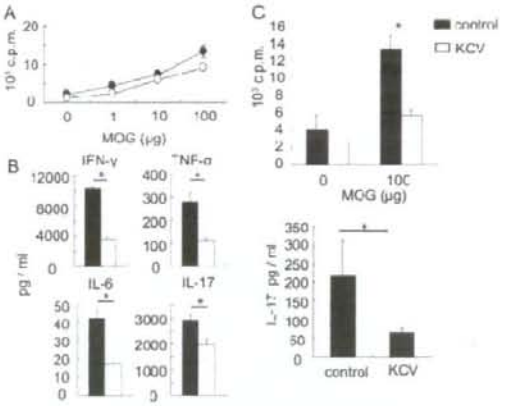

Figure 3. Reduced MOG-specific responses in dLN and MLN cells from KCV-treated mice. **A:** Effect of KCV on the lymphocyte proliferative responses. Draining lymph nodes (dLNs) were removed from control or KCV-treated mice 11 days after immunization with MOG (35–55) and the total lymphoid cells (1×10^6) were stimulated with varying doses of MOG (35–55) peptide for 72 hours. Proliferative responses were assessed by [^{3}H] thymidine incorporation. Data are from one of three independent experiments, showing the mean of triplicate samples. **B:** Effect of KCV treatment on MOG (35–55)-reactive T cells in the dLN. Supernatants were collected after stimulating the dLN cells of day 11 with 100 μmol/L MOG (35–55) peptide *in vitro* for 72 hours. Cytokine concentration was measured by cytometric bead array or ELISA as described in Materials and Methods. Data represent the mean ± SEM of duplicated samples from one of three separate experiments ($n = 2$ mice). $^*P < 0.05$ (two-tailed Student's *t*-test.) **C:** Effect of KCV treatment on MOG (35–55)-reactive T cells in the MLN. Whole MLN cells were isolated from control or KCV-treated mice ($n = 2$) 11 days after EAE induction. The cells were stimulated with MOG (35–55) as conducted for dLN cells and proliferative responses (upper panel) and IL-17 production (lower panel) were measured. IL-17 was measured by using ELISA. Data represent the mean ± SEM of triplicate samples from one of two independent experiments ($n = 2$ mice). $^*P < 0.05$ (two-tailed Student's *t*-test).

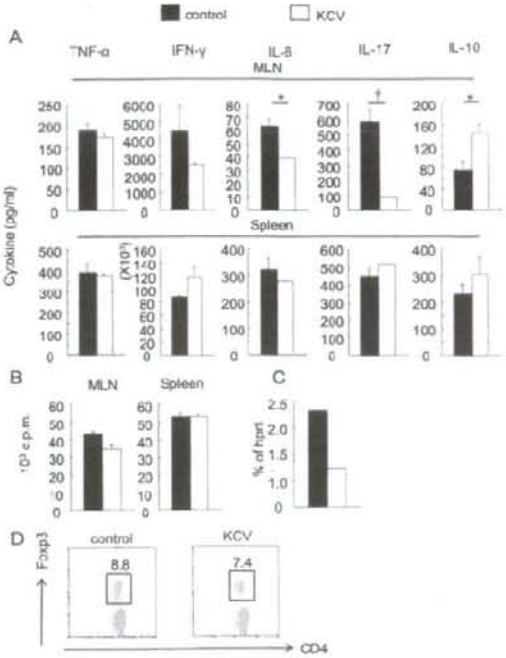

Figure 4. Decreased production of inflammatory cytokines from MLN cells after oral KCV treatment. **A:** Cytokine production from MLN T cells of naïve mice after KCV treatment. Mice were continuously given KCV-containing or control water for 7 days. Then MLN cells and splenocytes were isolated and stimulated by immobilized anti-CD3. MLNs (top panels) or splenocytes (bottom panels) from control or KCV-treated mice (unprimed) were stimulated with immobilized anti-CD3 antibody for 72 hours. Cytokines in the supernatants were measured by using cytometric bead array or ELISA. Data are from a representative out of three independent experiments ($n = 2$ mice). $^*P < 0.05$, $^\dagger P < 0.001$, (Student's *t*-test). **B:** Proliferative responses of MLN cells after anti-CD3 stimulation. MLN cells and splenocytes were prepared as described in (**A**). Proliferative responses were assessed by [^{3}H] thymidine incorporation. Data represent the mean ± SEM of triplicate samples from one out of three independent experiments ($n = 2$ mice). $^*P < 0.05$ (Student's *t*-test). **C:** Reduction of RORγt expression after KCV treatment. Total RNA was isolated from pooled MLN cells prepared from control mice or from mice given oral KCV treatment for 7 days. RORγt mRNA was estimated by quantitative RT-PCR and all data were normalized to hprt ($n = 2$ mice). **D:** Intracellular expression of Foxp3 for gated CD4$^+$ T cells derived from MLNs. Mice were given control or KCV-containing water for 1 week. Dot plots are gated on CD4$^+$ T cells. Data are representative of three independent experiments showing similar results.

in naïve wild-type mice. First we compared the ability of the MLN cells to produce pro-inflammatory cytokines on stimulation with plate-bound anti-CD3 antibody. Proliferative responses of the MLN cells were not affected or slightly suppressed at most by KCV treatment. Interestingly, MLN cells from KCV-treated mice secreted significantly lower amounts of IL-6 and IL-17 compared with those from control mice, whereas production of TNF-α and IFN-γ was not significantly suppressed (Figure 4, A and B). In contrast, splenocytes from both groups of mice showed essentially similar result following stimulation with anti-CD3 (Figure 4, A and B). Recently, Ivanov et al showed that an orphan nuclear receptor RORγt is the key transcription factor that orchestrates the differentiation of the Th17 cell lineage.[9] They also showed that Th17 cells tend to accumulate in the mucosa of the small intestine. Quantitative RT-PCR analysis revealed a lower expression of RORγt in the MLN cells from KCV-treated mice as compared with control mice (Figure 4C). We also found that the MLN cells from KCV-treated mice secreted significantly greater amounts of IL-10 than those from control mice (Figure 4A), suggesting that the mesenteric T cells would acquire less inflammatory properties after the antibiotic treatment.

Next we examined whether this treatment may alter the composition of lymphocytes in the MLN. We found that the total number of MLN cells was almost equal in KCV-treated and control mice (data not shown). Furthermore, flow cytometric analysis demonstrated that the proportion of dendritic cells, macrophage/monocytes, B cells, conventional CD4$^+$ and CD8$^+$ T cells, NK cells, and NKT cells in the MLN did not change after treatment with KCV (data not shown). These data indicate that the antibiotic treatment protocol does not exhibit any cytotoxic effect on the mesenteric lymphocyte populations, although it remarkably alters the cytokine profile of T cells. We also examined the frequency of Foxp3$^+$ regulatory CD4$^+$ T cells in the MLN. Although recent studies have revealed the presence of reciprocal developmental pathways between Th17 cells and Foxp3$^+$ regulatory T cells,[31] we

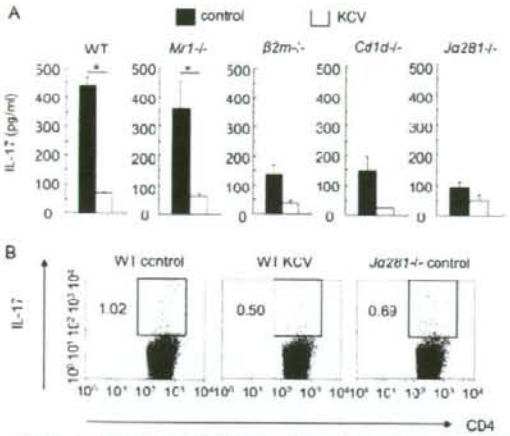

Figure 5. A role of Vα14 iNKT cells in the regulation of mesenteric Th17 cells. **A:** IL-17 production by the MLN T cells of mice lacking invariant iNKT or MAIT cells. After 1 week of KCV treatment, MLN cells were isolated from control or KCV-treated mice, including wild-type (WT), *Mr1*$^{-/-}$, *β2m*$^{-/-}$, *Cd1*$^{-/-}$, or *Jα281*$^{-/-}$ mice. The cells were stimulated with immobilized anti-CD3 antibody for 72 hours. IL-17 in the supernatant was measured using ELISA. Data are a representative of two independent experiments ($n = 2$ mice). *$P < 0.05$ (Student's *t*-test). **B:** Th17 cells in MLNs in KCV-treated or iNKT deficient mice. MLN cells were isolated from wild-type mice (WT control), KCV-treated wild-type mice (WT KCV), or iNKT cell-deficient *Jα281*$^{-/-}$ mice and stimulated for 5 hours with phorbol 12-myristate 13-acetate and ionomycin in the presence of GolgiPlug. We conducted surface labeling with the indicated antibody and αGalCer-loaded CD1 days dimer as well as intracellular IL-17 staining. Dot plots are gated on CD4$^+$ T cells devoid of tNKT cells. Data are representative of two independent experiments ($n = 2$ mice).

could not detect any increase of CD4$^+$Foxp3$^+$ T cells in the MLN cells after KCV treatment (Figure 4D).

