+$ NK cells. These features of the cells are reminiscent of NK type 2 (NK2) cells, which can be induced *in vitro* in the presence of IL-4 and of anti-IL-12 antibodies (Peritt *et al.*, 1998). The NK2 cells induced from PBMCs of healthy subjects inhibit the generation of IFN-γ-secreting Th1 cells from the PBMCs of the same subjects (Takahashi *et al.*, 2001), leading us to postulate that NK2-like cells detected in MS-rem may play a regulatory role. While the NK2-like features were found to be lost in patients at acute relapsing state, they tended to be restored along with clinical recovery. Obviously, these results do not imply that clinically diagnosed MS-rem represents a homogeneous condition. In fact, the parameters characteristic for NK2-like cells (i.e. up-regulation of IL-5 mRNA and an increased frequency of $CD95^+$ cells) showed a substantial variance in MS-rem, indicating their heterogeneity.

More recently, we have noticed that MS-rem can be divided into two subgroups, '$CD95^+$ NK-high' and '$CD95^+$ NK-low', according to the frequency of $CD95^+$ cells among NK cells. Here, we demonstrate that these two groups significantly differ in the responsiveness to MBP *ex vivo* in an NK-cell-depleted condition. Namely, NK-deleted PBMCs from '$CD95^+$ NK-high' multiple sclerosis responded rapidly to MBP, as assessed by the frequency of IFN-γ-secreting $CD4^+$ T cells at 8 h after stimulation with MBP, whereas those from the '$CD95^+$ NK-low' or from healthy subjects responded only marginally. Moreover, we showed that NK cells from a '$CD95^+$ NK-high' multiple sclerosis could inhibit the antigen-driven secretion of IFN-γ by MBP-specific TCC established from the same patient. These results demonstrate, for the first time to our knowledge, that NK cell depletion leads to augmentation of memory T cell response to an autoantigen in human, and that an elaborate interplay between NK cells and MBP-specific memory T cells may be involved in the regulation of multiple sclerosis in '$CD95^+$ NK-high' patients.

Material and methods

Subjects

To clarify the heterogeneity among patients with MS-rem regarding NK cell phenotype, we first examined 30 patients with MS-rem (male/female = 11/19; aged 37.7 ± 11.1 years) for the lymphoid cell expression of CD95. As a control for multiple sclerosis, we examined 26 healthy sex- and age-matched subjects (male/female = 11/15; aged 39.9 ± 12.2 years). Furthermore, for a new cohort of 14 patients with MS-rem (male/female = four/10; aged 39.2 ± 10.7 years) (Table 1) and 14 healthy subjects (male/female = five/nine; aged 35.3 ± 8.0 years), we conducted the cytokine secretion assay as well as flow cytometer analysis for the frequency of $CD95^+$ NK cells. Two of the patients were examined again after a 1-year interval.

Written informed consent was obtained from all patients and healthy volunteers and the study was approved by the Ethics Committee of the National Center of Neuroscience (NCNP). All patients fulfilled standard criteria for the diagnosis of relapsing–remitting multiple sclerosis (Poser *et al.*, 1983; McDonald *et al.*, 2001). The clinical status of multiple sclerosis (MS-rem or MS-rel) was operationally determined as described previously (Takahashi *et al.*, 2001). In brief, we selected MS-rem patients for study who had been clinically stable without any immunosuppressive medications for >3 months, and had shown no sign of new lesions as assessed by a recent MRI scan with gadolinium enhancement. None of our patients represented the pure optic-spinal form of multiple sclerosis (Misu *et al.*, 2002), which may be rather unique to Japanese populations.

Table 1 *List of the PBMC samples examined for the frequency of memory Th1 cells*

Information on patients			
PBMC code	Age (years)/sex	$CD95^+$ NK frequency	EDSS#
#1	43/M	High	2.5
#2	30/F	High	2.5
#3	53/M	High	1.0
#4	39/F	High	3.5
#5	28/F	High	1.0
#6*	35/M	Low	2.0
#7**	57/F	Low	3.0
#8	31/M	Low	1.0
#9	29/F	Low	3.0
#10	38/F	Low	2.0
#11	59/F	High	3.5
#12*	36/M	High	2.0
#13**	58/F	High	3.0
#14	33/F	High	6.5
#15	29/F	Low	1.0
#16	45/F	Low	4.0

The samples marked with * or ** are derived from the same patients, with an interval of 1 year between samples. The phenotype of both of these patients changed from '$CD95^+$ NK-low' to '$CD95^+$ NK-high'. M = male; F = female; EDSS = Expanded Disability Status Scale.

Reagents

Anti-CD3-FITC or -ECD, anti-CD4-PC5, anti-CD8-FITC, anti-CD16-Phytoerythrin, and anti-CD56-PC5 or -PE mAbs were purchased from IMMUNOTECH (Marseille, France). Anti-CD57-FITC, anti-CD69-PE, anti-CD94-FITC, anti-CD95-FITC, -Cych or -PE, anti-CD158a-FITC, anti-NKB1-FITC, and anti-HLA-DR-FITC mAbs were purchased from BD PharMingen (San Jose, CA, USA). Human MBP was purified with a modification of previously described methods (Deibler *et al.*, 1972, 1995).

Cell preparation and NK cell deletion

Shortly after drawing peripheral blood, PBMCs were separated by density gradient centrifugation with Ficoll-Hypaque™ PLUS (Amersham Biosciences, Uppsala, Sweden). They were washed three times in phosphate-buffered saline (PBS), and resuspended at 1×10^6 cells/ml in AIM-V culture medium (Invitrogen Corp., Carlsbad, CA, USA) containing 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies, Rockville, MD, USA). NK cells were depleted from the PBMCs with either CD56- or CD16-MicroBeads (Miltenyi Biotech, Gradbach, Germany), following the protocol provided by the manufacturer.

T cell clones

$CD4^+$ TCC were generated from a '$CD95^+$ NK-high' multiple sclerosis patient (HLA-DRB1*1502) by repeated selection against human whole MBP with modification of a previously described method (Pette *et al.*, 1990). The TCC proliferated and secreted Th1 cytokines specifically in response to MBP, and the proliferative response and cytokine production was greatly reduced in the presence of antibodies against HLA-DR. The DR-restricted clone cells were grown in AIM-V medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

T-cell stimulation with MBP

To assess the presence of memory MBP-reactive T cells in the peripheral blood, fresh PBMCs or NK-deleted PBMCs were stimulated for 8 h with 10 µg/ml MBP in 96-well round-bottomed plates at 2×10^5 cells/well, and then analysed for the presence of IFN-γ-secreting cells using the cytokine secretion assay. To evaluate the regulatory function of NK cells from '$CD95^+$ NK-high' multiple sclerosis, resting cells of MBP-specific TCC (2×10^4 cells/well) were stimulated with 10 µg/ml MBP in the presence of X-irradiated (5000 rad) autologous total PBMCs or $CD56^+$ NK-deleted PBMCs (1×10^5 cells/well) for 8 h prior to the cytokine secretion assay, and for 60 h to determine the proliferation of the TCC. To assess cell proliferation, we counted incorporation of [^{3}H]thymidine (1 µCi/well) during the final 12 h with a beta-1205 counter (Pharmacia, Uppsala, Sweden).

Cytokine secretion assay

We used a commercial kit from Miltenyi Biotech to identify T cells secreting IFN-γ. The principle of this assay has been described previously (Manz *et al.*, 1995). Briefly, cells were stained with IFN-γ capture antibody 8 h after stimulation with MBP or ovalbumin (OVA), then washed and cultured again for 45 min. They were stained with PE-conjugated IFN-γ detection antibody, together with anti-human CD3-FITC and -CD4-PC5, then washed and resuspended in PBS containing propidium iodide (PI) (BD PharMingen). Samples were analysed using flow cytometry.

Cytokine bead array

The levels of IL-2, -4, -5, -10, tumour necrosis factor (TNF)-α and IFN-γ in the culture supernatants were measured by cytokine bead array (CBA) (BD PharMingen), in which six bead populations with distinct fluorescence intensities are coated with capture antibodies specific for each cytokine (Cook *et al.*, 2001). The cytokine capture beads were mixed with the PE-conjugated detection antibodies and then incubated with recombinant standards or supernatant samples to form sandwich complexes. After washing the beads, sample data were acquired using the flow cytometer and were analysed with the BD CBA Analysis Software® (BD PharMingen).

Results

An increased frequency of $CD95^+$ NK cells distinguishes a subgroup of multiple sclerosis

As we have reported previously (Takahashi *et al.*, 2001), whereas proportions of $CD3^-$ $CD56^+$ NK cells in fresh PBMCs weakly express CD95 on their surface, the frequency of $CD95^+$ NK cells is significantly elevated in MS-rem as compared with healthy subjects or MS-rel. We have further noticed that MS-rem can be divided into two subgroups according to the frequency (%) of $CD95^+$ cells among NK cells (Fig. 1A; see also the left panels in Figs 1B and 2A, showing the distinction between $CD95^+$ and $CD95^-$ cells). When we determined the mean + 2 SD value for healthy subjects (35.86%) as an upper boundary for healthy subjects,

