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Differential Expression of CD11c by Peripheral Blood NK Cells Reflects Temporal Activity of Multiple Sclerosis¹

Toshimasa Aranami, Sachiko Miyake, and Takashi Yamamura²

Multiple sclerosis (MS) is an autoimmune disease, showing a great degree of variance in temporal disease activity. We have recently demonstrated that peripheral blood NK cells biased for secreting IL-5 (NK2 bias) are associated with the remission state of MS. In this study, we report that MS patients in remission differentially express CD11c on NK cell surface (operationally defined as CD11c[^hi] or CD11c[^lo]). When we compared CD11c[^hi] or CD11c[^lo] patients, the expression of IL-5 and GATA-3 in NK cells supposed to endow a disease-protective NK2 phenotype was observed in CD11c[^hi] but not in CD11c[^lo] patients. In contrast, the CD11c[^hi] group showed a higher expression of HLA-DR on NK cells. In vitro studies demonstrated that NK cell stimulatory cytokines such as IL-15 would up-regulate CD11c expression on NK cells. Given previous evidence showing an association between an increased level of proinflammatory cytokines and temporal disease activity in MS, we postulate that inflammatory signals may play a role in inducing the CD11c[^hi] NK cell phenotype. Follow-up of a new cohort of patients showed that 6 of 10 CD11c[^hi] MS patients developed a clinical relapse within 120 days after evaluation, whereas only 2 of 13 CD11c[^lo] developed exacerbated disease ($p = 0.003$). As such, a higher expression of CD11c on NK cells may reflect the temporal activity of MS as well as a loss of regulatory NK2 phenotype, which may allow us to use it as a potential biomarker to monitor the immunological status of MS patients. *The Journal of Immunology*, 2006, 177: 5659–5667.

Multiple sclerosis (MS)³ is a chronic inflammatory disease of the CNS, in which autoreactive T cells targeting CNS Ags are presumed to play a pathogenic role (1). A large majority of the patients with MS (~70%), known as relapsing-remitting MS, would develop acute exacerbations of disease between intervals of remission. It is currently believed that relapses are caused by T cell- and Ab-mediated inflammatory reactions to the self-CNS components, and could be controlled at least to some degree by anti-inflammatory therapeutics, immunosuppressants, or plasma exchange.

The clinical course of MS varies greatly among individuals, implicating difficulties to predict the future of each patient. For example, patients who had been clinically inactive in the early stage of illness could abruptly change into active MS accompanying frequent relapses and progressive worsening of neurological conditions. There are a number of unpredictable matters in MS, including an interval between relapses, responsiveness to remedy and the prognosis in terms of neurological disability. To provide better quality of management of the patients, searches of appropriate biomarkers are currently being warranted (2).

We have recently shown that surface phenotype and cytokine secretion pattern of peripheral blood NK cells may reflect the disease activity of MS (3, 4). A combination of quantitative PCR and flow cytometry analysis has revealed that NK cells in clinical remission of MS are characterized by a higher frequency of CD95[^−] cells as well as a higher expression level of IL-5 than those of healthy subjects (HS) (3). As IL-5-producing NK cells, referred to as NK2 cells (5), could inhibit Th1 cell activation in vitro (3), we interpreted that the NK2 bias in MS may contribute to maintaining the remission state of MS. More recently, we have found that MS patients in remission can be further divided into CD95[^hi] and CD95[^lo], according to the frequency of CD95[^+] cells among NK cells (4). Notably, memory T cells reactive to myelin basic protein, a major target Ag in MS, were increased in CD95[^hi] patients, compared with CD95[^lo]. Of note, CD95[^hi] NK cells exhibited an ability to actively suppress the autoimmune T cells, whereas those from CD95[^lo] patients did not. These results suggest that NK cells may accommodate their function and phenotype to properly counterregulate autoimmune T cells in the remission state of MS.