A Role of Vα14 iNKT Cells in the Regulation of Mesenteric Th17 Cells that Are Vulnerable to KCV Treatment

Recent studies have revealed that MR1-restricted invariant Vα19-Jα33 T cells, also referred to as mucosal associated invariant T (MAIT) cells, are preferentially distributed to gut LP and are strikingly influenced by the presence of gut flora.[23,32] We have recently shown that the MAIT cells could play a regulatory role in EAE.[33] Because of their dependence on commensal flora[23,32] we speculated that the antibiotic treatment might suppress the Th17 cell-mediated EAE disease by using the regulatory function of MAIT cells triggered by a change of flora. To verify this idea, we treated MAIT cell-deficient *Mr1*$^{-/-}$ mice as well as wild-type B6 mice with oral KCV, and examined the ability of the MLN cells to produce IL-17 after anti-CD3 stimulation. Contrary to our speculation, the results showed that the MLN cells from *Mr1*$^{-/-}$ mice and wild-type mice produced an equivalent amount of IL-17 either before or after KCV treatment (Figure 5A), indicating that MAIT cells do not play a major role in the suppression of Th17 cells by KCV treatment. However, in additional experiments using *β2-microglobulin*$^{-/-}$ (*β2m*$^{-/-}$) mice, we found that the baseline production of IL-17 by the MLN T cells after anti-CD3 stimulation was remarkably diminished in the mice, whereas the mesenteric T cells from *β2m*$^{-/-}$ mice and wild-type mice produced a similar amount of IL-17 after KCV treatment. Accordingly, oral KCV causes only a marginal reduction of IL-17 in *β2m*$^{-/-}$ mice, indicating that class I-restricted T cells other than MAIT cells play a critical role in the KCV-induced suppression of the Th17 cells within MLN.

Then we explored a possible role of Vα14 iNKT cells restricted by CD1 days, an MHC class 1b molecule. As is widely known, iNKT cells produce a variety of regulatory cytokines after recognizing glycolipid antigens such as α-galactocylceramide (α-GalCer) in association with CD1 days. Numerous reports have supported the role of iNKT cells in the regulation of autoimmunity.[22,34,35] We, therefore, repeated our above experiments using *Cd1*$^{-/-}$ mice,[24] which do not express either iNKT cells or non-invariant type II NKT cells,[36] as well as with *Jα281*$^{-/-}$ mice,[25] in which iNKT cells alone are specifically deleted. In these iNKT cell-deficient mice, we again found a great reduction in the baseline production of IL-17 from the MLNs after anti-CD3 stimulation. Furthermore, effects of oral KCV on the Th17 cells were only marginal, if any, in the mice (Figure 5A), raising a possibility that the host immune system may sense the change of gut flora by using iNKT cells.

It is now known that IL-17 secreting CD4$^+$ MLN cells comprise not only Th17 cells but also CD4$^+$ Vα14 iNKT cells.[37] To evaluate the alteration of mesenteric Th17 cells with accuracy, we next evaluated the proportion of IL-17$^+$ CD4$^+$ T cells after excluding iNKT cells by gating. By analyzing the MLN cells from wild-type mice (WT control), KCV-treated wild-type mice (WT KCV), or iNKT cell-deficient *Jα281*$^{-/-}$ mice (Figure 5B), we have confirmed that the number of IL-17$^+$ CD4$^+$ T cells corresponding to Th17 cells is reduced in the KCV-treated wild-type mice and in the iNKT cell-deficient *Jα281*$^{-/-}$ mice. We also noticed that IL-17$^+$ iNKT cells are 15 times lower than IL-17$^+$ CD4$^+$ T cells in wild-type mice (data not shown).

Oral KCV Treatment Inhibits Production of Th17-Promoting Cytokines in the Intestinal Lamina Propria

Next we sought to identify a primary event that would take place in the intestinal immune system following oral KCV treatment. Because the vast majority of Th17 cells in the MLNs appear to depend on iNKT cells (Figure 5A), we evaluated the number and function of iNKT cells in the MLNs. However, neither reduction nor increase of iNKT cells was found in the MLNs after the antibiotic treatment (data not shown). In addition, the MLN cells from KCV-treated mice and from control mice produced similar levels of cytokines in response to α-GalCer (data not shown). These results indicate that as seen with CD4$^+$Fox3$^+$ T cells (Figure 4D), iNKT cells in the MLN are not significantly influenced by the status of gut flora. Therefore, we postulate that local accumulation of regulatory cells is probably not the mechanism for the reduction of Th17

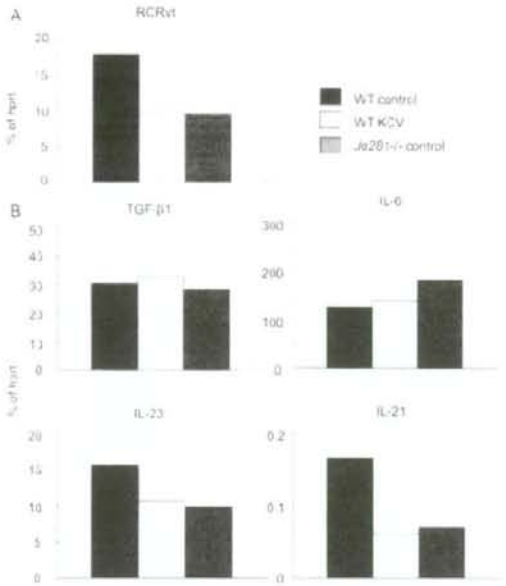

Figure 6. Reduced expression of Th17-promoting cytokines in the intestinal lamina propria lymphocytes from KCV-treated mice as well as iNKT-deficient mice. **A:** RORγt expression in the intestinal LP after treatment with oral KCV. Total RNA was isolated from pooled LPLs prepared from wild-type control B6 mice (WT control), KCV-treated wild-type B6 mice (WT KCV), and control $J\alpha281^{-/-}$ mice ($J\alpha281^{-/-}$ control) ($n = 2$). Wild-type KCV were given oral KCV for 7 days before the analysis. RORγt mRNA was estimated by quantitative RT-PCR and all data were normalized to hprt. Data are representative of two independent experiments. **B:** Expression of Th17-promoting cytokines in the intestinal LPL. Total RNA was isolated from LPLs of the three groups of mice as described in (A). Expression of TGFβ−1, IL-6, IL-23, and IL-21 mRNA was estimated by quantitative RT-PCR and all data were normalized to hprt. Data are a representative of two independent experiments.

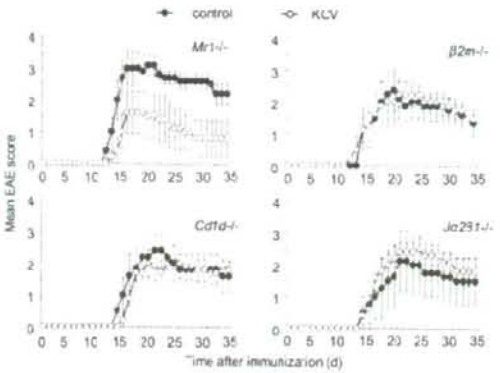

Figure 7. Suppressive effect of oral KCV treatment on EAE is abolished in Vα14 iNKT-deficient mice. $Mr1^{-/-}$, $\beta 2m^{-/-}$, $Cd1^{-/-}$, and $J\alpha281^{-/-}$ mice were treated with KCV as described in Materials and Methods. After immunization of mice with MOG (35–55) clinical EAE scores of mice were assessed. Data represent mean score ± SEM from two independent experiments ($n = 4$ or 5 mice).

cells in the MLN of KCV-treated mice (Figures 3 and 4). By using quantitative RT-PCR, we also measured mRNA expression of TGF-β, IL-6, IL-21, and IL-23 in the MLNs, which play key roles in the development or maintenance of Th17 cells in the intestine.[7,31,38,39,40] However, expression of these Th17-promoting cytokines did not change after KCV treatment (data not shown). Taking these results together, we assumed that the reduction of Th17 cells in the MLNs might result from a primary event that takes place upstream to the MLNs. Therefore, we shifted our attention from MLNs to intestinal LPLs.