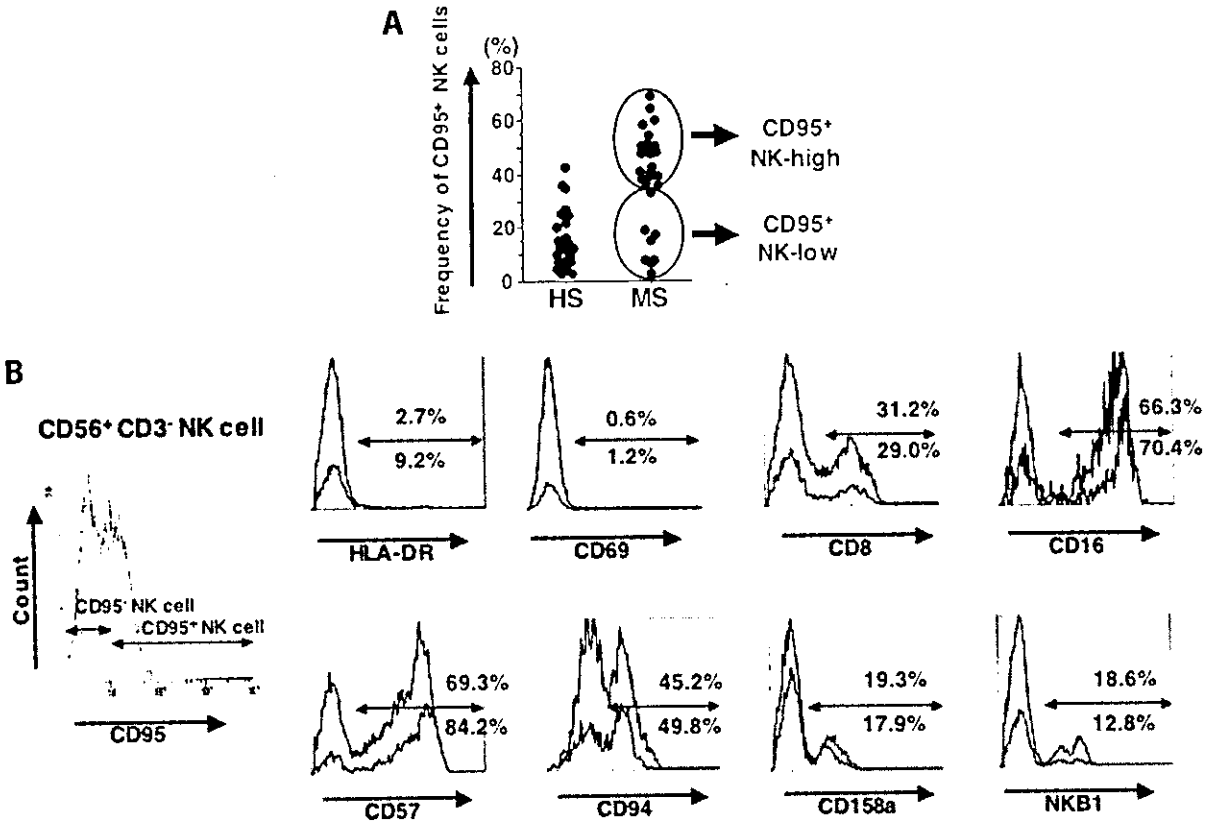

Fig. 1 Characterization of CD95$^+$ NK cells from 'CD95$^+$ NK-high' multiple sclerosis. (**A**) Multiple sclerosis patients in remission (MS-rem) can be subgrouped into 'CD95$^+$ NK-high' and 'CD95$^+$ NK-low'. Freshly isolated PBMCs from 26 healthy subjects or 30 MS-rem were stained with the combination of anti-CD3-FITC, -CD56-PC5 and -CD95-PE, and evaluated for the frequency of CD95$^+$ cells in the CD56$^+$CD3$^-$ NK cell population (the fluorescence intensity for CD95 expression is shown in the histograms in **B** and Fig. 2A). Note that flow fluorocytometric analysis was completed within 2 h after drawing blood in order to avoid spontaneous death of CD95$^+$ cells. (**B**) Comparison of CD95$^+$ versus CD95$^-$ NK cells in the expression of various surface molecules. We stained the PBMCs from 'CD95$^+$ NK-high' patients with the panel of antibodies for surface molecules expressed by NK cells. Red lines represent the histogram gated for CD95$^-$ NK cells and blue lines for CD95$^+$ NK cells. Values in red and in blue represent the positive percentage in CD95$^-$ and CD95$^+$ cells, respectively. As indicated, CD95$^+$ NK cells did not differ significantly from CD95$^-$ NK cells in the staining pattern for each antibody regarding the proportion of positive cells as well as the mean fluorescence intensity. Shown are the results of a representative case.

three-quarters of MS-rem had a percentage value higher than this boundary. We defined these patients in remission with a higher frequency of CD95$^+$ cells in NK cells as 'CD95$^+$ NK-high' multiple sclerosis, and the rest as 'CD95$^+$ NK-low' (Fig. 1A). In contrast to CD56$^+$ NK cells, CD3$^+$CD56$^-$ T cells and CD3$^+$CD56$^+$ NK T cells were not different between healthy subjects and multiple sclerosis patients as regards the frequency of CD95$^+$ cells (data not shown), which directed our attention to the analysis of CD56$^+$ NK cells.

Because NK cells from MS-rem were found to express a larger amount IL-5 mRNA, and since they were neither defective in cytolytic function nor reduced in number (Takahashi *et al.*, 2001), we hypothesized that the CD95 expression may reflect an activation state of the NK cells. To test this hypothesis, we compared the CD95$^+$ and CD95$^-$ NK populations derived from 'CD95$^+$ NK-high' patients by flow cytometry. Histogram plot analysis for the proportion of positive cells and for mean fluorescence intensity showed that the two populations are analogous in the expression of HLA-DR, CD69, CD8, CD16, CD57, CD94, CD158a and NKB1 (Fig. 1B). Whereas HLA-DR and CD69 molecules are regarded as cell activation markers, few populations of CD95$^+$ NK cells from multiple sclerosis or healthy subjects expressed these molecules. These results do not support the idea that the CD95$^+$ NK cells are in a state of activation, nor do they indicate that the CD95$^+$ cells represent a unique subset of monoclonal or oligoclonal origin. It has recently been suggested that CD56^{bright} NK cells may represent a distinct subset (Jacobs *et al.*, 2001). However, we saw no difference in the proportion of CD56^{bright} cells between CD95$^+$ and CD95$^-$ NK cells (data not shown).

CD56$^+$ NK cell depletion induces the rapid activation of MBP-reactive memory T cells in PBMCs from 'CD95$^+$ NK-high' multiple sclerosis

We have previously shown that the CD95$^+$ NK cells found in multiple sclerosis patients resemble the NK cells that can be induced in culture in the presence of IL-4 and anti-IL-12

mAb [referred to as 'NK2-like cells' according to the definition by Peritt *et al.* (1998)]. We also found that Peritt's NK2 cells induced *in vitro* inhibited the induction of IFN-γ-secreting T cells from peripheral T cells after stimulation with phorbol myristate acetate and ionomycin (Takahashi *et al.*, 2001). Based on these observations, we speculated that NK cells might prohibit Th1 cell activation in the remission of multiple sclerosis in an antigen-non-specific manner, and contribute to maintaining the remission. However, it remained an open question as to whether the NK2-like cells found in MS-rem would indeed regulate pathogenic autoimmune T cells *in vivo*. To investigate functions of NK cells in MS-rem, we evaluated the effect of NK cell depletion on the peripheral T cell response to MBP, a major target antigen of multiple sclerosis (Bielekova *et al.*, 2000). In brief, we depleted $CD56^+$ cells from the PBMCs with a magnetic sorter, and then stimulated the NK-deleted populations as well as whole PBMCs with MBP *in vitro* for 8–24 h. Subsequently, we detected the antigen-responsive T cells based on the secretion of IFN-γ (Manz *et al.*, 1995). The preparatory experiments revealed that 8 h of stimulation provides an optimal condition yielding a low background (0–0.03%). This novel assay enables us to selectively detect memory-type Th1 cells that can respond rapidly to antigen, whereas previous assays that depend on long-term cultures (Pette *et al.*, 1990; Martin *et al.*, 1992) evaluate not only memory but also naive T cells. Of note, there is a general consensus that peripheral blood of multiple sclerosis patients contains MBP-reactive T cells that are activated and/or differentiated into memory T cells (Allegretta *et al.*, 1990; Martin *et al.*, 1992; Zhang *et al.*, 1994; Lovett-Racke *et al.*, 1998; Scholz *et al.*, 1998).

We examined 16 PBMC samples from 14 MS-rem patients (nine samples from '$CD95^+$ NK-high', and seven from '$CD95^+$ NK-low') and 14 healthy subjects (see Table 1). When freshly isolated PBMCs were stimulated with MBP before NK cell depletion, four MS-rem and five healthy subjects samples showed a marginal response to MBP (0.01–0.03% increase of IFN-γ-positive cells among $CD4^+$ T cells). We did not find any significant response to MBP with the other PBMC samples. In contrast, when cells were stimulated with MBP after deleting $CD56^+$ NK cells, a significant response with a stimulatory index >3 was detected in seven of the nine '$CD95^+$ NK-high' samples, and a marginal response was detected in two (Fig. 2A and B). Of note, none of the NK-deleted samples from the '$CD95^+$ NK-low' patients and healthy subjects showed a definitive response to MBP. The difference for the '$CD95^+$ NK-high' versus the '$CD95^+$ NK-low' or healthy subjects was statistically significant (Fig. 2B). These *ex vivo* experiments have revealed that the '$CD95^+$ NK-high' patients may possess a higher number of T cells that can rapidly respond to MBP (MBP-specific memory T cells), compared with '$CD95^+$ NK-low' MS-rem or healthy subjects. In other words, they provide strong evidence for clonal expansion of memory autopathogenic T cells in the '$CD95^+$ NK-high' patients. However, as we could demonstrate an increase of the memory autoimmune T cells only after depleting NK cells, we interpreted that the potentially hazardous autoimmune T cells are being controlled by counter-regulatory NK cells in the '$CD95^+$ NK-high' patients. Of note, previous studies relying on alternative assays have revealed the presence of MBP-reactive T cells with activated and/or memory phenotypes at similar high frequencies in not all, but a major portion, of multiple sclerosis patients (Allegretta *et al.*, 1990; Zhang *et al.*, 1994; Bieganowska *et al.*, 1997; Lovett-Racke *et al.*, 1998; Scholz *et al.*, 1998; Illés *et al.*, 1999).