Recently, a distinct population of NK cells that express CD11c, a prototypical dendritic cell (DC) marker, was identified in mice (6, 7). As the CD11c[^+] NK cells exhibited both NK and DC functions, they are called as “bitypic NK/DC cells.” CD11c associates with integrin CD18 to form CD11c/CD18 complex and is expressed on monocytes, granulocytes, DCs, and a subset of NK cells. Although precise functions are unclear, it has been reported that CD11c is involved in binding of iC3b (8), adhesion to stimulated endothelium (9) or phagocytosis of apoptotic cells (10). The initial purpose of this study was to evaluate CD11c expression and function of CD11c[^+] NK cells in MS in the line of our research to characterize NK cells in MS. On initiating study, we noticed that there was no significant difference between MS and HS in the frequency of CD11c[^+] NK cells. However, expression levels of CD11c were significantly higher in MS. We further noticed that up-regulation of CD11c is seen in some, but not all, patients with MS. So we have operationally classified MS into CD11c[^lo] and CD11c[^hi].

In this study, we demonstrate that IL-5, characteristic of NK2 cells (5), were significantly down-regulated in CD11c[^hi] than

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³ Abbreviations used in this paper: MS, multiple sclerosis; HS, healthy subject; DC, dendritic cell; MFI, mean fluorescence intensity; ECD, energy-coupled dye.
CD11c<sub>low</sub> NK cells. In contrast, expression of HLA-DR class II molecule was up-regulated in CD11c<sub>high</sub> NK cells. Notably, both CD11c and HLA-DR on NK cells were reproducibly induced in vitro in the presence of IL-15 (11) or combination of inflammatory cytokines, known to be increased in the blood of MS (12–14). Furthermore, we found that the remission state of CD11c<sub>high</sub> is unstable in comparison to CD11c<sub>low</sub>, as judged by an increased number of the patients who exacerbated during the 120 days after examining NK cell phenotypes. These results suggest that the CD11c<sub>high</sub> group of patients may be in more unstable condition than CD11c<sub>low</sub>, presenting with reduced regulatory functions of NK cells.

Materials and Methods

**Subjects**

Twenty-five patients with relapsing-remitting MS (15) (male (M)/female (F) = 8/17; age = 37.7 ± 11.1 (year old)) and 10 sex- and age-matched HS (M/F = 37; age = 39.9 ± 12.2 (year old)) were enrolled for studying NK cell phenotypes. All the patients were in the state of remission at examination as judged by magnetic resonance imaging scanning and clinical assessment. They had not been given immunosuppressive medications, or corticosteroids for at least 1 mo before examination. They had relatively mild neurological disability (expanded disability status scale <4) and could walk to the hospital without any assistance during remission. The same neurologist followed up the patients regularly (every 3–4 wk) and judged the occurrence of relapse by using magnetic resonance imaging and clinical examinations. Information on NK cell phenotype or other immunological parameters was never given to either the neurologist or the patients at the time of evaluation. To precisely determine the onset of relapse, patients were allowed to take examination within a few days after a new symptom appeared. Written informed consent was obtained from all the patients and the Ethics Committee of the National Center of Neuroscience (NCNP) approved the study.

**Reagents**

Mouse IgG1 isotype control-PE, anti-CD3-energy-coupled dye (ECD), anti-CD4-PE, anti-CD8-PC5, anti-CD56-PC5, anti-CD69-PE, and anti-HLA-DR-FITC mAbs were purchased from Immunotech. Anti-CD11c-PE and anti-CD95-FITC were purchased from BD Pharmingen. Recombinant human cytokines were purchased from PeproTech. AIM-V (Invitrogen Life Technologies) was used for cell culture after supplementing 2 mM t-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen Life Technologies).

**Cell preparation and NK cell purification**

PBMC were separated by density gradient centrifugation with Ficoll-Hypaque PLUS (Amersham Biosciences). To purify NK cells, PBMC were treated with NK isolation kit II (Miltenyi Biotech) twice, according to the manufacturer’s protocol. Briefly, PBMC were labeled with a mixture of biotin-conjugated mAbs reactive to non-NK cells and magnetic microbead-conjugated anti-biotin mAbs. The magnetically labeled non-NK cells were depleted with auto-MACS (Miltenyi Biotech) and this procedure always yielded >95% purity of NK cells when assessed by the proportions of CD3<sup>-</sup> CD56<sup>-</sup> cells with flow cytometry.