Notably, Th17 cells constitutively inhabit LP,[9] and more iNKT cells are detected in LP than in MLN (our unpublished data). We first confirmed that RORγt expression was significantly reduced in the LPLs from KCV-treated wild-type mice as compared with those from control wild-type mice (Figure 6A), indicating that a reduced number of Th17 cells could be traced upstream to the LP. Moreover, the LPLs from iNKT cell deficient $J\alpha281^{-/-}$ mice showed a reduced expression of RORγt, again indicating the importance of iNKT cells for the maintenance of Th17 cells. We further quantified mRNAs of TGF-β1, IL-6, IL-23, and IL-21 expressed by LPLs by RT-PCR. Compared with the LPLs from control wild-type mice, those from KCV-treated wild-type mice and from $J\alpha281^{-/-}$ mice showed a reduced expression of IL-21 (Figure 6B). Expression of IL-23 was also reduced in KCV-treated wild-type mice as well as in $J\alpha281^{-/-}$ mice. These results support our postulation that LPLs are primarily influenced by the antibiotic treatment, resulting in a downstream decrease in the number of Th17 cells.

Suppressive Effect of KCV Treatment on EAE Is Abolished in iNKT-Cell Deficient Mice

The *ex vivo* experiments have demonstrated that Th17 cells in the MLN and LP are affected by KCV treatment in association with suppressed signs of EAE. Moreover, we showed that the KCV effects on Th17 cells could not be seen in the absence of iNKT cells. Although the results indicate an intimate relationship between Th17 cells and iNKT cells in the intestinal immune system, it does not necessarily imply that altering gut flora would suppress the development of EAE in a way dependent of iNKT cells. To make this point clear, we examined the effects of oral KCV treatment on the development of EAE induced in iNKT cell-deficient mice ($\beta 2m^{-/-}$, $Cd1^{-/-}$, $J\alpha281^{-/-}$) as well as in MAIT cell-deficient mice ($Mr1^{-/-}$) (Figure 7). First, we noted that clinical EAE induced in $Mr1^{-/-}$ mice was significantly suppressed by KCV treatment, which coincides with the fact that the mesenteric Th17 cells are not affected by the absence of MAIT cells (Figure 6A). In contrast, suppressive effects of oral KCV was almost completely abolished in $\beta 2m^{-/-}$, $Cd1^{-/-}$, and $J\alpha281^{-/-}$ mice (Figure 7), allowing us to conclude that iNKT cells play a key role in the KCV-induced suppression of EAE.

Health Status of KCV-Treated Mice

We have observed that antibiotic treatment tended to cause loose stool in the KCV treated mice. However, this happened in both wild-type mice and iNKT cell-deficient mice, which does not validate speculation on any relation

with the EAE disease suppression by KCV. Furthermore, KCV-treatment did not cause a significant change in body weight. We also examined the histology of gut lumen, and found that KCV treatment did not cause any pathological changes.

Discussion

The present study has experimentally demonstrated that altering gut flora by non-absorbing antibiotics could lead to protection against autoimmune disease EAE. Although the suppressive effect of antibiotics on EAE has been previously described,[41] the prior study did not address the possible contribution of the altered gut flora and has correlated the EAE suppression with an altered Th1/Th2 balance. In contrast, the present study has linked the antibiotic effects with a reduced number of Th17 cells in the gut-associated immune system. Most notably, the immunomodulatory effects of KCV could not be seen in iNKT cell-deficient mice, as assessed by the number of mesenteric Th17 cells or by severity of EAE. Comparison of wild-type and iNKT cell-deficient mice revealed that iNKT cells in the wild-type mice are able to promote the maintenance of mesenteric Th17 cells in the steady state, whereas the disease promoting ability of iNKT cell is impaired by KCV treatment. Given that oral administration of synthetic glycolipid ligands stimulatory for iNKT cells could alter the manifestation of autoimmune diseases,[22,42] one may speculate that oral KCV treatment leads to the appearance or disappearance of glycolipid ligands in the intestinal content that critically influence the function of iNKT cells.

The mucosal sites continuously sample foreign materials mainly via M cells in Peyer's patch and dendritic cells (DCs) in the LP.[43] The DCs in the LP would present orally applied antigens, migrate and enter the MLN.[44,45] Therefore, we wondered if the MLN might serve as the primary site where a contraction of Th17 cells takes place via mechanisms involving regulatory cells or changes of local cytokine milieu. However, the antibiotic treatment did not influence iNKT cells or Foxp3$^+$ regulatory T cells in the MLNs. Cytokines needed for promoting Th17 cell development and survival were not altered either, indicating that a critical event causing a reduction of Th17 cells probably takes place upstream. Consistent with this idea, we showed that expression of IL-21 and IL-23 in the LPLs was significantly suppressed in KCV-treated mice and iNKT cell-deficient mice. The role of IL-21 in the development of Th17 cells[39] has been demonstrated in mice lacking IL-6, the cytokine originally identified as a crucial promoter of Th17 cells. Intriguingly, it has recently been reported that IL-21 plays a critical role in the regulation of Th17 cells involved in gut inflammation.[38] Taken together, we suggest that the suppression of IL-21 and IL-23 may be a primary event after KCV treatment, which leads to the reduction of mesenteric Th17 cells. It is known that both iNKT cells and Th17 cells are able to produce IL-21.[46,47] Given that iNKT cells in the MLNs were not altered after KCV treatment, we speculated that iNKT cells within LP may numerically or functionally be altered, which could account for the reduced IL-21 in the LPLs. However, because of technical limitations, we have not definitively demonstrated that this is the case. Although a recent report using IL-21 knockout mice showed that IL-21 is not essential for the development of Th17 cells *in vitro* and *in vivo*,[48] it does not exclude the role of IL-21 in wild-type mice.

It is arguable that the reduced Th17 cells in the MLN cells from KCV-treated mice may result from a direct or indirect effect of KCV on DCs. However, flow cytometric analysis did not reveal any difference between KCV-treated and control mice with regard to the surface levels of MHC class II, CD80 or CD86 on the MLN-DCs (data not shown). In addition, there was no alteration of CD103 on the MLN-DCs that is described as an inducer of Foxp3$^+$ regulatory T cells.[49]

Although we have so far focused on analysis of Th17 cells and iNKT cells in the gut immune system, we cannot overlook that dLN cells from KCV-treated mice produced a lower amount of IFN-γ in response to MOG (35–55) indicating that Th1 cells in the dLNs could be also affected by KCV treatment. Interestingly, a concomitant reduction of Th1 cells and Th17 cells has recently been demonstrated in EAE mice treated with anti-IL-6 receptor antibody, which was used for aiming at specific suppression of Th17 cells.[50] These homologous results suggest the possible induction of regulatory T cells in the dLN that may regulate both Th1 and Th17 cells. Although Foxp3$^+$ regulatory T cells are qualified suppressors, total number of the CD4$^+$Foxp3$^+$ T cells in dLN was not altered after KCV treatment. It is possible that MOG (35–55) specific regulatory T cells might be selectively induced by altering gut flora. It is obvious that further studies are needed to clarify the total picture of NKT cell-dependent suppression of EAE by altering gut flora.

There is a clear tendency for an increased incidence of immune-mediated disorders in developed countries.[51] Although this increase has often been linked with improved hygiene, a number of studies have suggested a role for commensal flora affected by life style.[52] This is an attractive idea, in particular for inflammatory bowel disease, where the target is the gut and is inhabited by pathogenic Th17 cells as well as regulatory cells such as MAIT cells. In contrast, much less attention has been paid on the role of commensal flora in the development of the CNS autoimmune disease MS. The present study emphasizes that the repertoire of the immune system is greatly regulated by gut flora, which has broad implications for understanding the pathogenesis of autoimmune disease and allergy, and could be applied for future studies. However, it is too early to suggest that antibiotic treatment will be beneficial for MS. Indeed, altering gut flora could trigger or prevent the development of autoimmune conditions. Future studies coping with such variables as timing, duration, choice of antibiotics used for treatment will not only give us deeper understanding on the interaction between gut flora and Th17 cells, but also provide important information related to the human health.

Acknowledgments

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Synthetic Glycolipid Ligands for Human *i*NKT Cells as Potential Therapeutic Agents for Immunotherapy

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Abstract: Invariant natural killer T (*i*NKT) cells are an attractive therapeutic target in autoimmune diseases, since they play a major role in immune regulation. *i*NKT cells recognize glycolipid antigens presented by CD1d molecules that resemble the non-polymorphic MHC class I protein. α-galactosylceramide (α-GalCer) isolated from marine sponge has long been used as a prototype *i*NKT cell ligand in the laboratory. As α-GalCer is the most efficacious ligand for *i*NKT cells, its potential to treat autoimmune disease has been evaluated in animal models. Previous studies showed that α-GalCer effectively suppressed disease in some autoimmunity models, but not in others. This inconsistency may be attributed to the ability of α-GalCer to induce the production of both proinflammatory Th1 and anti-inflammatory Th2 cytokines by *i*NKT cells. To overcome this issue, we and other groups have synthesized new, unnatural glycolipids by modifying the structure of α-GalCer. These efforts have led to an identification of glycolipid compounds that provoke the production of Th2 (but not Th1) cytokines by *i*NKT cells. Among these novel ligands, an α-GalCer analogue named OCH, which contains a truncated sphingosine chain, induces a Th2 biased response by murine *i*NKT cells. Here we describe that OCH also polarizes human *i*NKT cells towards Th2, which opens up a new avenue for the clinical application of glycolipid compounds in treating of autoimmune diseases such as multiple sclerosis. The pursuit of synthetic glycolipid antigens has the great potential to lead to a better understanding of the regulatory effects of human *i*NKT cells and development of a new therapeutic agent for autoimmune diseases.