We conducted the same assay with a foreign antigen OVA in three of the '$CD95^+$ NK-high' (PBMC codes #3, #4 and #5 in Table 1) and one of the '$CD95^+$ NK-low' samples (#6). However, OVA-reactive T cells could not be detected in any sample of the fresh or NK-deleted PBMCs (data not shown). Because NK cells cannot discriminate T cells with different antigen specificities, the negative response to OVA in the four multiple sclerosis patients was interpreted to mean that they do not possess clonally expanded memory T cells reactive to OVA.

Depletion of $CD16^+$ NK cells also allows detection of MBP-reactive memory T cells in PBMCs from '$CD95^+$ NK-high' multiple sclerosis

Although we used anti-CD56 magnetic beads to deplete NK cells in the above experiments, the method would also deplete $CD3^+CD56^+$ NK T cells that may possibly play a role in the regulation of autoimmunity. To evaluate the possible contribution of $CD3^+CD56^+$ NK T cells, we next depleted NK cells from PBMCs from two '$CD95^+$ NK-high' patients on the basis of their expression of CD16. We found that after treatment with CD16-MicroBeads, almost all of $CD56^+$ NK cells are deleted, but $CD56^+CD3^+$ NKT cells remain largely untouched (Fig. 3A). However, like $CD56^+$-cell-deleted PBMCs, the $CD16^+$-cell-deleted PBMCs responded to MBP, as assessed by the induction of IFN-γ-secreting $CD4^+$ T cells (Fig. 3B). The responses found in the two patients were considered significant with regard to both percentage increase of IFN-γ-secreting cells (0.08% and 0.04%) and the stimulatory index (9.0 and 5.0) obtained after MBP stimulation. This result indicates that responsible cells to regulate autoimmune T cells in '$CD95^+$ NK-high' multiple sclerosis are not $CD56^+CD3^+$ NK T cells but NK cells.

Unfortunately, it remains unclear whether only $CD95^+$ NK cells play a regulatory role in '$CD95^+$ NK-high' multiple sclerosis or whether $CD95^-$ cells could also exhibit regulatory functions in the patients. We attempted to compare directly the function of $CD95^+$ and $CD95^-$ populations. However, isolation of $CD95^+$ NK cells with a cell sorter invariably induced cell activation as revealed by the expression of various activation markers. Furthermore, the isolated cells tended to die rapidly, probably due to CD95 ligation by the antibody (data not shown).

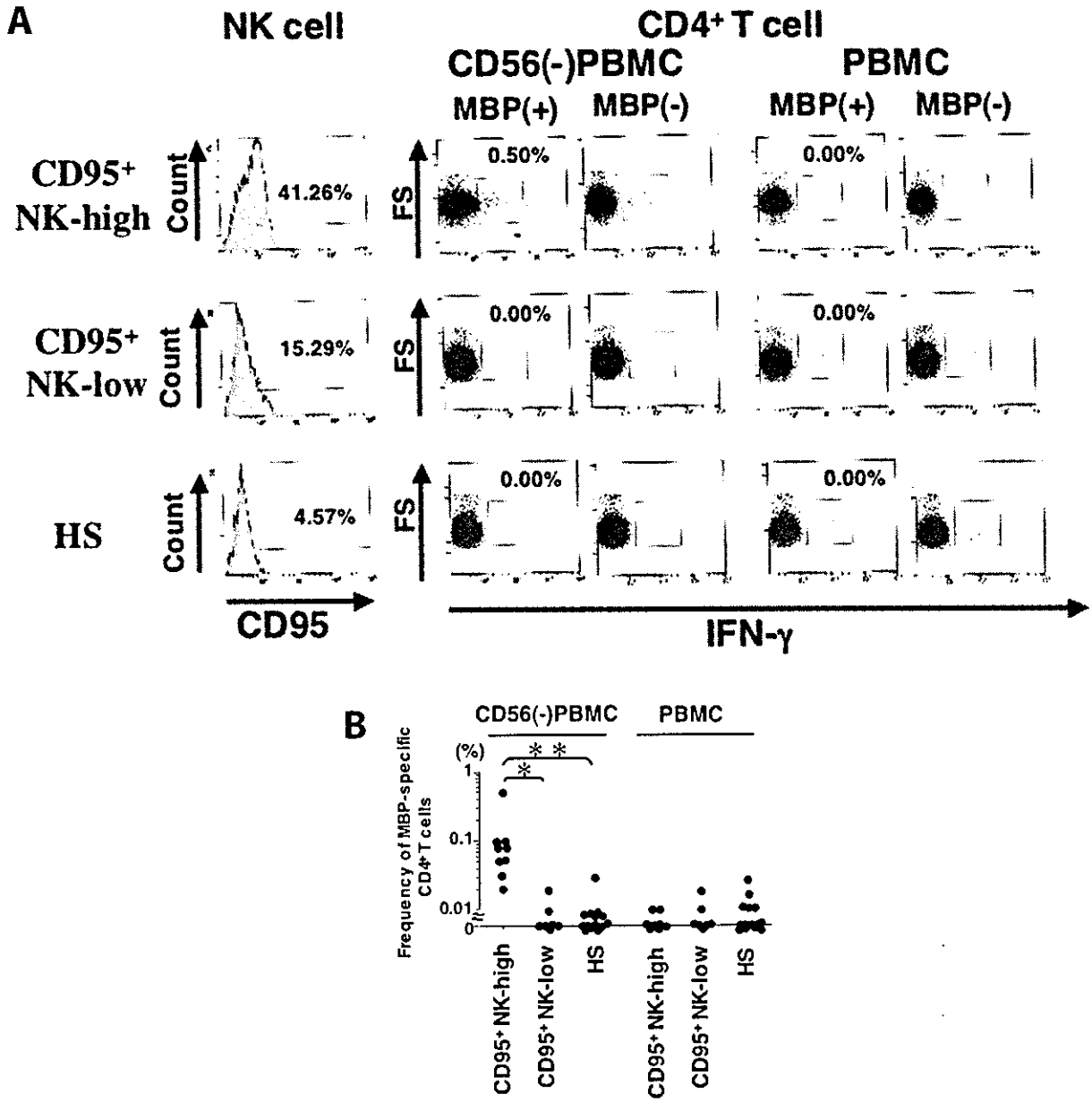

Fig. 2 Evidence for the role of NK cells in the regulation of MBP-reactive memory T cells in 'CD95$^+$ NK-high' multiple sclerosis. (**A**) IFN-γ secretion assay for NK-cell-deleted PBMCs and freshly isolated PBMCs. Whole PBMCs or PBMCs depleted for CD56$^+$ NK cells [CD56(−) PBMC] from the 'CD95$^+$ NK-high' multiple sclerosis (n = 9), 'CD95$^+$ NK-low' multiple sclerosis (n = 7) or healthy subjects (n = 14) were stimulated with 10 μg/ml of human MBP for 8 h for the IFN-γ secretion assay. The cells were also stained with anti-CD4-PC5 and -CD3-FITC, and the CD4$^+$CD3$^+$ and PI$^-$ cells were gated for analysis. Here we show representative results from 'CD95$^+$ NK-high' (*top*), 'CD95$^+$ NK-low' (*middle*) and healthy subjects (*bottom*). The IFN-γ-secreting CD4$^+$ T cells are shown as red dots; blue dots represent IFN-γ-negative cells. The histograms demonstrate the level of CD95 expression on the fresh CD56$^+$ NK cells from each individual, and the attached values show the frequency of CD95$^+$ cells. (**B**) Frequency (%) of MBP-reactive memory T cells among CD4$^+$ T cells. By using the cytokine secretion assay, we determined the frequency of IFN-γ-positive cells among CD4$^+$ T cells in each individual after culture with or without MBP. Here we plot the Δ% values [(%) with MBP − (%) without MBP], which represent the frequency of MBP-reactive CD4$^+$ T cells in each subject. Kruskal–Wallis test with Scheffe's F *post hoc* test was used for statistical analysis. $^*P < 0.05$; $^{**}P < 0.02$.

NK cells from 'CD95$^+$ NK-high' multiple sclerosis inhibit IFN-γ production by MBP-reactive T cell clones

To analyse how the NK cells from 'CD95$^+$ NK-high' multiple sclerosis efficiently control autoimmune T cell responses, we established three MBP-specific TCC from a 'CD95$^+$ NK-high' patient. These TCC proliferated and secreted IFN-γ, TNF-α, IL-2 and IL-5 in response to MBP presented by irradiated, fresh autologous PBMCs. Using the proliferation response and cytokine secretion by