**Flow cytometry**

To evaluate the expression of CD11c, CD95, or other surface molecules on NK cells, PBMC were stained with anti-CD3-ECD, anti-CD56-PC5, and FITC- or PE-conjugated mAbs against molecules of our interest and were analyzed with EPICS flow cytometry (Beckman Coulter). Mean fluorescence intensity (MFI) of CD11c was measured on gated CD11c<sup>-</sup> fraction or whole NK cells.

**Stimulation of purified NK cells with proinflammatory cytokines**

Purified NK cells (1 × 10<sup>6</sup>/well) were stimulated in the presence or absence of IL-4, IL-8, IL-12, IL-15, IL-18, IL-23, TNF-α, and GM-CSF or combination of IL-12, IL-15, and IL-18 for 3 days. We analyzed CD11c expression after staining the cells with anti-CD11c-PE, anti-CD3-PECy7, and anti-CD56-PC5. The concentration of IL-12 was at 10 ng/ml, and those of the other cytokines were at 100 ng/ml.

**RT-PCR**

Total RNA were extracted with a RNeasy Mini kit (Qiagen) from purified NK cells, and the cDNA were synthesized with Super Script III first strand systems (Invitrogen Life Technologies) according to the manufacturer’s protocol. For quantitative analysis of IL-5, IFN-γ, GATA-3, and T-bet, the LightCycler quantitative PCR system (Roche Diagnostics) was used. Rel-ative quantities of mRNA were evaluated after normalizing each expression levels with β-actin expression. PCR primers used were as follows: β-actin-sense, AGAGATGGGCAACGCGTCTT; and -antisense, ATTT GCGGTGGACGATGGAG; IFN-γ-sense, CAGGTCTTCACTAGTGA GCC; and -antisense, GCTTGTCAAGCATCTG; IL-5-sense, GCA CACTGGAGACGTCACCT; and -antisense, CACTCCTGTTCATTAC CACC; GATA-3-sense, CTAGGAAACTCTTGTCAGGG; and -antisense, CTGGTACTTGAGGCACTCTT; T-bet-sense, GGAGGACACCGACTA ATTTGGGA; and -antisense, AAAGAAGACGCAGACCCAGTGAA.

**Statistical analysis of remission rate**

We set the first episode of relapse after blood sampling as an end point, although we followed clinical course of each patient for up to 120 days, regardless of whether they developed relapses. No patients developed second relapse during the 120 days. When the neurologist prescribed corticosteroids without knowing any information on the NK cell phenotype, the patient was considered as the dropout at that time point. Remission rate was calculated as Kaplan-Meier survival rate, and statistical difference between CD11c<sub>low</sub> and CD11c<sub>high</sub> was evaluated with the log-rank test.

**Results**

**CD11c on NK cells is up-regulated in MS remission**

First, we confirmed that PBMC from healthy individuals and MS contain CD11c<sup>+</sup> NK cells (Fig. 1), which constitute a major population of whole NK cells. We then noticed that proportion of CD11c<sup>+</sup> NK cells as well as its levels of expression greatly varied among individuals, particularly in MS. To examine this issue further, we systemically examined 25 MS patients in remission and 10 HS for NK cell expression of CD11c. Whereas 20–80% of NK cells are CD11c<sup>+</sup> in HS (Fig. 1c), almost all NK cells were CD11c<sup>+</sup> in some MS patients (Fig. 1, a and e). However, reflecting a great degree of variance, comparison between HS and MS did not reveal a significant difference (Fig. 1c). In contrast, when we measured the MFI of CD11c expression on CD11c<sup>+</sup> NK cells, it was significantly higher in MS as compared with HS (Fig. 1a). This difference was also noticed when MFI of CD11c was measured for all the NK cell populations (Fig. 1b). It was interesting to know whether the levels of CD11c expression may correlate with NK cell functions. Therefore, we operationally divided the MS patients into CD11c<sub>low</sub> and CD11c<sub>high</sub> subgroups (Fig. 1a), by setting the border as (the average + 2 × SD) of the values for HS.