Keywords: Glycolipid, synthetic α-galacotosylceramide analogues, autoimmune disease, *i*NKT cells, Th1-Th2.

1. *i*NKT CELLS

Autoimmune diseases generate persistent tissue-specific damage and affect millions of people worldwide, leading to numerous social and economical problems. Thus, investigation of mechanisms by which autoimmunity develops and identification of novel therapeutic targets for treating autoimmune diseases are one of the major research themes in life science, as well as in pharmaceutical research. Recent research has revealed that the pathogenesis of autoimmune diseases such as multiple sclerosis (MS) may be caused by an alteration in the function of immune regulatory cells [1,2]. In fact, it was observed that the development of autoimmune diseases could be accompanied by functional changes amongst $CD25^+$ regulatory T cells [3] and invariant natural killer T (*i*NKT) cells [4,5]. Based on these data, one could argue that the restoration of regulatory cell function or the promotion of regulation by other cell types are ideal strategies for combating autoimmune diseases.

*i*NKT cells are a unique subset of T lymphocytes that display regulatory functions mainly *via* production of cytokines. They bear a distinctive T cell receptor (TCR) α chain encoded by an invariant Vα14-Jα18 rearrangement in mice or Vα24-JαQ in humans. The invariant TCRα chain pairs with a restricted repertoire of TCRβ chains, comprising Vβ8.2, Vβ7, and Vβ2 in mice or Vβ11 in humans [6,7,8]. Unlike conventional T cells that recognize peptide antigens bound to major histocompatibility complex (MHC) molecules, *i*NKT cells instead recognize glycolipid antigens bound to CD1d molecules. CD1d is a MHC class I-like molecule, which is expressed by monocytes, dendritic cells, and B cells. Optimally activated *i*NKT cells rapidly secrete large amounts of both inflammatory and anti-inflammatory cytokines and as *i*NKT cells produce such regulatory cytokines, it is supposed that they may play a critical role in the regulation of both innate and acquired immunity. Recent studies have addressed how *i*NKT cells can be activated during infectious diseases, tumor immunity, and autoimmunity: it appears that under certain conditions *i*NKT cells recognize an endogenous glycolipid bound to CD1d before secreting cytokines [9,10].

Frequencies of *i*NKT cells among peripheral lymphocytes are much lower in human than in mice [7,8]. However, human and mouse *i*NKT cells do appear to share similar characteristics in their function and activity. Human *i*NKT cells are mainly comprised of two subsets: $CD4^-CD8^-$ (double negative, DN) and $CD4^+$. Whereas the DN *i*NKT cells predominantly produce proinflammatory Th1 cytokines upon stimulation, the $CD4^+$ subset can release both Th1 and Th2 cytokines upon activation [11,12]. This unique ability to produce cytokines with antagonizing functions raises the possibility that *i*NKT cells can play an important role in the maintenance of the immune homeostasis. As *i*NKT lack TCR diversity and mount such rapid responses to antigens, one may speculate on their role in eradicating neoplasm or combating bacterial [13,14], viral [15-17], and parasite infection [18]. In addition, recent studies demonstrated that *i*NKT cells can modulate the pathogenesis of various autoimmune diseases [19-24]. However, whether *i*NKT cells play a protective or pathogenic role in autoimmunity appears to be influenced by a number of factors that require further characterization [20].

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2. GLYCOLIPID LIGANDS FOR *i*NKT CELLS

2.1. Microbial and Self Ligands

To understand the function of *i*NKT cells, the search for microbial and self ligands is essential. Recent studies have shown that glycosphingolipids (GSLs), bacterial components from the cell wall of *Sphingomonas*, are vital ligands for *i*NKT cells [13,14,25]. Among the GSLs, monoglycosylceramide (GSL-1) (Fig. **1A**) seems to act as the most potent stimulator of *i*NKT cells [26]. The GSLs may act as a substitute for lipopolysaccharide (LPS) in LPS-negative bacteria and it is accepted that the GSLs-stimulated *i*NKT cells participate in clearing such bacterial infections. *Borrelia burgdorferi* glycolipid I (BbGL-I) and II (BbGL-II) (Fig. **1A**) are also reported to activate both murine and human *i*NKT cells to elaborate IL-4 and IFN-γ [27]. In addition, glycolipids derived from *Sphingomonas wittichii* and *Borrelia burgdorferi* [28] are shown to stimulate IL-17 production by a particular subset of *i*NKT cells and *Mycobacterium bovis* (BCG) is also reported to activate a subpopulation of *i*NKT cells [29]. These results support the hypothesis that *i*NKT cells play a protective role following infection with particular bacteria that express *i*NKT cell ligands.

During investigation of *i*NKT cell ligands, glycosylphosphatidylinositol [30] and cellular phospholipids [31] were identified as possible endogenous ligands for *i*NKT cells. Recent analysis has suggested that Isoglobotrihexosylcera-

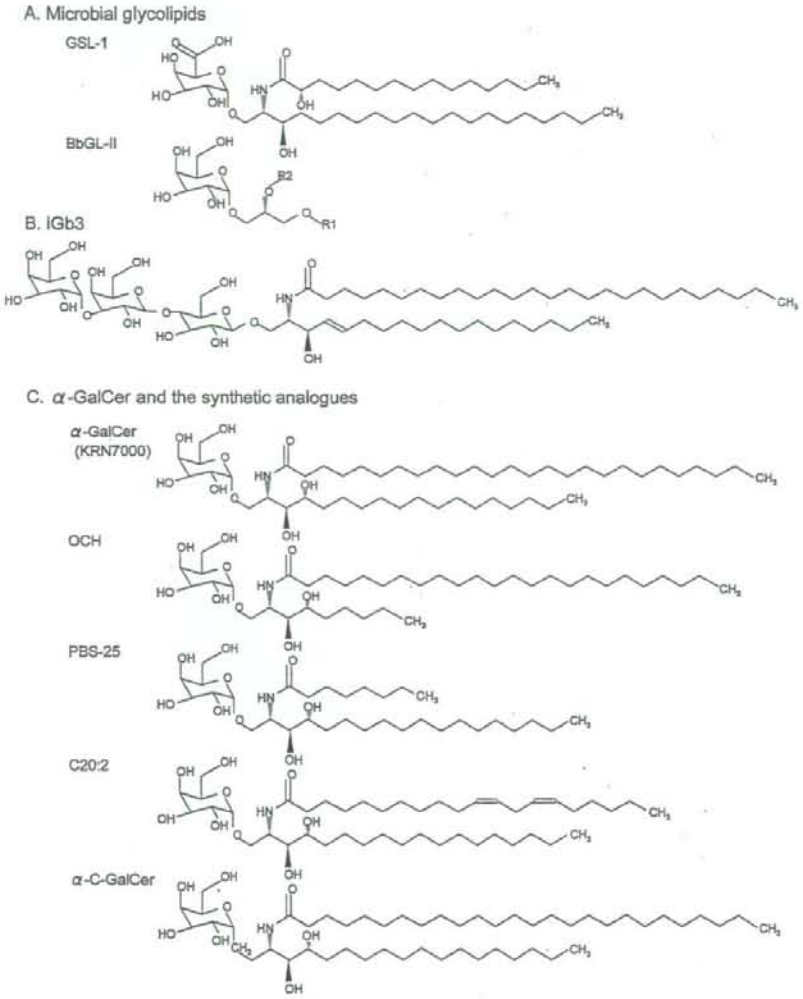

Fig. (1). Chemical structures of glycolipids and the analogues.