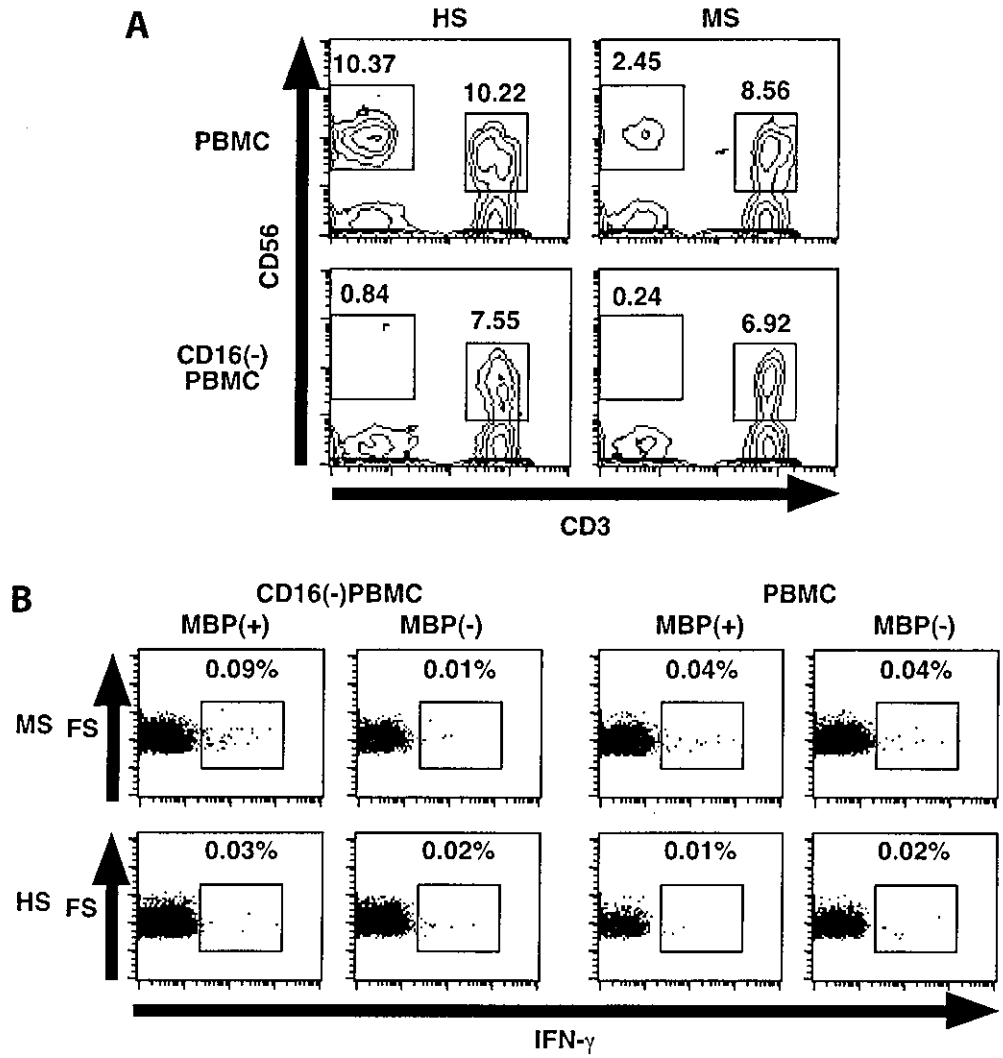

Fig. 3 Depletion of CD16$^+$ cells also allows detection of MBP-specific memory T cells in 'CD95$^+$ NK-high' multiple sclerosis. (**A**) Changes in the frequency of CD56$^+$ NK cells and CD56$^+$ NKT cells after deleting CD16$^+$ cells. Using CD16 microbeads, we deleted CD16$^+$ cells from PBMCs from two 'CD95$^+$ NK-high' patients and from two healthy subjects. The cells were stained with anti-human CD3-FITC and anti-CD56-PC5 to check the proportion of CD56$^+$ NK cells and CD56$^+$ T cells before and after CD16$^+$ cell depletion (upper versus lower panels). Shown are the results of a representative pair of multiple sclerosis and healthy subjects. (**B**) CD16$^+$-cell-depleted PBMCs from 'CD95$^+$ NK-high' multiple sclerosis responded rapidly to MBP. Using the same PBMC samples (CD16$^+$ or CD16$^-$), we conducted the IFN-γ secretion assay as described in Fig. 2A. This figure shows the result of the representative pair of multiple sclerosis patients and healthy subjects.

the TCC as read-out, we compared the whole PBMCs and the NK cell-deleted PBMCs for the ability to present whole MBP to the autologous TCC. We found that the whole PBMCs did not differ from the NK-deleted PBMCs in the ability to induce MBP-driven proliferation of TCC (Fig. 4A). However, the proportion of IFN-γ-secreting T cells among the TCC increased significantly when the NK cell-depleted PBMCs were used as antigen presenting cells (APC) (Fig. 4B). We also noticed a significant elevation of IFN-γ in the culture supernatant along with the increase of IFN-γ-secreting T cells (Fig. 4C). However, neither TNF-α nor IL-2 production was enhanced by NK cell depletion. These results support the view that NK cells from 'CD95$^+$ NK-high' multiple sclerosis regulate autoimmune T cells by inhibiting the T cell production of IFN-γ.

Discussion

It is generally held that relapse of multiple sclerosis represents the destructive CNS inflammation triggered by recently activated autoimmune T cells. In other words, pathogenic autoimmunity is apparently active during clinical relapse, which can be objectively defined by clinical status as well as MRI findings. In contrast, remission of multiple sclerosis, which is chiefly determined by exclusion of active inflammation in the CNS, may probably cover a wider range of disease states.

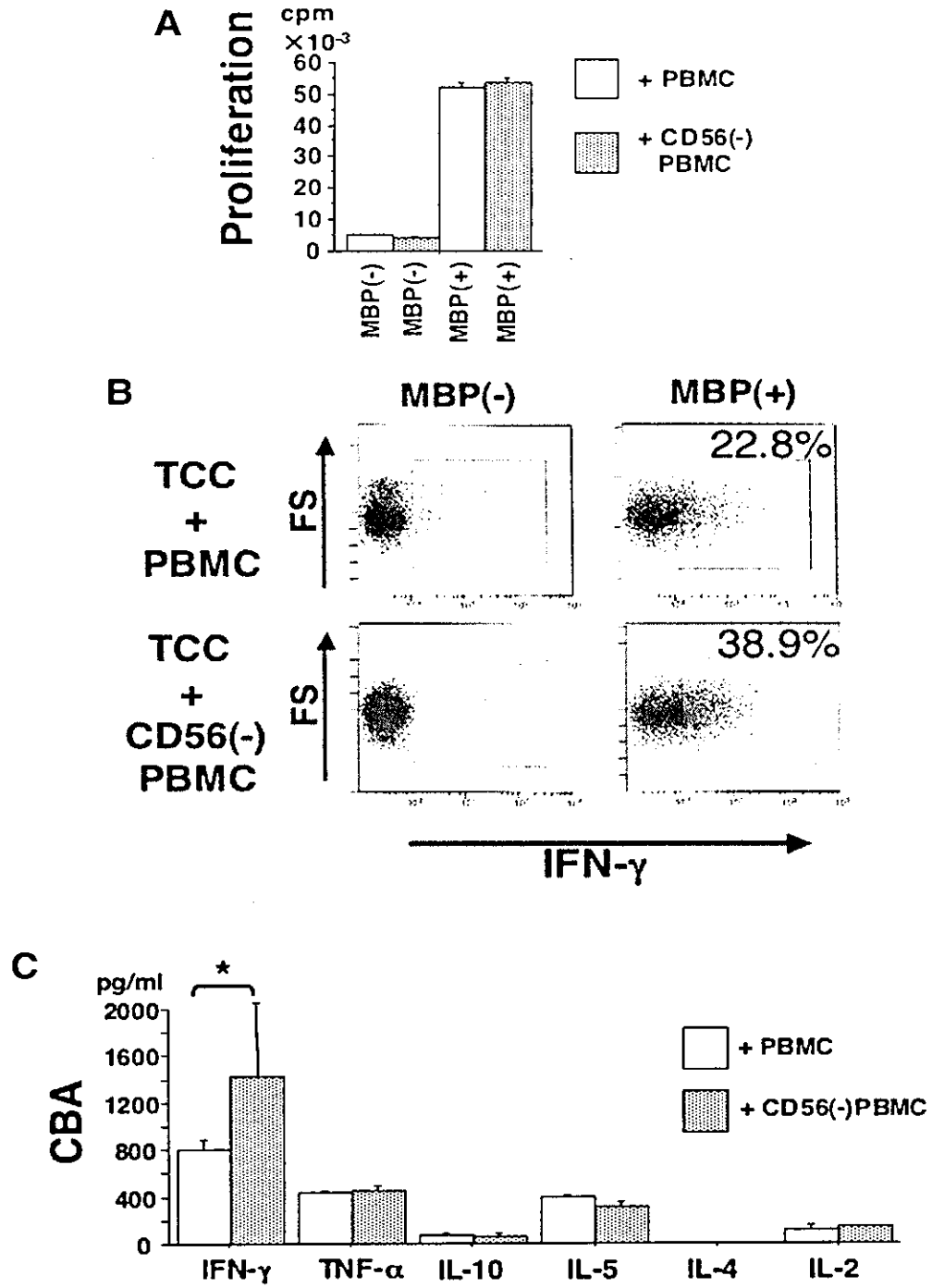

Fig. 4 Depletion of NK cells augments the antigen-presenting potential of PBMCs from 'CD95$^+$ NK-high' multiple sclerosis. (**A**) Effect of NK-cell deletion on the proliferation of MBP-specific TCC. We established three MBP-specific TCC from a 'CD95$^+$ NK-high' patient, and evaluated the proliferative response of the clone cells to MBP (10 μg/ml) in the presence of fresh autologous PBMCs [+ PBMC] or NK-deleted PBMCs [+ CD56 (−) PBMC]. This is a representative result of three TCC, which yielded essentially the same results. Data represent mean ±SD of quadruplicate cultures. (**B**) Effect of NK cell deletion on IFN-γ secretion by the MBP-specific TCC. MBP-specific TCC were cultured with or without MBP for 8 h in the presence of autologous PBMCs (upper panels) or of the autologous PBMCs depleted for CD56$^+$ NK cells (lower panels). We then conducted the cytokine secretion assay to detect IFN-γ-positive cells. Red dots indicate IFN-γ-secreting cells among CD4$^+$CD3$^+$PI$^-$ cells; blue dots represent IFN-γ-negative CD4$^+$CD3$^+$PI$^-$ T cells. The values (%) represent the frequency of IFN-γ-secreting cells among CD4$^+$CD3$^+$PI$^-$ cells. We conducted the assay with three TCC, which yielded essentially the same results. FS = forward scatter. (**C**) Effect of NK cell depletion on cytokine release by TCC into culture medium. The TCC were stimulated with MBP for 48 h in the presence of autologous PBMCs or NK-depleted PBMCs. Then we measured the concentrations of IFN-γ, TNF-α, IL-10, IL-5, IL-4 and IL-2 in the supernatants, using ELISA and CBA. Both assays yielded essentially the same results, and here we show the result of a CBA assay. Data represent mean ± SD. The Mann–Whitney *U*-test was used for statistical analysis. *$P < 0.05$. We conducted the assay with three TCC, which yielded essentially the same results.