**CD11c<sub>high</sub> NK cells express HLA-DR more brightly than CD11c<sub>low</sub> NK cells**

It was previously reported that infection with certain viruses would accompany up-regulation of CD11c on NK cells (16). This raises a possibility that the increased expression of CD11c in CD11c<sub>high</sub> MS may reflect an activation state of NK cells caused by some sort of stimuli. To verify this hypothesis, we examined surface expression of cell activation markers (CD69 and HLA-DR). Although CD69, an early activation marker, was not detectable on NK cells (Fig. 2a), NK cells from MS, particularly CD11c<sub>high</sub> MS, significantly overexpressed HLA-DR on surface (Fig. 2). Interestingly, HLA-DR expression was also up-regulated on CD4<sup>+</sup> T cells from CD11c<sub>high</sub> MS compared with those from HS (data not shown). These results indicate that NK cells and T cells are differentially activated in CD11c<sub>high</sub> MS, CD11c<sub>low</sub> MS, and HS.

**Absence of NK2 bias in CD11c<sub>high</sub> MS**

We have previously reported that a higher level of IL-5 expression (NK2 bias) is one of the characteristics of NK cells of MS in...
remission (3). Although the mechanism for NK2 bias in MS remains to be further studied, up-regulation of GATA-3 has recently been reported in the induction of NK2 cells in mice (17). To explore the possible difference in the functions of CD11c\textsuperscript{high} and CD11c\textsuperscript{low} NK cells, we isolated NK cells from CD11c\textsuperscript{high} or CD11c\textsuperscript{low} group of patients and measured the mRNA levels of representative cytokines IFN-\(\gamma\) and IL-5 as well as corresponding transcription factors T-bet and GATA-3. As shown in Fig. 3, mRNA expression of both IL-5 and GATA-3 was significantly higher in CD11c\textsuperscript{low} MS compared with HS or CD11c\textsuperscript{high} MS, indicating that NK2 bias thought to be characteristic of MS remission is restricted to CD11c\textsuperscript{low} MS. In contrast, there were no differences in mRNA expression of IFN-\(\gamma\) and T-bet among these three groups. Because NK cells from CD11c\textsuperscript{high} patients expressed HLA-DR most brightly, we speculate that NK2 bias associated with CD11c\textsuperscript{low} MS would attenuate when NK cells are further activated or differentiated.

NK cell stimulatory proinflammatory cytokines induce up-regulation of CD11c

We next attempted to explore the mechanism(s) for up-regulation of CD11c on NK cells in CD11c\textsuperscript{high} MS. Because both NK cells and CD4\textsuperscript{+} T cells overexpress HLA-DR in CD11c\textsuperscript{high}, it is probable that immune signals influencing both innate and acquired immunity are operative. So we hypothesized that cytokine signals that have been implicated in the pathogenesis of MS may play a role. We cultured NK cells from HS in the presence or absence of cytokine(s) for 3 days, and evaluated the CD11c expression (MFI). We focused our attention to IL-12, IL-15, and IL-18, which are known to stimulate NK cells with or without help of other cytokines. Notably, they are reportedly elevated in the serum or blood lymphocytes of MS patients as compared with HS (11–14, 18, 19), and prior studies suggest that they may play an important role in autoimmune diseases (20–24). As shown in Fig. 4, although IL-12 and IL-18 showed only a marginal effect on purified NK cells, IL-15 consistently induced 2- to 3-fold up-regulation of CD11c compared with control culture without addition of cytokines. As IL-12 and IL-18 were reported to synergistically work in various settings (25, 26), we then examined whether combinations of these cytokines may induce CD11c. Combination of IL-15 and IL-12 or of IL-15 and IL-18 did not augment the CD11c expression to the level higher than that could be induced by IL-15 alone. However, the combination of IL-12 and IL-18 did up-regulate CD11c compared with control culture without addition of cytokines. As IL-12 and IL-18 were reported to synergistically work in various settings (25, 26), we then examined whether combinations of these cytokines may induce CD11c. Combination of IL-15 and IL-12 or of IL-15 and IL-18 did not augment the CD11c expression to the level higher than that could be induced by IL-15 alone. However, the combination of IL-12 and IL-18 did up-regulate CD11c on NK cells, which was comparable to the effect of IL-15 alone (Table I). Additionally, we tested the effects of several cytokines involved in differentiation of DC (TNF-\(\alpha\), GM-CSF, IL-4) (27), or known to up-regulate CD11c in granulocytes (IL-8) as controls (28) in the same assay. These cytokines showed no significant effect (Table I).