(A) GSL-1 derived from Sphingomonas bacteria, and BbGL-II derived from *Borrelia burgdorferi*. R1, *sn*-1 position of glycerol; R2, *sn*-2 position of glycerol (e.g. BbGL-IIa, R1 = C16:0, R2 = C18:1). **(B)** Endogenous ligand iGb3. (C) α-GalCer (KRN7000) [40] and the synthetic analogues OCH [49], PBS-25 [57], C20:2 [58], α-C-GalCer [60].

mide (iGb3) may be a natural ligand for *i*NKT cells (Fig. **1B**) [32-34] and further evidence indicates that it is likely that iGb3 functions as a physiological ligand for *i*NKT cells, which is required for their development. This hypothesis is supported by data demonstrating that a lack of β-hexoaminidase B, an enzyme which degrades iGb4 into iGb3, results in a severe impairment of thymic *i*NKT cell production in mice [35]. Furthermore, *i*NKT cell number was greatly reduced in mice that lacked a small lysosomal protein, Niemann-Pick type 2, which is involved in loading iGb3 onto CD1d [34]. However, it has recently been documented that the presence of iGb3 could not be detected in either human or murine lymphoid organs [36], casting doubt on the requirement of iGb3 for differentiation of *i*NKT cells. In addition, another research group has shown that *i*NKT cells normally develop in the thymus of iGb3 synthase-deficient mice [37]. Moreover, a defect in *i*NKT cells has been reported in multiple mouse models of lysosomal glycosphingolipids storage disease, irrespective of the specific genetic defect of lipid species stored; suggesting that storage of glycosphingolipids may disrupt the presentation of endogenous ligands by CD1d and non-specifically affect *i*NKT cell selection [38]. Therefore, it still remains unknown as to whether or not iGb3 is truly the exclusive ligand for *i*NKT cells.

2.2. α-GalCer

α-GalCer was originally isolated from glycolipid extracts isolated from marine sponge *Agelas mauritianus* during an attempt to seek substances with anti-tumor activity [39]. Studies carried out to explore the biological activity of α-GalCer against immune cells demonstrated that α-GalCer was a potent agonist for *i*NKT cells [40]. KRN7000 (Fig. **1C**), an analogue of α-GalCer modified for optimal productive condition, has been widely utilized for experimental and clinical studies. Although α-GalCer is a potent pharmacological activator of *i*NKT cells, it does not appear to be an endogenous ligand for *i*NKT cells. In fact, sugar moieties in mammalian glycolipids are essentially linked in β-anomeric position [7,40]; and the presence of lipids with α-anomeric sugar such as α-GalCer has not been confirmed in mammals. Therefore, it is very likely that activation of *i*NKT cells by α-GalCer is qualitatively different from that by natural endogenous antigens with β-anomeric sugar.

Following activation with α-GalCer, *i*NKT cells release large amounts of IL-2, IL-4, IL-5, IL-10, IFN-γ, and TNF-α and, on a per cell basis, the amount of these cytokines secreted by activated *i*NKT cells is much greater than by conventional T cells. This rapid and abundant cytokine production by *i*NKT cells may be accounted for by the presence of pre-existing transcripts for these cytokines in naïve *i*NKT cells [41-43]. In support of this, histone hyperacetylation of IL-4 and IFN-γ promoters could take place dynamically possibly following recognition of unknown endogenous ligands, resulting in the pre-activated phenotypes of *i*NKT cells.

Cytokines released from activated *i*NKT cells can act as intercellular mediators in the entire immune network. For example, when α-GalCer is injected into mice intraperitoneally, serum levels of IFN-γ rise immediately and then persist for several hours [44,45]. Studies have revealed that not only *i*NKT cells, but also NK cells, are major producers of IFN-γ in mice injected with α-GalCer [45,46] and that the NK cell production of IFN-γ is preceded by cellular cross-talk between *i*NKT cells, dendritic cells (DCs) and NK cells. It is now known that the IFN-γ produced by *i*NKT cells plays a role in this cross-talk, although we can not ignore the roles of other molecules such as costimulatory molecules expressed by DCs, as well as IL-12 produced by DCs [9,47,48].

2.3. Synthetic α-GalCer Analogues

Although α-GalCer is a potent agonist for *i*NKT cells; stimulation of *i*NKT cells with α-GalCer induces production of both Th1 and Th2 cytokines. Given that Th1 cells principally counteract with Th2 cells *via* production of cytokines, the non-selective induction of counteracting cytokines could lower the value of α-GalCer as an immunomodulatory agent. In fact, our attempts to treat an animal model of MS, experimental autoimmune encephalomyelitis (EAE), with α-GalCer were successful only when we used mice lacking IFN-γ in which *i*NKT cells production of cytokines is biased for Th2 [44]. A selective activator of Th2 cytokines from *i*NKT cells is more preferable as a therapeutic agent for treatment of autoimmune diseases. Therefore, we screened synthetic α-GalCer analogues for their ability to activate and polarize *i*NKT cell cytokine production, and identified OCH as a first synthetic compound that could be regarded as a Th2 selective inducer (Fig. **1C**) [49]. OCH has a shorter sphingosine chain compared with α-GalCer, and provokes a relatively weak proliferative response of *i*NKT cells. Critically, OCH stimulation of *i*NKT cells *in vitro* or *in vivo* induced much lower levels of IFN-γ secretion, as compared to α-GalCer stimulation; whereas similar levels of IL-4 were produced after OCH or α-GalCer stimulation of *i*NKT cells. These data suggest that, owing to its ability to polarize *i*NKT responses towards Th2, OCH may have a greater efficacy than α-GalCer in suppressing inflammatory pathology mediated by Th1 cells. Indeed, although injecting α-GalCer did not ameliorate EAE induced in wild-type B6 mice, we demonstrated that in this disease model OCH had a significant preventive and therapeutic effect [49].

Crystal structure analysis showed that two lipid tails of α-GalCer are bound to highly hydrophobic groove of CD1d [50]. Subsequent analysis showed that the amount of IFN-γ triggered by an α-GalCer analogue was positively correlated with the length of its sphingosine chain. In contrast, there was no clear correlation between IL-4 production by *i*NKT cells and the length of sphingosine chain [51]; indicating that the shorter lipid tail may offer a structural basis for the Th2 inducing glycolipid OCH. Based on this premise, we speculated that a selective deviation towards Th2 cytokine production following OCH stimulation may correlate with an unstable association between this glycolipid and CD1d due to its shorter sphingosine chain. Consistently, α-GalCer analogues bearing a shorter sphingosine chain tended to have a shorter half-life in the binding to CD1d, indicating that TCR ligation by OCH is likely to be less persistent than that by α-GalCer. Intriguingly, TCR stimulation of *i*NKT cells with anti-CD3 antibody for a shorter time period (a few hours) induced a detectable amount of IL-4, but not of IFN-γ *in vitro*. In fact, IFN-γ production by *i*NKT cells required TCR

stimulation for a longer period of time. Taken together, we postulated that the differential cytokine profiles triggered by α-GalCer or OCH may result from a differential duration of TCR stimulation, due to the differences in length of their lipid tail that correlate with their binding stability to the CD1d molecule.

A previous study using a protein synthesis inhibitor indicated that cytokines produced by *i*NKT cells could be divided into two groups based on their dependency on *de novo* protein synthesis. IFN-γ belongs to the group of cytokines that would require *de novo* proteins synthesis, whereas IL-4 represents the group of cytokines that do not require additional synthesis of proteins. Our study has shown that protooncogene c-Rel, a member of the nuclear factor κB (NF-κB) family of transcription factors, is an important molecule in α-GalCer-induced transcription of the IFN-γ gene [51]. We have proposed a model in which the longer sphingosine chain of α-GalCer confers the ability to continuously stimulate *i*NKT cells, inducing long lasting calcium influx, which results in a sustained nuclear residence of nuclear factor of activated T-cells (NF-AT), as well as c-Rel protein synthesis. The sequence of events leads to the activation of IFN-γ promoter, enabling *i*NKT cells to produce IFN-γ. In contrast, the rather sporadic stimulation of *i*NKT cells by OCH induces a short-lived nuclear residence of NF-AT, followed by an insufficient c-Rel expression, which prohibits an efficient production of IFN-γ from *i*NKT cells. McCarthy *et al.* confirmed that shortening the phytosphingosine chain increased the rate of lipid dissociation from CD1d molecule and induced less sustained TCR signals [52]. In addition, they also demonstrated there was a decreased affinity of TCR to CD1d-bound OCH. Both the lower stability of the association of OCH and CD1d and the lower affinity of TCR and OCH/CD1d complex may contribute to the less sustained stimulation of TCR.