The present results show that multiple sclerosis patients in remission can be divided at least into two subgroups, 'CD95$^+$ NK-high' and 'CD95$^+$ NK-low', based on the frequency of CD95$^+$ cells among NK cells. Furthermore, our functional analysis combining NK cell deletion and stimulation with MBP has indicated that the two subgroups differ significantly with regard to the responsiveness of the MBP-specific memory T cells to MBP in the absence of NK cells. Namely, after deleting CD56$^+$ NK cells, we saw a rapid induction of IFN-γ-secreting, anti-MBP T cells in 'CD95$^+$ NK-high' multiple sclerosis, whereas such a rapid response to MBP was not seen in 'CD95$^+$ NK-low' multiple sclerosis or healthy subjects. This result is in harmony with the previous results that clonally expanded MBP-specific T cells can be detected in a majority of multiple sclerosis patients (Zhang *et al.*, 1994; Smeltz *et al.*, 1999), and indicates that patients with an increased number of the autoimmune T cells may have the 'CD95$^+$ NK-high' phenotype during remission. Thus, the frequency of CD95$^+$ NK cells correlates with the frequency of MBP-reactive memory T cells and may serve as a useful marker to evaluate the immunological status of multiple sclerosis during remission.

The role of NK cells in the regulation of MBP-specific T cells was further strengthened by the demonstration that deletion of CD16$^+$ cells also enabled detection of memory MBP-specific T cells. Because we confirmed that depletion of the CD16$^+$ cells would greatly reduce the number of NK cells but did not significantly reduce CD56$^+$CD3$^+$ NK T cells, the role of the NK T cells in the regulation was excluded.

We have previously described that the 'CD95$^+$ NK-low' phenotype could also be seen in multiple sclerosis patients during relapse. However, the 'CD95$^+$ NK-low' phenotype in MS-rel was not persistent, but the 'CD95$^+$ NK-high' phenotype could be regained in a month or so along with clinical recovery. This fact raised the possibility that 'CD95$^+$ NK-low' MS-rem may represent an active state of multiple sclerosis, contrary to our speculation. To evaluate this possibility, we examined three patients with MS-rem for the 'CD95$^+$ NK-high/low' phenotype every 4–6 weeks, and found that they maintained the 'CD95$^+$ NK-low' phenotype for longer than several months (data not shown). This is in a striking contrast to the transient appearance of the 'CD95$^+$ NK-low' phenotype during relapse. Together with the clinical observations that these patients were in a very stable condition with minimal neurological disability, we estimate the disease condition in 'CD95$^+$ NK-low' MS-rem to be truly inactive and distinct from MS-rel.

It is of note that IFN-γ-secreting T cells could be identified as early as 8 h after stimulation with MBP in the absence of NK cells. This result implies that the NK cells should interact with the autoimmune T cells shortly after antigen stimulation to regulate very early T cell response. To account for such a rapid regulation by NK cells, we speculate that the regulatory NK cells may detect the subtle change of the autoimmune T cells during the early stage of activation. At present, very little is known about the molecular basis of T cell–NK cell interaction. However, it is obvious that NK cells must interact with T cells in an antigen-non-specific fashion, as they do not express highly variable receptors like T cell antigen receptors. Our results indicate that attempts to identify the ligand and receptors involved in T cell–NK interactions are very rewarding.

It is currently speculated that activation of autoimmune T cells could occur in response to microbial proteins whose sequence has a significant homology to the self-peptide (Steinman, 2001). We predict that the increased MBP-reactive Th1 cells in the 'CD95$^+$ NK-high' patients will most likely respond to microbial peptides mimicking MBP from time to time. However, counter-regulatory NK cells would maintain the clinical silence by actively suppressing activation of the autoimmune T cells that might lead to destructive CNS inflammation (Fig. 5). We then imagine that the clinical silence in the 'CD95$^+$ NK-high' patients could readily be disrupted when NK cells are numerically or functionally altered by exogenous or endogenous factors independent of multiple sclerosis (Wu *et al.*, 2000). In contrast, the clinical remission in 'CD95$^+$ NK-low' multiple sclerosis appears to be stable, as they are expected to possess much lower numbers of MBP-specific memory T cells, which does not necessitate the active

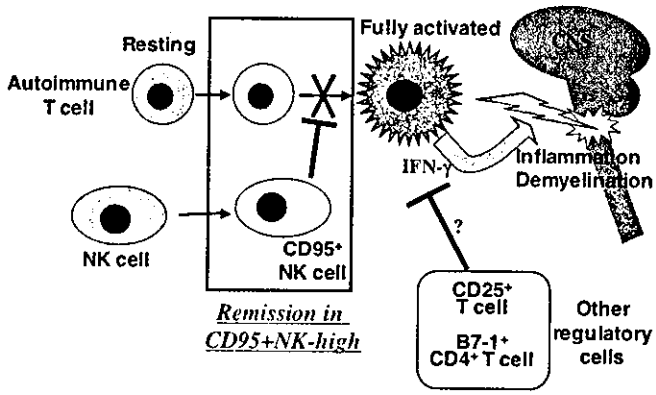

Fig. 5 The role of NK cells in 'CD95$^+$ NK-high' multiple sclerosis. As described in the text, the 'CD95$^+$ NK-high' patients are characterized by a concurrent increase of memory autoimmune T cells and CD95$^+$ NK cells. In the sense that memory autoimmune T cells cannot be detected in other patients in remission ('CD95$^+$ NK-low') even after NK cell depletion, we describe the immunological status of the 'CD95$^+$ NK-high' as a 'smouldering' state rather than 'remission'. Given that T cell recognition is much more promiscuous than previously anticipated, we imagine that autoimmune T cells in the 'CD95$^+$ NK-high' patients would respond to exogenous self-mimicking peptides from time to time. However, our results indicate that the CD95$^+$ NK cells could detect the early sign of T cell activation and then interact with autoimmune T cells to prohibit their full activation. Once this delicate control by NK cells is disrupted, the autoimmune T cells could be fully activated in response to the self-mimicking peptides. The fully activated T cells may be controlled by other regulatory cells such as CD4$^+$CD25$^+$ T cells (Sakaguchi *et al.*, 2001) or B7-1$^+$CD4$^+$ T cells (Kipp *et al.*, 2000). However, it is difficult to predict how efficiently the regulatory T cells may control the activated autoimmune T cells in individual cases.

engagement of regulatory NK cells. If these premises hold true, we may consider that the 'CD95$^+$ NK-high' patients are at a greater risk than 'CD95$^+$ NK-low' of developing relapses when exposed to potentially dangerous microbes that have cross-reactive epitopes. To describe the immunological status in 'CD95$^+$ NK-high', which seems to be more active than the 'CD95$^+$ NK-low', it might be appropriate to use the term 'smouldering' state rather than 'remission'.

After determining the presence of the 'CD95$^+$ NK-high' and 'CD95$^+$ NK-low' phenotypes in the patients with MS-rem, an important question might be whether the 'CD95$^+$ NK-high/-low' phenotype correlates with some clinical parameters or disease course. We speculated that 'CD95$^+$ NK-low' might be clinically less active than 'CD95$^+$ NK-high', when evaluated retrospectively. However, it might take time and would require a large number of patients to verify this postulate, taking the heterogeneity and chronic nature of the illness into consideration. Furthermore, it is of note that the 'CD95$^+$ NK-high' or '-low' phenotype appears to be interchangeable. For example, two of the patients who were examined for the memory T cell frequency showed the 'CD95$^+$ NK-low' phenotype in the first examination, but were found to have the 'CD95$^+$ NK-high' phenotype when examined 1 year later (Table 1). The phenotype switch in these patients was associated with an increase in the frequency of MBP-reactive memory T cells. We speculate that activity of multiple sclerosis may have been increased in these patients during the 1-year interval, although it is too early to draw any conclusions from the analysis of two patients.

Conversely, we have recently seen an opposing phenotype switch (from the 'CD95$^+$ NK-high' to 'CD95$^+$ NK-low') in two other patients. The frequency of CD95$^+$ cells among NK cells was >46.0% in both cases in the initial examinations, but the latest test showed normal values (27.4% and 10.0%). Although the patients appeared to be in the state of remission at the last examination, they developed serious signs of acute exacerbation 2 days later. As stated above, a transient switch from 'CD95$^+$ NK-high' to 'CD95$^+$ NK-low' could occur during relapse. Therefore, we speculate that the phenotype switch from 'high' to 'low' may be triggered by the very early events leading to clinical relapse. However, it is also possible that the reduction of the CD95$^+$ NK cells might have been triggered by multiple sclerosis-independent factors, such as infection or stress, and that this led to the occurrence of the relapse in these patients. This speculation is supported by the fact that a number of physiological conditions can alter NK cell number and/or function, and that CD95$^+$ NK cells tend do die more rapidly in culture than CD95$^-$ NK cells (our unpublished data). In future, it will be worthwhile to examine more systematically whether the phenotype switch may be the earliest marker to detect occurrence of relapse.

As Japanese neurologists have traditionally stressed that multiple sclerosis in Japan might be quite unique in immunopathology, it is theoretically possible that the regulatory function of CD95$^+$ NK cells reflects the uniqueness of Japanese multiple sclerosis and that the T cell–NK cell interaction is not operative in Caucasian multiple sclerosis. However, recent studies suggest that the frequency of pure optic-spinal form of multiple sclerosis linked with Japanese patients (Misu *et al.*, 2002) is drastically declining, possibly due to change in life-style or environmental factors in Japan (Yamamura, 2002; Houzen *et al.*, 2003). Reflecting this fact, the patients randomly recruited in this study did not have optic-spinal multiple sclerosis, and all had brain lesions similar to those found in Western multiple sclerosis. We therefore speculate that our experimental results will be reproduced in Caucasian patients in the future.