CD11c\textsuperscript{high} MS relapsed earlier

Given the significant difference in activation status and cytokine phenotype of NK cells as well as HLA-DR expression by CD4\textsuperscript{+} T cells, it was particularly interesting to know whether CD11c\textsuperscript{low} and CD11c\textsuperscript{high} MS may follow a different clinical course. A new cohort of
13 CD11clow and 10 CD11chigh MS patients listed in Table II were followed for up to 120 days. In this preliminary exploration, we set the first episode of relapse after blood sampling as an end point. When the neurologist prescribed corticosteroids without knowing any information on the NK cell phenotype, the patient was considered as the dropout at that time point. Remission rate was calculated as Kaplan-Meier survival rate, and statistical difference between CD11clow and CD11chigh MS was evaluated with the log-rank test (Fig. 5a). At entry, there was no significant difference in the age and disease duration between CD11clow and CD11chigh MS (Table II). On analyzing the collected data after completing the study, we found that 8 patients developed a single relapse during the observation period and that the proportion of patients who have had relapse during the follow-up period was greatly higher in CD11chigh MS (6 of 10, 60%) than in CD11clow MS (2 of 13, 15.3%). Furthermore, the log-rank test revealed that CD11chigh MS relapsed significantly earlier than CD11clow MS (p = 0.003), suggesting a possible role of CD11c as a temporal marker for predicting relapse within months after examination. We also explored whether the difference between CD11chigh and CD11clow could be influenced by age or sex. When we selected a group of patients younger than 38.5 years old (the mean age of all the patients), a significantly earlier relapse in CD11chigh than CD11clow MS was confirmed in this group of patients (p = 0.0067, Fig. 5b). In the rest of the patients (<38.5 years old), the difference was less clear and not significant (p = 0.095). In female patients, CD11chigh MS relapsed significantly earlier than CD11clow MS (p = 0.035, Fig. 5c), whereas this tendency was not statistically significant in male patients (p = 0.083). By examining the patients’ medical records, we also found that the duration from the last relapse tended to be shorter in CD11chigh than CD11clow MS (14.7 ± 12 mo in CD11chigh vs 26.7 ± 24.3 mo in CD11clow) and that the mean number of relapses per year was higher in CD11chigh MS (0.9 ± 0.6 in CD11chigh vs 0.5 ± 0.5 in CD11clow). These are consistent with the postulate that CD11chigh MS might be immunologically more active than CD11clow MS (Table II).

Alteration of CD11c expression in the course of MS

We previously described that NK cells may lose NK2 phenotype during relapse (3). It is interesting to know whether the CD11c phenotype also changes in the course of MS. During the follow-up period of 120 days, 8 patients developed a relapse. We were able to take blood samples at relapse before treatment with corticosteroid and then compared the relapse samples with the samples obtained during remission at initiation of the study. As shown in Fig. 6, we saw an obvious tendency that the levels of CD11c expression would decline during relapse (p < 0.05). HLA-DR expression on NK cells was also reduced in some patients during relapse, but the difference between remission and relapse samples was not statistically significant.

Expression pattern of CD95 vs CD11c on NK cells in MS

In a previous study, we showed that MS patients could be divided into CD95high and CD95low according to the frequency of CD95+ cells among NK cells (4). Additionally, we examined whether expression of CD11c and CD95 may independently reflect the status of MS. We found no significant correlation between CD95 (%) and CD11c (MFI) on NK cells in MS (r = 0.29, p = 0.16 with Spearman’s correlation coefficient by rank test), indicating that expression of CD95 and CD11c on NK cells may be regulated independently. By setting the upper limits of CD95+ (%) and CD11c MFI as (the average + 2 × SD) of HS (CD95: 44.6%, CD11c: 5.04),
we then examined whether there is a correlation between CD11c CD95 phenotype and clinical conditions (Fig. 7). Naturally, all the healthy subjects were plotted in the left lower quadrant (CD95lowCD11clow). In contrast, MS patients were plotted in all the four quadrants with differential proportions of patients who have no relapse during 120 days: CD95lowCD11clow; 3/3 (100%), CD95lowCD11chigh; 1/2 (50%), CD95highCD11clow; 8/10 (80%), CD95highCD11chigh; 2/7 (28.6%). Although the data for CD95low subjects (lower left and lower right) need to be omitted due to the limited sample size, we found that the difference between CD95highCD11clow and CD95highCD11chigh in remission rate was significant with log-rank test ($p < 0.028$). Provided that CD95high patients possessed an increased frequency of memory autoreactive T cells (4), this result is consistent with the idea that when comparable numbers of autoimmune T cells are present in the peripheral circulation, remission of MS is more stable in patients with CD11clow NK cells.