Activation of *i*NKT cells with α-GalCer promotes vigorous IFN-γ production by bystander cells such as NK cells. It is now thought that differential effects of α-GalCer and OCH injected *in vivo* could be partly explained by a reduced ability of OCH to stimulate production of IFN-γ from NK cells, partly due to lack of IFN-γ provided by *i*NKT cells. It is likely that the differential outcome following α-GalCer and OCH stimulation is also generated by differentially altered DCs function. In fact, we found that *in vivo* injection of OCH induces only one tenth of IL-12 in the serum of mice, as compared with α-GalCer injection [45]. Simultaneous injection of stimulating anti-CD40 antibody and IFN-γ with OCH restored IL-12 induction and administration of IL-12 together with OCH led to IFN-γ production by NK cells [45]. Therefore, the lower IL-12 production is to be linked with a lower expression of CD40L on *i*NKT cells, following OCH stimulation, although the lower levels of IFN-γ produced by *i*NKT cells and NK cells may also play a role [53–55]. In summary, OCH induces a lower amount of IFN-γ from *i*NKT cells due to the less sustained stimulation of TCR. In the context of the elaborate cellular network *in vivo*, OCH stimulation of *i*NKT cells is less efficient at cross-activating DCs and NK cells, due to less CD40L expression on the surface of *i*NKT cells and a reduced secretion of IL-12 by DCs, leading to a lower production of IFN-γ by NK cells.

An acquisition of Th2-biasing ability seems to be a general consequence of truncations in the acyl or sphingosine chains of α-GalCer [56]. PBS-25 is a synthetic compound with short of acyl chains (C8) compared with α-GalCer bearing a C26 fatty acid chain (Fig. **1C**) [57]. Thus, *i*NKT cells stimulated with this ligand would show a Th2 profile compared with those stimulated with α-GalCer. However, the mechanism of Th2-biased cytokine production mediated by PBS-25 seems different from that mediated by OCH, since, like α-GalCer, PBS-25 binds stably to CD1d [58]. Apart from the lipid chain truncation of α-GalCer, α-GalCer analogue, C20:2, which is distinguished by unsaturation of the fatty acid portion, has been reported to be a Th2 skewing ligand [59]. C20:2 contains a C20 fatty acid with cis-unsaturations at positions 11 and 14 (Fig. **1C**) [59,60]. Although C20:2 elicits a Th-2-biased response similar to those induced by OCH, the mechanisms of preferentially inducing Th2 cytokines appears to be different from OCH and remains to be clarified. Conversely, synthetic C-glycoside analogue of α-GalCer (α-C-GalCer) (Fig. **1C**) elicits Th1 type responses of *i*NKT cells even at very low concentrations and exhibits a more potent anti-malaria and anti-tumor effect compared to α-GalCer [61]. To overcome the poor solubility of α-GalCer *in vivo*, a pegylated derivative of α-GalCer (α-GalCerMPEG) has been designed and is found to be a more efficacious compound than α-GalCer [62], although the mechanisms underlying the Th1-biased response remain unclear.

3. THE EFFECT OF SYNTHETIC GLYCOLIPID ANTIGENS ON HUMAN *i*NKT CELLS

Early studies have documented that there is a reduced number of *i*NKT cells in the peripheral blood of patients suffering from systemic sclerosis [63], MS [5,64,65], and other autoimmune disease conditions [66-68]. However, inconsistent results (decreased [69,70], normal [71] or increased [72] numbers of *i*NKT cells) obtained in type 1 diabetes patients by three independent groups has led to considerable argument on the role of *i*NKT cells in autoimmunity. Moreover, *i*NKT cells derived from patients with atherosclerosis [73] appear to participate in promotion of the diseases.

The potent therapeutic efficacy of synthetic α-GalCer analogues has been reported in various autoimmune disease models: EAE [49,74-76], type 1 diabetes mellitus [77-81], arthritis [82-84], lupus [85-89] and experimental inflammatory bowel disease [90,91]. Since mouse and human *i*NKT cells share similar characteristics in function and antigen recognition, these promising results in disease models raised the possibility of the development of *i*NKT cell-mediated immunotherapy for human autoimmune diseases. However, treatment with α-GalCer sometimes exacerbated EAE [74,75] and lupus models [88,89] probably due to Th1 responses elicited by *i*NKT cell stimulation. To avoid such a deleterious effect in human, it is obvious that choice of glycolipids for clinical application requires systemic evaluation using human *i*NKT cells.

The frequency of *i*NKT cells in the peripheral blood is approximately 0.1 % in healthy human individuals (Fig. **2A**) [5,7]. Such a low cell number might raise some concern as to

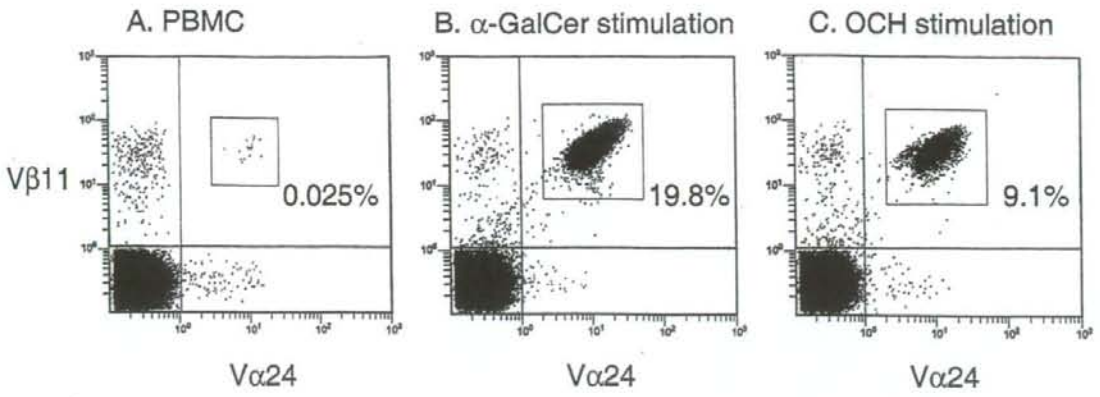

Fig. (2). Identification of Vα24$^+$Vβ11$^+$ NKT cells in PBMCs and α-GalCer/OCH-stimulated cultures.

PBMCs obtained from healthy subjects were stained with anti-Vα24 and -Vβ11 mAbs. This combination of antibodies has been widely used for identifying human *i*NKT cells . (**A**) The percentage value represents the frequency of the Vα24$^+$Vβ11$^+$ NKT cells among total lymphocytes. (**B** and **C**) α-GC- or OCH-stimulated cultures on day 7 were stained with those mAbs. The data shown here are representative out of five samples.

whether or not *i*NKT cells are relevant targets for human diseases. However, stimulating human peripheral blood mononuclear cells (PBMCs) with α-GalCer leads to a remarkable expansion of *i*NKT cells *in vitro* (Fig. **2B**). Furthermore, we have found that OCH also expands *i*NKT cells *in vitro*, although its potency to induce proliferation of human *i*NKT cells was inferior to that of α-GalCer (Fig. **2C**), suggesting that OCH could be an efficient ligand for human *i*NKT cells even if the binding affinity of OCH to human CD1d is weaker than that of α-GalCer as found in rodents. To further analyze the responsiveness of *i*NKT cells to OCH in detail, we have generated CD4$^+$ and DN *i*NKT cell clones using a single cell sorting technique. We established four CD4$^+$ and four DN *i*NKT cell clones from two healthy individuals by stimulating a single sorted CD4$^+$ NKT cell and DN NKT cell with human rIL-2, human rIL-7, and PHA-P in the presence of irradiated autologous PBMCs. All clone cells positively stained with 6B11, a specific mAb to Vα24-JαQ junction. We also confirmed that the clone cells express the invariant Vα24-JαQ rearrangement by performing DNA direct sequencing. All of the *i*NKT cell clones were found to proliferate upon stimulation with either α-GalCer or OCH in the presence of CD1d-transfected cells as APCs. As the APCs do not express conventional MHC class I or class II molecules, the clone cells should recognize glycolipid antigens in a CD1d-dependent manner. We have also generated *i*NKT cell clones using α-GalCer as a primary stimulant. These *i*NKT cell clones have maintained their reactivity to α-GalCer but were unable to generate any responses to OCH. Although not as yet proven experimentally, we speculate that an initial stimulation of *i*NKT cells with a strong agonist such as α-GalCer might heighten the threshold for signals needed to activate *i*NKT cells, and therefore, a weaker ligand like OCH may not be able to induce a detectable response upon restimulation.