In summary, we have revealed that multiple sclerosis patients in remission have either 'CD95$^+$ NK-high' or 'CD95$^+$ NK-low' phenotype, and that 'CD95$^+$ NK-high' patients have a higher frequency of memory autoimmune T cells and have more active multiple sclerosis than 'CD95$^+$ NK-low' patients. Our *ex vivo* assay has demonstrated that 'CD95$^+$ NK-high' patients possess NK cells that actively inhibit activation of memory autoimmune T cells. In the sense that clinical silence depends on the functional regulatory NK cells, the condition of 'CD95$^+$ NK-high' is thought to be so unstable, as could be expressed by the term 'smouldering'. As such, evaluation of the NK cell functions and phenotypes in multiple sclerosis gives us a new insight into the autoimmune pathogenesis of multiple sclerosis, encouraging further efforts to clarify the NK cell–T cell interactions.

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Natural killer T cells accelerate atherogenesis in mice

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We have investigated the potential role of CD1d-restricted natural killer T (NKT) cells in the development of atherosclerosis in mice. When fed an atherogenic diet (AD), NKT cell–deficient CD1d$^{-/-}$ mice had significantly smaller atherosclerotic lesions than AD-fed C57BL/6 (wild-type [WT]) mice. A significant reduction in atherosclerotic lesions was also demonstrated in AD-fed, low-density lipoprotein receptor–deficient (Ldlr$^{-/-}$) mice reconstituted with CD1d$^{-/-}$ bone marrow cells compared with the lesions observed in Ldlr$^{-/-}$ mice reconstituted with WT marrow cells. In addition, repeated injections of α-GalCer or the related glycolipid OCH to apolipoprotein E knockout (apoE$^{-/-}$) mice during the early phase of atherosclerosis significantly enlarged the lesion areas compared with mice injected with vehicle control. However, administering α-GalCer to apoE$^{-/-}$ mice with established lesions did not significantly increase the lesion area but considerably decreased the collagen content. Atherosclerosis development in either AD-fed WT or apoE$^{-/-}$ mice was associated with the presence of Vα14Jα18 transcripts in the atherosclerotic arterial walls, indicating that NKT cells were recruited to these lesions. Thioglycolate-elicited macrophages pulsed with oxidized low-density lipoproteins expressed enhanced CD1d levels and induced NKT cells to produce interferon-γ, a potentially proatherogenic T-helper 1 (T_H1) cytokine. Collectively, we conclude that NKT cells are proatherogenic in mice. (Blood. 2004;104:2051-2059)

Introduction

Atherosclerosis is an inflammatory vascular disease that involves components of the innate and acquired immune systems.[1-3] Several studies have suggested that lymphocytes, which are detected in atherosclerotic lesions in humans and mice,[4,5] play a proatherogenic role.[6-8] Recently, the role of distinct lymphocyte subsets in the development of atherosclerosis has been evaluated. For example, emerging evidence indicates that T-helper 1 (T_H1) cells are proatherogenic,[9] whereas T_H2 cells are antiatherogenic.[10,11] These observations are further supported by the finding that T_H1 cytokines (eg, interferon-γ [IFN-γ and interleukin-12 [IL-12]) are important in the progression of atherosclerosis[12-15] and that, among T_H2 cytokines, IL-10 is antiatherogenic.[16] On the other hand, recent studies have suggested that B cells play a protective role in atherogenesis.[17,18]

Natural killer T (NKT) cells are a unique subset of lymphocytes that have surface markers and functions of T cells and NK cells.[19-23] Several characteristics of NKT cells suggest that they may play a role in the atherogenic process. Most NKT cells express an invariant Vα14Jα18 T-cell receptor (TCR)–Vα chain paired with a restricted set of TCR-Vβ chains. These classical NKT cells recognize lipid antigens presented by the major histocompatibility complex (MHC) class 1–like molecule CD1d, produce copious amounts of IFN-γ and IL-4 on activation,[22] and constitutively express Fas-ligand.[23] Moreover, NKT cells play a protective role in several autoimmune diseases, infections, and tumor progression/metastasis.[20] Protective effects of NKT cells and their ligands in autoimmunity are largely attributed to their capacity to promote T_H2 immune responses.[24,25] However, in some situations, NKT cells can contribute to the development of T_H1 immune responses as well.[26] Therefore, it was difficult to predict whether NKT cells would play a proatherogenic or an antiatherogenic role.[2]

To date, few studies have investigated the role of CD1d and CD1d-dependent T cells in atherogenesis. CD1d-expressing cells are present in human atherosclerotic plaques,[27] suggesting that NKT cells may be recruited to the lesions. Furthermore, treatment of apolipoprotein E knockout (apoE$^{-/-}$) mice,[28] a model of severe

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atherosclerosis, with lipopolysaccharide (LPS) resulted in NKT cell recruitment to the atherosclerotic plaques.[29] However, whether NKT cells are directly involved in the development or the regulation of atherosclerosis remains to be investigated.

In the present study, we compared atherosclerotic lesions induced by an atherogenic diet (AD) between NKT cell–deficient $CD1d^{-/-}$[30] and wild-type C57BL/6 (WT) mice and between low-density lipoprotein receptor–deficient ($Ldlr^{-/-}$) mice[31] reconstituted with bone marrow (BM) cells from $CD1d^{-/-}$ mice and $Ldlr^{-/-}$ mice reconstituted with BM of WT mice. Moreover, we examined whether NKT cell ligands (α-galactosylceramide[32] (α-GalCer) and OCH[33]) could modulate atherogenesis in $apoE^{-/-}$ mice. Our findings consistently demonstrated that NKT cells played a proatherogenic role. Possible mechanisms underlying the proatherogenic role of NKT cells are discussed.

Materials and methods

Glycolipids

α-GalCer (Pharmaceutical Research Laboratories, Kirin Brewery, Gunma, Japan) and OCH were dissolved in either 0.5% polysorbate-20 at 220 μg/mL or dimethyl sulfoxide at 100 μg/mL, respectively, and were further diluted with phosphate-buffered saline (PBS) before use.

Mice

Female WT (Japan SLC, Hamamatsu, Japan), $CD1d^{-/-}$[30] (Vanderbilt University, Nashville, TN), $Jα18^{-/-}$[34] (Chiba University, Chiba, Japan), $Ldlr^{-/-}$, and $apoE^{-/-}$ (The Jackson Laboratory, Bar Harbor, ME) mice with the C57BL/6 genetic background were used throughout the study. WT and $CD1d^{-/-}$ mice were fed a regular chow diet or the atherogenic diet (AD) (15% fat, 1.25% cholesterol, and 0.5% cholic acid; Nihon-nohsan, Yokohama, Japan) from 10 to 30 weeks of age. All animal care and experimental procedures conformed to the regulations of the Committee of Hokkaido University on Animal Experimentation.

BMT

Bone marrow transplantation (BMT) was performed with lethally irradiated (9.5 Gy) $Ldlr^{-/-}$ mice as recipients, as previously described.[35] Briefly, recipient mice were injected with T cell–depleted BM cells (5×10^6) from WT mice (Thy1.1 in BMT protocol, referred to as [WT→$Ldlr^{-/-}$]), $CD1d^{-/-}$ mice ([$CD1d^{-/-}$→$Ldlr^{-/-}$]), or $Ldlr^{-/-}$ mice ([$Ldlr^{-/-}$→$Ldlr^{-/-}$]). Treated mice were administered oxytetracyclin (Pfizer Japan, Tokyo, Japan) in drinking water for 4 weeks and then placed on the AD for 5 weeks. Reconstitution was assessed by evaluating thymocyte expression of CD1d for $CD1d^{-/-}$ donors and both Thy1.1 (donor) and Thy1.2 (recipient) for WT donors using flow cytometry.

Induction of atherosclerotic lesions

Early-phase studies. $ApoE^{-/-}$ mice were divided into 4 groups (n = 10 each): 1 group received intraperitoneal (intraperitoneal) injections of 0.1 μg/g body weight (BW) α-GalCer; 1 group received its vehicle; and the remaining groups were administered 0.3 μg/g BW OCH or its vehicle, respectively. Injections were started at 8 weeks of age and repeated every 2 weeks. At 13 weeks of age, mice were killed and used for experiments. Blood samples were consecutively collected from the retro-orbital plexus at 0, 2, 5, 12, 24, 48, and 72 hours after injection of either α-GalCer or OCH, and the levels of IFN-γ and IL-4 were quantitated using enzyme-linked immunosorbent assay (ELISA; Biosource, Camarillo, CA).

Late-phase studies. Ten mice received intraperitoneal injections of 0.1 μg/g BW α-GalCer or vehicle every week starting from 8 weeks of age. One week after the eleventh injection, mice were killed and used for experiments.

Serum chemistry

Amounts of total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglyceride concentrations in sera were determined with colorimetric assay kits (Kyowa Medex, Tokyo, and Serotekku, Sapporo, Japan). Individual serum alanine aminotransferase and total bilirubin were quantitated using the Fuji Drychem system (Fujifilm Medical, Osaka, Japan).