Discussion

Blood examination of systemic autoimmune diseases such as systemic lupus erythematosus usually exhibits measurable abnormalities such as elevation of autoantibodies, which is useful for evaluating activity of disease. In contrast, patients with MS do not accompany such systemic abnormalities in laboratory tests except

**FIGURE 3.** IL-5 and GATA-3 mRNA are increased in CD11c\textsuperscript{low} but not in CD11c\textsuperscript{high} MS. Total RNAs were extracted from purified NK cells of HS ($n = 8$), CD11c\textsuperscript{low} ($n = 9$), or CD11c\textsuperscript{high} MS ($n = 8$). mRNA expression of IL-5 (a), GATA-3 (b), IFN-\(\gamma\) (c), and T-bet (d) was evaluated by quantitative PCR. The data are normalized to endogenous \(\beta\)-actin expressions in the same samples. ANOVA was used for statistical analysis. Horizontal bars indicate the mean values, *, $p < 0.05$; **, $p < 0.01$.

**FIGURE 4.** CD11c expression on NK cells is upregulated with addition of IL-15. a, Purified NK cells were cultured in the absence or presence of IL-12, IL-18, or IL-15. Three days later, the cells were stained with anti-CD11c-PE, -CD3-ECD, and -CD56-PC5 mAb. CD11c expression on NK cells (CD3\(^+\)CD56\(^-\) cells) is demonstrated as single histogram. Values indicate CD11c MFI of CD11c\textsuperscript{+} fractions. A representative of three independent experiments is shown. b, Data are expressed as mean fold increase of CD11c MFI (the MFI in the presence of cytokine/the MFI in the absence of cytokine) + SD from three independent experiments. ANOVA was used for statistical analysis. ***, $p < 0.01$. 


in unusual cases. It is currently recognized that autoreactive T cells might be activated and expanded to various degrees in the peripheral blood and peripheral lymphoid organs of MS even during remission (1–4). In fact, our previous work suggests that a higher number of memory autoreactive T cells is linked with unstable disease course (4). If we are able to accurately evaluate the immune status of each patient with a relatively simple test, it should be most helpful in treatment and management of MS. In this line, it is currently of particular importance to identify measurable indicators which would serve as clinically appropriate biomarkers in MS (2).

This study has clarified for the first time to our knowledge that CD11c expression on peripheral NK cells is significantly up-regulated in a major proportion of patients with MS in remission. To obtain insights into the mechanism and the biological meaning of the NK cell expression of CD11c in autoimmune disease MS, we have attempted to clarify the difference between CD11c\textsuperscript{high} and CD11c\textsuperscript{low} patients regarding phenotypes of NK cells, cytokine profile, and temporal clinical activity. We also explored which inflammatory cytokines might induce CD11c on NK cells. According to the NK cell expression of CD11c, we have classified the patients with MS in remission into CD11c\textsuperscript{high} and CD11c\textsuperscript{low}. Most notably, NK2 phenotype characterized by predominant IL-5 production was seen in CD11c\textsuperscript{low} patients, but not in CD11c\textsuperscript{high}. Consistently, the CD11c\textsuperscript{high} patients were found to be clinically more active than CD11c\textsuperscript{low} as judged by the remission rate during the 120 days after examination. These results indicate that up-regulation of CD11c on NK cells would reflect the temporal disease activity and therefore could be used to identify patients who are likely to exacerbate within months. It has been reported that CD11c\textsuperscript{+} NK cells in mice could serve as APCs (6, 7). However, we could not reveal Ag presenting capacity of human CD11c\textsuperscript{+} NK cells (data not shown).