When we evaluated cytokine production profiles of single cell-sorted clones, we saw a tendency for CD4$^+$ *i*NKT cell clones to produce greater amounts of cytokines than DN *i*NKT cell clones (Fig. **3A**). Following α-GalCer stimulation, CD4$^+$ *i*NKT cell clones produced large amounts of IFN-γ and TNF-α. They also produced modest amounts of IL-2, IL-4, IL-5 and IL-10. DN *i*NKT clones produced only a trace amount of these cytokines. Compared with α-GalCer, OCH stimulation induced production of lower amounts of Th1 cytokines (IFN-γ, TNF-α, and IL-2) by CD4$^+$ *i*NKT cell clones. However, the levels of Th2 cytokines (IL-4, IL-5 and IL-10) were not altered from those induced by α-GalCer. On the other hand, DN *i*NKT cells produced mainly Th1 cytokines (IFN-γ and TNF-α) when stimulated with α-GalCer. The response of DN *i*NKT cells to OCH was much lower as compared with that of CD4$^+$ *i*NKT cells. To evaluate the ability of each ligand to polarize *i*NKT cells towards Th1 or Th2, we have calculated an "OCH/α-GalCer ratio" for each cytokine by dividing quantities of cytokine produced after OCH stimulation by those following α-GalCer stimulation. Thus a higher OCH/α-GalCer ratio for a given cytokine implies that OCH is more efficacious for inducing this cytokine, as compared with α-GalCer. The results showed that the ratios for Th2 cytokines were higher than those for Th1 cytokines (Fig. **3B**), supporting that OCH stimulation also polarize human CD4$^+$ *i*NKT cells towards Th2 cytokine production. Interestingly, that Goff *et al.* also demonstrated the

A.

		IFN-γ	TNF-α	IL-2	IL-4	IL-5	IL-10
CD4⁺-1	OCH	1600*	1972	N. D.	644	1621	259
	α-GC	6357	5949	48	887	695	290
	No Ag	78	66	N. D.	372	120	N. D.
CD4⁺-2	OCH	5562	9155	N. D.	424	70	1122
	α-GC	19772	23121	1567	1794	62	476
	No Ag	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.
CD4⁺-3	OCH	3025	4578	N. D.	1080	158	399
	α-GC	4163	11046	2972	1764	133	125
	No Ag	104	219	N. D.	64	N. D.	N. D.
CD4⁺-4	OCH	2570	10262	N. D.	540	3940	657
	α-GC	9708	37318	305	1448	3589	908
	No Ag	N. D.	20	N. D.	N. D.	N. D.	N. D.
DN-5	OCH	173	293	N. D.	26	1202	24
	α-GC	2167	5852	N. D.	325	9033	536
	No Ag	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.
DN-6	OCH	159	22	N. D.	N. D.	23	N. D.
	α-GC	1690	85	N. D.	N. D.	259	N. D.
	No Ag	N. D.	20	N. D.	N. D.	N. D.	N. D.
DN-7	OCH	150	102	N. D.	N. D.	38	N. D.
	α-GC	728	120	N. D.	N. D.	58	N. D.
	No Ag	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.
DN-8	OCH	264	38	N. D.	N. D.	45	N. D.
	α-GC	1490	54	N. D.	N. D.	179	N. D.
	No Ag	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.

*All values are expressed in pg/ml. The detection limit is 20pg/ml in this assay.
Ag: antigen, N. D.: Not detected

B.

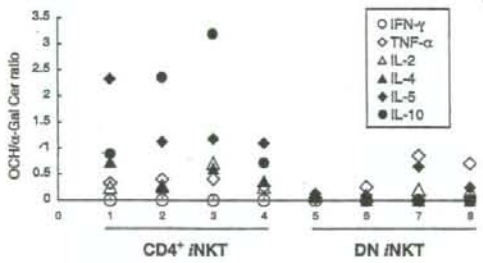

Fig. (3). Cytokine profile of CD4⁺ and DN *i*NKT cell clones.

(**A**) Four CD4⁺ *i*NKT clones and three DN *i*NKT clones were established from two donors respectively. In short, CD4⁺ and DN Vα24⁺ Vβ11⁺ cell were sorted by flowcytometry into single wells of a 96-well culture plate. Each well contained a mixture of irradiated (100Gy) autologous PBMCs at 1 x 10^5 cells/well supplemented with human rIL-2 (20 units/ml), human rIL-7 (10 ng/ml) and PHA-P (1μg/ml) in complete medium. To generate *i*NKT cell clones, the culture cells were re-stimulated three times with PHA, rIL-2 and rIL-7 in the presence of irradiated APC every 18~20 days, thereafter the purity of *i*NKT cells in the culture was more than 99%. These *i*NKT cells were confirmed to bear invariant TCR consisting of Vα24/JαQ by direct DNA sequence and Vβ11 by flowcytometry. CD4⁺ and DN *i*NKT cell clones were stimulated with α-GalCer or OCH in the presence of immature DC. Th1 cytokines (IFN-γ, TNF-α and IL-2) and Th2 cytokines (IL-4, IL-5 and IL-10) in the supernatants were examined using cytometric bead array. Four CD4⁺ clones from two donors and four DN clones from two donors were analyzed. To discern any alteration in cytokine secretion profile following OCH stimulation, the OCH/α-GalCer ratio for each cytokine was defined according to the following equation: the value (pg/ml) in α-GalCer-stimulation/ the value (pg/ml) in α-GalCer-stimulation. (**B**) Number 1~8 represents CD4⁺-1~DN-8 *i*NKT cell clone. The OCH/α-GalCer ratio of Th2 cytokines (IL-4, IL-5 and IL-10) are shown in filled black dots and similar ratios for Th1 cytokines (IFN-γ, TNF-α and IL-2) are depicted as open dots. CD4⁺ *i*NKT cell clones (No. 1-4) tended towards Th2, but DN *i*NKT cell clones (No.5-8) showed no alteration of cytokine profile.

Th2-biased cytokine production from human and murine *i*NKT cells when stimulated with α-GalCer analogue with short sphingosine chain [56].

4. CONCLUDING REMARKS

Targeting a single molecule that plays a crucial role in mediating inflammatory processes is a popular strategy to develop a new drug for autoimmune diseases. Although such a highly selective drug could powerfully suppress the development of disease, it is important to note that it is potentially dangerous to suppress the self-defensive immune system, particularly when considering responses against concurrent infections and the development of malignant tumors. This concern is particularly serious in the case of autoimmune diseases, as life-long treatment with conventional drugs

could prolong life expectancy to the normal level. In contrast, targeting immune regulatory cells that are defective in autoimmune diseases is conceptually less harmful. Among known regulatory cells, *i*NKT cells are a promising target since they recognize glycolipid antigen bound to monomorphic molecule CD1d. The advantage of glycolipid over peptide is obvious in that the glycolipid drug could elicit uniform responses in highly heterogeneous populations. On the other hand, peptide therapy needs to be individually designed for each patient considering the human MHC polymorphism. Since α-GalCer was identified as an *i*NKT cell ligand in 1997, the search for alternative ligands has greatly enriched our understanding of the biology of *i*NKT cells, and has led to the identification of novel therapeutic ligands such as OCH.

ABBREVIATIONS

APC	=	Antigen presenting cell
BbGL	=	*Borrelia burgdorferi* glycolipid
CHX	=	Cyclohexamide
DC	=	Dendritic cell
DN	=	Double negative
EAE	=	Experimental autoimmune encephalomyelitis
GM-CSF	=	Granulocyte/macrophage colony stimulating factor
GSL	=	Glycosphingolipid
IFN	=	Interferon
iGb3	=	Isoglobotrihexosylceramide
IL	=	Interleukin
*i*NKT	=	Invariant Natural Killer T
LPS	=	Lipopolysaccharide
mAb	=	Monoclonal antibody
MHC	=	Major histocompatibility complex
MS	=	Multiple sclerosis
NF-AT	=	Nuclear factor of activated T-cells
NF-κB	=	Nuclear factor kappa B
NK	=	Natural killer
PBMC	=	Peripheral blood mononuclear cell
rIL	=	Recombinant interleukin
T-bet	=	T-box expressed in T cells
TCR	=	T-cell receptor
Th	=	T helper
TNF	=	Tumor necrosis factor

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Overcoming drug resistance induced by P-glycoprotein on lymphocytes in patients with refractory rheumatoid arthritis

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ABSTRACT

Objective: P-glycoprotein (P-gp), a member of the ATP-binding cassette transporter family, causes drug resistance by exclusion of intracellular drugs. Here, we elucidate the clinical relevance of P-gp expression on lymphocytes to drug resistance in patients with rheumatoid arthritis (RA).

Methods: P-gp expression on lymphocytes from 20 normal volunteers and 100 RA patients was analysed by flow cytometry. Drug exclusion analysis of lymphocytes was conducted by radioisotope-labelled dexamethasone.

Results: P-gp was overexpressed on RA lymphocytes compared with normal lymphocytes. P-gp expression levels were higher in partial responders with a Disease Activity Score (DAS) 28-3 of >5.1 despite taking at least two disease-modifying antirheumatic drugs (DMARDs) or one DMARD and corticosteroids for at least 2 years. P-gp expression levels correlated with DAS28-3. Intracellular dexamethasone levels (IDLs) in RA lymphocytes decreased according to P-gp expression. Tacrolimus, a P-gp inhibitor, restored IDLs in RA lymphocytes. P-gp overexpression in patients with highly active RA was suppressed by methotrexate but enhanced by corticosteroids. Furthermore, infliximab (3 mg/kg) resulted in improvement of RA disease activity, reduction of P-gp and recovery of IDLs.