Quantitative analyses of atherosclerotic lesion areas

Atherosclerotic lesions were analyzed as previously described.[35] In brief, the basal portion of the heart and proximal aortic root were excised and embedded in OCT compound and frozen in liquid nitrogen. Eight serial cryosections of 10-μm thickness at 80-μm intervals throughout the aortic sinus were stained with oil red O (Sigma, St Louis, MO) and hematoxylin. Lesion images were captured with an Olympus BX50 microscope (Tokyo, Japan) equipped with a Fujix HC-300Z/OL digital camera (Fijifilm, Kanagawa, Japan) and Photograb-300 SH-3 software (Fujifilm). Captured images were further analyzed with Scion Image software (Scion, Frederick, MD). For advanced lesions, the entire aorta was examined using the en face method, as described elsewhere.[36]

Characterization of atherosclerotic lesions

Immunohistochemistry was performed on 8-μm thick cryosections, as previously described.[37] Rat monoclonal antibodies (mAbs) to mouse macrophages (MOMA-2; Serotec, Oxford, United Kingdom), hamster antimouse CD3 (BD Biosciences, San Jose, CA), anti–α-smooth muscle actin (DAKO, Glostrup, Denmark), rat antimouse IFN-γ (BioSource), rat antimouse IL-10 (Endogen, Woburn, MA), biotinylated secondary antibodies to the respective primary reagents, and streptavidin–horseradish peroxidase (DAKO) were used for detection. Signals were developed with DBA kits (Vector Laboratories, Burlingame, CA). The number of $CD3^+$ cells per cross-section of lesion area was counted at × 400 magnification. Elastica-Masson staining was performed to analyze the composition of the lesion using 3 aortic cross-sections per animal from 10 animals. The percentage of collagen-rich matrix areas among the total lesion areas was defined as collagen contents. Total cell numbers per lesion were also counted.

RT-PCR

WT (fed the chow diet or the AD), $apoE^{-/-}$, and $Jα18^{-/-}$ mice were killed after overnight fasting. After whole body perfusion with cold RNase-free PBS, aortae from the ascending portion to the end of the thoracic aorta were removed, dissected longitudinally, and washed meticulously in cold PBS to remove attached hematocytes and tissue fragments outside the aortae. RNA extraction and reverse transcription–polymerase chain reaction (RT-PCR) were performed as described previously.[38]

Flow cytometry

Splenocytes were prepared by lysing red blood cells with Tris-NH_4Cl solution. Hepatic mononuclear cells (HMNCs) were isolated using 33% Percoll (Amersham Pharmacia Biotech, Piscataway, NJ), as previously reported.[39] Cells were incubated with 2.4G2 mAb (anti-FcγR) to block nonspecific staining and were stained with a combination of the following mAb conjugates: for lymphocytes—biotinylated anti-Thy1.1 (OX7), fluorescein isothiocyanate (FITC) anti-Thy1.2 (Coulter, Miami, FL), anti-CD1d (1B1), anti-TCRβ (H57-597), and phycoerythrin (PE) anti-NK1.1 (PK136) (all from BD Biosciences, except Thy1.2); for macrophages—biotinylated anti-$H\text{-}2K^b$ (AF6-88.5), anti-$I\text{-}A^b$ (AF6-120.1), anti-CD40 (3/23), and antimouse (BALB/c) immunoglobulin G2aκ (IgG2aκ) (G155-178; BD Biosciences), FITC anti-CD1d (1B1) and -rat IgG2b (LODNP57; Immunotech, Marseille, France), and PE anti–Mac-1 (CL8941; Cedarlane, Hornby, Ontario, Canada). Streptavidin-allophycocyanin (APC) (BD Biosciences) was used for detection of biotinylated mAb. Mouse CD1d/α-GalCer tetramers were prepared as previously described.[40] Cells were incubated with FITC anti-TCRβ and PE anti-NK1.1 and then with APC α-GalCer–loaded CD1d tetramers. Propidium iodide (Sigma) positive cells were

electronically gated out from the analysis, and stained cells were analyzed using a FACSCalibur flow cytometer, as described elsewhere.[38]

In vitro culture of splenocytes from AD- or chow-fed WT mice treated with α-GalCer

Splenocytes were obtained from either AD- or chow-fed WT mice 2 to 12 hours after intravenous injection with 0.1 μg/g BW α-GalCer. Cells were suspended in RPMI 1640 supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 5×10^{-5} M 2-mercaptoethanol (culture medium) and were cultured in 24-well plates at 5×10^6/mL for 1.5 hours without additional stimulation. Culture supernatants were harvested and quantitated for IL-4 levels with ELISA kits (Biosource) and for IFN-γ and IL-10 with Cytometric Bead Array kits (BD Biosciences) by flow cytometry, according to the manufacturer's instructions.

Response of HMNCs to oxidized low-density lipoprotein in vitro

Peritoneal cells were harvested from young WT or CD1d$^{-/-}$ mice 4 days after intraperitoneal injection of 4.05% thioglycolate. Cells were suspended at a concentration of 2×10^6/mL in culture medium, incubated at 37°C for 24 to 48 hours with LDL, oxidized LDL (OxLDL) (10 and 50 μg/mL; Biomedical Technologies, Stoughton, MA), or vehicle alone, and used for flow cytometric analysis. For cytokine analysis, the peritoneal cells (2×10^5/well) were cultured in 96-well plates at 37°C for 2 hours and were washed to remove nonadherent cells. Adherent cells were incubated at 37°C for 48 hours with LDL or OXLDL. After incubation, each well was washed 3 times, and the adherent macrophages were irradiated with 30 Gy x-rays. HMNCs isolated from WT mice (2×10^5/well) were cultured with these macrophages in the presence of recombinant human IL-2 (1000 U/mL; Takeda Chemical Industries, Osaka, Japan) for 24 hours. The supernatant was quantitated for IFN-γ and IL-4 levels with ELISA kits (Biosource).

Statistical analysis

Results were expressed as mean ± SE. Statistical analysis was performed using the Student *t* test or the Mann-Whitney *U* rank sum test. All data analyses were performed using Statview software (Abacus Concepts, Berkeley, CA). Values with $P < .05$ were considered statistically significant.

Results

Development of atherosclerotic lesions in CD1d$^{-/-}$ mice

WT (n = 20) and CD1d$^{-/-}$ (n = 18) mice were fed on the AD for 20 weeks. All mice on the AD appeared generally to be in good health throughout the study except for the development of diet-induced liver steatosis and its consequential liver damage. When the sizes of atherosclerotic lesions in aortae were compared, the lesions in CD1d$^{-/-}$ mice were smaller than those in WT mice (Figure 1A). Mean lesion areas in CD1d$^{-/-}$ mice (2292 ± 397 μm^2) were significantly smaller than those in WT mice (5627 ± 580 μm^2) ($P = .014$) (Figure 1B). These findings demonstrate that CD1d deficiency reduces atherosclerotic lesions. Concerning serum lipid profiles, total cholesterol, HDL cholesterol, and triglyceride levels were not significantly different between WT (137.9 ± 8.0 mg/dL, 36.3 ± 1.6 mg/dL, and 57.5 ± 3.4 mg/dL, respectively) and CD1d$^{-/-}$ (140.2 ± 14.7 mg/dL, 38.1 ± 2.4 mg/dL, and 59.9 ± 4.4 mg/dL, respectively) mice. Histologic findings of liver sections stained with hematoxylin and eosin revealed typical steatosis to similar extents for WT and CD1d$^{-/-}$ mice fed on the AD (data not shown). Serum alanine aminotransferase and total bilirubin levels in WT and CD1d$^{-/-}$ mice also decreased within similar levels (WT, 111.3 ± 6.9 U/L, 0.6 ± 0.1 mg/dL; CD1d$^{-/-}$, 108.2 ± 14.5 U/L, 0.6 ± 0.1 mg/dL).

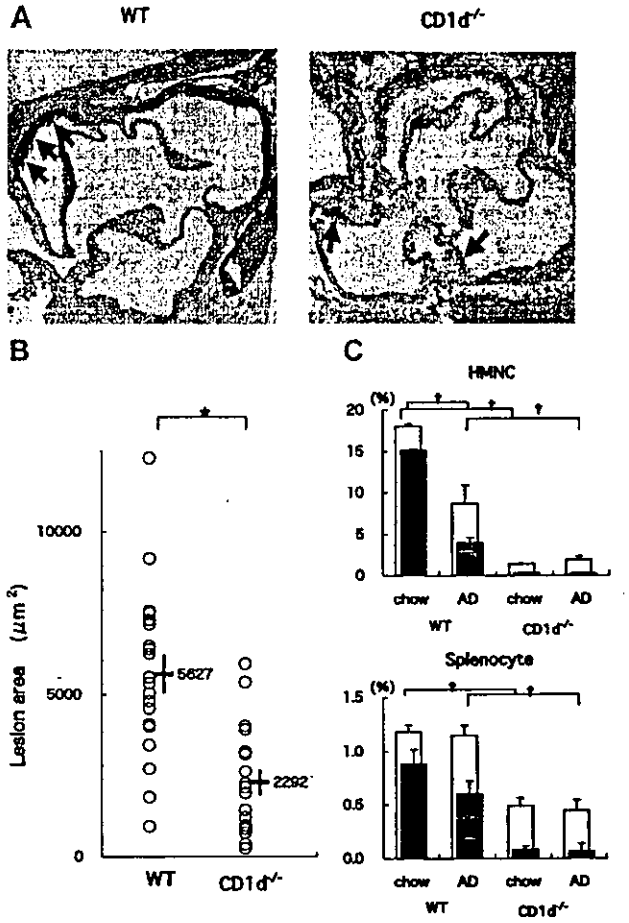

Figure 1. Atherosclerotic lesion areas in WT and CD1d$^{-/-}$ mice fed on the AD. (A) Representative histologic sections of WT and CD1d$^{-/-}$ mice fed on the AD. Arrows represent the oil red O–positive atherosclerotic lesions typically observed within the internal elastic lamina (original magnification, × 40). (B) Mean lesion areas of WT and CD1d$^{-/-}$ mice. Each symbol represents the lesion area of an individual mouse. Horizontal bars and numbers represent the mean of all mice within each group, and vertical bars represent SEM. (C) Prevalence of NKT cells in WT and CD1d$^{-/-}$ mice. HMNCs and splenocytes were prepared and stained with FITC anti-TCRαβ, PE anti-NK1.1, and APC-CD1d/α-GalCer tetramer, as described in "Materials and methods." Open columns represent the proportion of total NKT cells, and closed columns represent the proportion of CD1d/α-GalCer tetramer$^+$ cells. Each value represents the mean ± SE calculated from more than 5 experiments. Statistical analyses were performed with the Mann-Whitney *U* test. †$P < .01$ (for closed columns and open columns); *$P < .05$.