Regarding the mechanism of CD11c induction on NK cells, we have found that in CD11c\textsuperscript{high} patients, HLA-DR is concomitantly up-regulated with CD11c on NK cells (Fig. 2), which suggests that up-regulation of CD11c may represent an activation-induced change. After exploring the culture condition that may induce CD11c on NK cells, we have found that the addition of IL-15 or combination of IL-12 and IL-18 would increase the expression levels of CD11c on NK cells from healthy individuals. Because increased levels of these proinflammatory cytokines are detected in the blood samples of MS (11–13, 18, 19, 23), it is possible that in

### Table I. Effect of several cytokines on CD11c expression on NK cells

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\* Purified NK cells were stimulated with cytokines. Data are expressed as fold increase of CD11c MFI (the MFI in the presence of the indicated cytokines/the MFI in the absence of cytokines) in the presence of indicated cytokines. More than a 2-fold increase is highlighted (bold).

### Table II. Information on the patients whose clinical courses were followed for up to 120 days

<table>
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<th>Identification No.</th>
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<th>Sex</th>
<th>Disease Period (Years)</th>
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<th>Duration from the Last Relapse (mo)</th>
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<td>Mean + SD</td>
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| 14                 | High  | 39          | M   | 4.4                    | 2                        | 22                                 | 0.5                           |
| 15                 | High  | 31          | F   | 9.2                    | 11                       | 14                                 | 1.2                           |
| 16                 | High  | 46          | F   | 7.4                    | >20\textsuperscript{b}   | 2                                  | ND                            |
| 17                 | High  | 53          | F   | 2.1                    | 4                        | 5                                  | 1.9                           |
| 18                 | High  | 59          | F   | 4.9                    | 2                        | 19                                 | 0.4                           |
| 19                 | High  | 27          | M   | 9.3                    | 4                        | 9                                  | 0.4                           |
| 20                 | High  | 36          | F   | 2.7                    | 1                        | 19                                 | 0.4                           |
| 21                 | High  | 34          | F   | 3.8                    | 2                        | 43                                 | 0.5                           |
| 22                 | High  | 60          | F   | 3.4                    | 6                        | 10                                 | 1.8                           |
| 23                 | High  | 21          | F   | 1.8                    | 2                        | 4                                  | 1.1                           |
| Mean + SD          |       | 40.6 + 13.4 |     | 4.9 + 2.8              | 3.8 + 3.1                | 14.7 + 12.0                       | 0.9 + 0.6                     |

\* F, Female; M, male.

\textsuperscript{b} This value is eliminated from calculation of the mean.
vitro CD11c induction on NK cells may recapitulate the phenotypic alteration of NK cells in CD11c<sup>high</sup> patients. Interestingly, IL-18 is not only a cytokine able to facilitate IFN-γ production by NK cells in cooperation with IL-12 (25, 26) but is crucial in inducing pathogenic autoimmune responses (21). Furthermore, autoimmune encephalitogenic T cells can induce more serious disease upon adoptive transfer when they are preactivated in the presence of IL-12 and IL-18 (20). Taken together, these results allow us to speculate that the proinflammatory cytokines may be involved in the up-regulation of CD11c on NK cells. Although the relationship between serum cytokine concentration and levels of CD11c expression on NK cells should be estimated in future studies, a previous work (11, 29, 30) showing that a probable link between IL-15 and temporal disease activity, indicates that NK cell expression of CD11c is likely to correlate with the levels of cytokines.

In the Th cell differentiation, specific transcription factors have been identified that play a crucial role in inducing Th1 or Th2 cells. Namely, Th1 differentiation characterized by IFN-γ induction requires a transcription factor T-bet, whereas GATA-3 and c-maf act to promote Th2 cytokine production (31–33). Human NK cells cultured in the presence of IL-12 or IL-4 differentiate into NK1 or NK2 populations, reminiscent of Th1 and Th2 cells (5). Whereas NK1 cells produce IL-10 and IFN-γ, NK2 cells would serve as immune regulators by producing IL-5 and IL-13. Notably, up-regulation of GATA-3 has been reported in mouse NK2 cells (17), raising a possibility that Th cells and NK cells might share the same transcription factor for inducing the key cytokine. We have previously reported that IL-5 expression is one of the characteristics of NK cells in the remission state of MS (3). However, it was not excluded that overexpression of IL-5 could be restricted to a proportion of the patients. Here, we have addressed whether NK cells from CD11c<sup>low</sup> and CD11c<sup>high</sup> may differ with regard to expression levels of IFN-γ and IL-5 and of their transcription factors T-bet and GATA-3. By measuring the mRNAs, we found that expression levels of IL-5 and GATA-3 are elevated in CD11c<sup>low</sup> MS but not in CD11c<sup>high</sup> (Fig. 3). Furthermore, we showed that