Flow cytometric analyses of NKT cells

Using flow cytometry, we analyzed NKT cells in the liver, spleen, and peripheral blood of WT mice fed either the chow diet or the AD. NK1.1$^+$TCRβ^{int} (ie, NKT) cells represented 18.1% ± 2.6% of the HMNCs of WT mice on the chow diet (Figure 1C, top panel). Among NK1.1$^+$TCRβ^{int} cells, 84.2% ± 4.1% stained with α-GalCer–loaded CD1d tetramers. It should be noted that the mean proportion of total NK1.1$^+$TCRβ^{int} cells in HMNCs of AD-fed WT mice (8.7% ± 2.3%) was significantly lower than that in chow-fed WT mice ($P = .009$). This was attributed to the considerable reduction of CD1d/α-GalCer tetramer$^+$ cells in AD-fed mice. Proportions of CD1d/α-GalCer tetramer$^-$ cells remained unaltered among chow- and AD-fed animals. Similarly, a mild reduction in the prevalence of tetramer$^+$ NKT cells among splenocytes of AD-fed mice was noted (Figure 1C, bottom panel; $P = .07$), but the proportion of total NK1.1$^+$TCRβ^{int} cells was unchanged. In CD1d$^{-/-}$ mice, the proportion of NK1.1$^+$TCRβ^{int} HMNCs was markedly smaller than that in WT mice, and tetramer$^+$ cells were not detected. Of note, the proportion of NK1.1$^+$TCRβ^{int} cells in CD1d$^{-/-}$ mice was unaffected by

AD feeding (1.5% ± 0.3% on the chow diet compared with 1.9% ± 0.4% on the AD). Similar results were obtained with splenocytes of CD1d$^{-/-}$ mice. No significant changes were seen in conventional T-cell subsets (CD4$^+$, CD8$^+$), γδ T cells, and NK cells by AD feeding in WT and CD1d$^{-/-}$ mice (data not shown).

Production of cytokines by splenocytes from AD- or chow-fed WT mice treated with α-GalCer

Our results indicate that AD feeding quantitatively and qualitatively alters the Vα14 NKT cell population of WT mice. One hallmark of NKT cells is their capacity to rapidly produce cytokines on TCR engagement.[22,41] To examine whether AD feeding influences the functional status of NKT cells, we administered a synthetic glycolipid, α-GalCer, to AD- or chow-fed WT mice, and 2 or 12 hours later we measured the amounts of IFN-γ, IL-4, and IL-10 produced by splenocytes in vitro. Two hours after α-GalCer injection, IFN-γ levels were significantly higher in AD-fed WT splenocytes than in chow-fed WT splenocytes ($P = .034$) (Figure 2). In contrast, IL-4 and IL-10 levels were significantly lower in AD-fed WT splenocytes than in chow-fed WT splenocytes ($P = .021$, $P = .047$, respectively). At 12 hours, IFN-γ levels were slightly higher in AD-fed WT splenocytes ($P = .094$), amounts of IL-4 decreased to undetectable levels in both groups, and IL-10 levels were still significantly lower in AD-fed WT splenocytes ($P = .009$). Experiments using HMNCs from AD- and chow-fed WT mice showed similar results (data not shown). Because NKT cells (particularly CD1d/α-GalCer tetramer$^+$ NKT cells) were decreased in AD-fed WT mice (Figure 1C), these findings indicate that NKT cells in AD-fed WT mice exhibit an enhanced capacity to produce cytokines, especially IFN-γ. It should be noted that AD feeding of WT mice shifted the cytokine production pattern in response to α-GalCer stimulation toward a T_H1 profile. Importantly, it has been reported that T_H1 responses are proatherogenic.[10-14]

Development of atherosclerosis in Ldlr$^{-/-}$ mice reconstituted with BM cells from CD1d$^{-/-}$ or WT mice

Next, to examine whether NKT cell deficiency is directly related to the reduction of atherosclerotic lesions, [WT→Ldlr$^{-/-}$] and [CD1d$^{-/-}$→Ldlr$^{-/-}$] BM chimeric mice (n = 7 in each group) were prepared. Four weeks after BMT, almost all thymocytes from [CD1d$^{-/-}$→Ldlr$^{-/-}$] chimeras used in these experiments were CD1d$^-$ and, thus, of donor origin (Figure 3A). In addition,

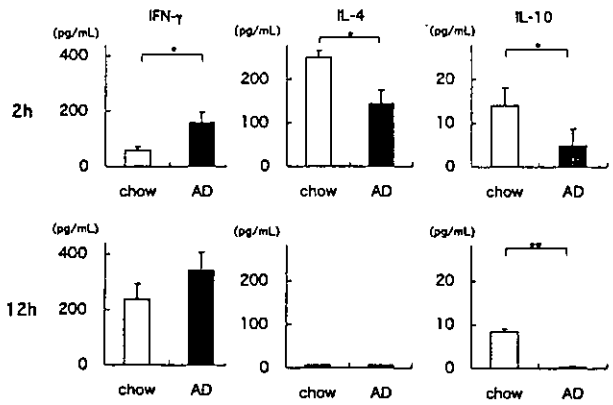

Figure 2. Production of cytokines by splenocytes from AD- or chow-fed WT mice treated with α-GalCer. Splenocytes were obtained from either AD- or chow-fed WT mice 2 or 12 hours after intravenous injection with 0.1 μg/g BW α-GalCer. Cells were cultured for 1.5 hours without additional stimulation. Culture supernatants were harvested, and IFN-γ, IL-4, and IL-10 levels were quantitated. Values are mean ± SE. Statistical analyses were performed using the Mann-Whitney *U* test. $^*P < .05$; $^{**}P < .01$.

thymocytes from [WT→Ldlr$^{-/-}$] chimeras were mostly Thy1.1$^+$ (donor) (donor chimerism = 99.0% ± 0.81%). AD feeding for 5 weeks led to similar levels of hypercholesterolemia in both groups (total cholesterol or HDL cholesterol, [WT→Ldlr$^{-/-}$]: 2147 ± 144 mg/dL or 15.0 ± 3.5 mg/dL; [CD1d$^{-/-}$→Ldlr$^{-/-}$]: 2207 ± 119 mg/dL or 15.9 ± 1.6 mg/dL, respectively). However, the atherosclerotic lesions in [CD1d$^{-/-}$→Ldlr$^{-/-}$] mice were significantly smaller than those in [WT→Ldlr$^{-/-}$] mice (Figure 3C-D). Immunohistochemistry revealed that the main components of the lesions were MOMA-2$^+$ macrophages in both groups (Figure 3E, upper). Notably, CD3$^+$ cells were significantly more abundant in [WT→Ldlr$^{-/-}$] mice than in [CD1d$^{-/-}$→Ldlr$^{-/-}$] mice (Figure 3E, middle, 3F; $P = .006$), and IFN-γ–positive cells, probably lymphocytes, were detected at more significant numbers in [WT→Ldlr$^{-/-}$] chimeras than in [CD1d$^{-/-}$→Ldlr$^{-/-}$] chimeras (Figure 3E, lower). There were no overt differences in the staining patterns of α-smooth muscle actin and IL-10 between these 2 groups (data not shown). Mice reconstituted with syngeneic BMT ([Ldlr$^{-/-}$→Ldlr$^{-/-}$]) showed the same atherosclerotic lesions as those in [WT→Ldlr$^{-/-}$] mice (data not shown).

Effects of NKT cell activation on the development of early atherosclerotic lesions in apoE$^{-/-}$ mice

To examine influences of NKT cell activation on the development of atherosclerosis, we administered α-GalCer or OCH to apoE$^{-/-}$ mice. ApoE$^{-/-}$ mice spontaneously develop severe atherosclerosis early in life.[8,9,35] It has been reported that α-GalCer and OCH activate NKT cells with differential patterns of cytokine production.[33]

In an early-phase study, apoE$^{-/-}$ mice were intraperitoneally injected 3 times with either 0.1 μg/g BW α-GalCer, 0.3 μg/g BW OCH or the respective vehicle at 8, 10, and 12 weeks of age. At 13 weeks of age, the mice were killed and examined for atherosclerotic lesions. No significant differences in physiologic status or serum lipid profiles were observed between experimental and control groups (α-GalCer or OCH vs their vehicle; data not shown). α-GalCer administration increased atherosclerotic lesion areas of apoE$^{-/-}$ mice compared with the vehicle control group (34 705 ± 5908 μm^2 vs 20 895 ± 2155 μm^2; $P = .039$) (Figure 4A-B). Major components of the atherosclerotic lesions in α-GalCer–treated mice included MOMA-2$^+$ macrophages (Figure 4C). OCH administration also increased atherosclerotic lesion areas compared with control (28 519 ± 2822 μm^2 vs 19 863 ± 1813 μm^2; $P = .048$) (Figure 4D). Lesion areas in the OCH-treated group, however, were relatively smaller than those in the α-GalCer–treated group. To determine a potential mechanism for the differences observed between the α-GalCer– and OCH-treated mice, we evaluated the sequential patterns of IFN-γ and IL-4 production in the serum after glycolipid injection. Both glycolipids induced robust cytokine production; however, though IL-4 production was similar, α-GalCer induced more IFN-γ than OCH, which is consistent with earlier findings (Figure 4E).[33]

Effects of long-term administration of α-GalCer on advanced atherosclerotic lesions in apoE$^{-/-}$ mice

In a late-phase study, we analyzed lesions in 19-week-old apoE$^{-/-}$ mice that had received 11 intraperitoneal injections of either α-GalCer or its vehicle. Again, no significant differences were observed in the physiologic status and serum lipid profiles between α-GalCer– and vehicle-treated mice (data not shown). Mean areas of lesions in the aortic sinus were slightly larger in the α-GalCer group than in the control group (197 448 ± 18 259 μm^2 vs 160 703 ± 16 320 μm^2) (Figure 5A). In