**FIGURE 5.** Rate of remission is lower in CD11c<sup>high</sup> MS. The first episode of relapse after blood sampling was set as an end point and clinical course of each patient was followed for up to 120 days. The remission rate was calculated in all (a), the younger (b), or female (c) patients as Kaplan-Meier survival rate, and statistical difference between CD11c<sup>low</sup> and CD11c<sup>high</sup> MS was evaluated with log-rank test at day 120. *p < 0.05; **p < 0.01.

**FIGURE 6.** Down-regulation of CD11c expression during relapse. a. Representative CD11c histograms from the same patient in remission (closed) and relapse (open). Values indicate CD11c MFI of CD11c<sup>+</sup> fractions. b. Comparison of NK cells from remission and relapse from the same patients (n = 6). The data obtained from the same patients are connected with lines. Wilcoxon signed-ranks test was used for statistical analysis. *p < 0.05.
indicates that CD95highCD11chigh MS may be most unstable sub-group of MS, among the patients whose clinical state could be followed up the clinical course of the patients after blood sampling. Although there was no significant difference in clinical parameters at examination of NK cells, we found that CD11chigh MS showed a significantly earlier relapse than CD11clow MS. This is consistent with our assumption that the absence of NK2 bias in CD11chigh MS should imply that regulatory NK cell functions are defective in this group of patients. When we analyzed expression of CD95 and CD11c on NK cells from MS, PBMC from MS or HS were stained with CD95-FITC, CD11c-PE, CD3-ECD, and CD56-PC5. After determining the proportion of CD95 cell (percent) and CD11c MFI for HS as (the average + two times SD) of HS, MS, MS patients who relapsed during the 120 days follow-up period.

neither IFN-γ nor T-bet was increased in CD11chigh MS. This suggests that NK cells from CD11clow are NK2-biased but those from CD11chigh are not, although MS in remission as a whole is NK2-biased as compared with control subjects. More recently, we have observed that stimulation with IL-15 or IL-12 plus IL-18 would decrease IL-5 and GATA-3 mRNA in purified NK cells with reciprocal up-regulation of CD11c (data not shown). This further supports a model that proinflammatory cytokines may play a crucial role in the absence of NK2 bias in CD11chigh MS.

To clarify the clinical differences between CD11chigh and CD11clow, we followed up the clinical course of the patients after blood sampling. Although there was no significant difference in clinical parameters at examination of NK cells, we found that CD11chigh MS showed a significantly earlier relapse than CD11clow MS. This is consistent with our assumption that the absence of NK2 bias in CD11chigh MS should imply that regulatory NK cell functions are defective in this group of patients. When we reanalyzed the data regarding various clinical parameters, we found that an earlier relapse in CD11chigh than CD11clow MS is more remarkable in the younger group (<38.5 years old) or in female patients. Furthermore, the duration from the last relapse tended to be shorter and the mean number of relapses per year higher in CD11chigh MS, supporting that CD11chigh MS is more active than CD11clow MS.

When we analyzed expression of CD95 and CD11c on NK cells simultaneously, we found that MS patients in remission could be divided into four subgroups (Fig. 7). When we compared clinical course after examination of NK cell phenotypes, we found that CD95highCD11chigh MS relapsed significantly earlier than CD95highCD11clow MS (p = 0.028 with log-rank test). This result indicates that CD95highCD11chigh MS may be most unstable subgroup of MS, among the patients whose clinical state could be judged as being in clinical remission.

In this study, we have demonstrated that MS patients differentially express CD11c on peripheral blood NK cells and a higher expression of CD11c on NK cells may reflect the temporal disease activity as well as functional alteration of regulatory NK cells. Our results have a clinical implication because of a lack of appropriate biomarker to monitor the immunological status in MS at present. To verify the reliability of this marker, longitudinal examination of CD11c expression on NK cells in the same patients should be performed in the future study.

**Disclosures**

The authors have no financial conflict of interest.

**References**