Conclusions: P-gp overexpression on lymphocytes might cause efflux of corticosteroids and DMARDs, P-gp substrates, from lymphocytes, resulting in drug resistance in patients with highly active RA. P-gp inhibition/reduction could overcome such drug resistance. Measurement of P-gp expression on lymphocytes could be a potentially useful marker for assessing drug resistance in RA, and may be suitable for selecting infliximab or DMARDs including tacrolimus for RA treatment.

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterised by progressive polyarthritis with occasional extra-articular involvement.[1] Progression of RA results in severe disability and loss of function with severe pain.[2] The ultimate goals of effective treatment are the control of abnormal immune system, erosive synovitis and prevention of joint destruction by administration of disease-modifying antirheumatic drugs (DMARDs) without delay. The main target of these DMARDs is the lymphocyte, but we often encounter refractory RA patients. Therefore, resistance to antirheumatic drugs in lymphocytes is one of the important issues to be overcome with regard to the treatment of RA.

Among the multiple mechanisms of multidrug resistance, overexpression of P-glycoprotein (P-gp), which is a 170-kDa product of the multidrug resistance (MDR)-1 gene, has emerged as the major molecule involved in multidrug resistance and prognosis during chemotherapy for various malignancies.[3–7] P-gp is a member of ATP-binding cassette transporter superfamily and functions as an energy-dependent transmembrane efflux pump. Overexpression of P-gp results in reduction of intracellular concentrations of substrates involving xenobiotics and various drugs such as vinca alkaloids, anthracyclines, verapamil, some DMARDs (eg, hydroxychloroquine, D-penicillamine, colchicines), and corticosteroids.[7–11] Thus, P-gp appears to be a double-edged sword, involved in protecting cells from these drugs and in the development of resistance to them.

In a previous study, we found that the transcription of MDR-1 gene in lymphocytes is induced by various lymphocyte-activation stimuli, eg, cytokines such as interleukin (IL)-2[12] and tumour necrosis factor (TNFα), and adhesion to extracellular matrix such as hyaluronan.[13] In this regard, immune stimuli, that play a critical role in the pathogenesis of RA,[14–20] might induce the expression of P-gp on RA lymphocytes. However, the expression of P-gp on lymphocytes, the regulatory mechanisms of P-gp expression, and clinical relevance of P-gp to drug resistance are not clear in RA.

The present study was designed to elucidate the relation between P-gp expression on lymphocytes in refractory RA patients and clinical features including drug resistance. We also determined the significance of evaluation of P-gp on peripheral blood lymphocytes on clinical decision-making regarding treatment strategies.

MATERIALS AND METHODS

Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs from 20 normal volunteers and 100 RA patients who fulfilled the American College of Rheumatology (ACR) revised criteria for RA were isolated by density gradient centrifugation using Lymphocyte Separation Medium 50494 (Pharmacia Biotech, Uppsala, Sweden) as described previously.[21 22] We confirmed by immunostaining that purified PBMCs contained more than 90% lymphocytes (CD4-, CD8- or CD19-positive cells) and less than 10% CD14-positive monocytes. The clinical activity of RA was assessed by the Disease Activity Score (DAS) 28-3 that was calculated by using tender joint counts, swollen joint counts, and erythrocyte sedimentation rate.[23 24] Table 1 summarises the demographic characteristics and clinical features of the RA patients.

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The study was approved by the institution human subjective research committee and informed consent was obtained from all the donors who were enrolled in the study.

Flow cytometry

Staining and flow cytometric analysis of PBMCs were conducted by standard procedures as described previously using a FACScan (Becton Dickinson, Mountain View, California, USA).[21 22] Briefly, PBMCs were initially plated onto a 96-well culture dish (2×10^5 cells/well) and incubated with polyclonal γ-globulin (10 μg/ml, Mitsubishi Welpharma Co., Osaka, Japan) to block Fc-receptors. These cells were then incubated with MRK-16 (100 μg/ml, Kyowa medex, Tokyo, Japan), a specific monoclonal antibody (mAb) against P-gp,[25] followed by the addition of fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG Ab (5 μg/ml, Fujisawa, Osaka, Japan) in FACS medium consisting of phosphate-buffered saline (PBS), 0.5% human serum albumin (HSA; Mitsubishi Welpharma), and 0.2% NaN_3 (Sigma Aldrich, Tokyo, Japan) for 30 min at 4°C. For the two-colour analysis, we incubated PBMCs with phycoerythrin (PE)-conjugated CD4mAb or CD19mAb (1.25 μg/ml, Fujisawa, Osaka, Japan) after blocking of free anti-mouse IgG-binding sites with irrelevant antibodies. The two-colour-stained cells were detected by electronic gating based on their CD4 or CD19 expression using a FACScan. Quantification of the cell surface antigens on one cell was performed using QIFIKIT beads (Dako, Kyoto, Japan) as described previously.[26] The data were used to construct a calibration curve of the mean fluorescence intensity vs anti-body-binding capacity. The cell specimen was analysed on the FACScan and the antibody-binding capacity calculated by interpolation on the calibration curve. When the green fluorescence laser detection was set at 500 nm in the FACScan, the antibody-binding capacity was equal to (202.98×exp (0.0092×mean fluorescence intensity), ($R^2 = 0.9995$)). Subsequently, the specific antibody-binding capacity was obtained after correcting for the background, and apparent antibody-binding capacity of the negative control anti-mouse IgG Ab. The specific antibody-binding capacity is the mean number of accessible antigenic sites per cell, referred to as antigen density and expressed in sites/cell.

Dexamethasone accumulation

^{14}C-Labelled *n*-butanol (1.61 mCi/mmol; Toho Biochemical, Tokyo, Japan) was diluted with unlabelled butanol (Sigma Aldrich Japan) at a concentration of 0.5 MBq/ml. ^{3}H-Labelled dexamethasone (40.0 Ci/mmol; Perkin, Boston, Massachusetts, USA) was dissolved in dimethylsulfoxide (DMSO; Nacalai Tesque, Tokyo, Japan) and then diluted in PBS (final concentration of DMSO 0.1%). PBMCs were suspended in PBS with 7 mM of dextrose for ATP supply, which is dispensable in this assay,[27] at a cell density of 5×10^6 cells/ml. The PBMCs were then incubated with 5.0×10^{-5} M of ^{14}C-labelled *n*-butanol and 3.0×10^{-8} M of ^{3}H-labelled dexamethasone for 20 min time range at 37°C.

Tacrolimus, which is effective for refractory RA patients,[28 29] had been reported to inhibit P-gp competitively.[29 30] The clinical safety dose of tacrolimus for RA patients is less than 3 mg/day and the mean (SD) blood concentration of tacrolimus (about 12 h after 3 mg dose of tacrolimus) is 5.3 (2.0) ng/ml (SD).[31] In preliminary studies, we prepared PBMCs that overexpressed P-gp by stimulation with IL-2. Tacrolimus inhibited the secretion of dexamethasone from these cells in a concentration-dependent manner, from 1–50 ng/ml, through a significant inhibition of P-gp by 10 ng/ml of tacrolimus (data not shown). The latter concentration was used in the following experiments. For competitive studies with tacrolimus, PBMCs were incubated with 10 ng/ml of tacrolimus (Fujisawa) for 15 min and then incubated with ^{14}C-labelled *n*-butanol and ^{3}H-labelled dexamethasone. Tacrolimus was dissolved in DMSO before diluting with PBS (final concentration of DMSO 0.03%). After incubation, 100 l of aliquots were layered on 80 l of a mixture of lauryl bromide and silicone oil (2:1 ratio; Nacalai Tesque) in an Eppendorf tube (Assist, Tokyo, Japan). After centrifugation at 10 000 rpm for 2 min, the aliquots were rapidly frozen in liquid nitrogen, the frozen tube was cut between medium–mixture bound areas, thereby obtaining the upper layer as the medium fraction and the lower layer as the cell fraction.

The cell fractions were melted with soluene-350 and 10 ml of Hionic-Fluor (Packard, Meriden, Connecticut, USA) was added. The medium fractions were mixed with 10ml of mixtures of toluene (Wako, Osaka, Japan), methanol (Wako), ethylene glycol monoethyl ether (Nacalai Tesque) and Permafluor (200:50:50:12 ratio; Packard). The radioactivity of each fraction was counted with a scintillation counter. The cell to medium ratio (C/M ratio), which is an index of intracellular and extracellular dexamethasone concentration ratio, was computed using the following formula: C/M ratio = ((^{3}H in cell fraction/^{14}C in cell fraction)/(^{3}H in medium fraction/^{14}C in medium fraction)).

Infliximab infusion

Eleven patients with refractory RA and P-gp-overexpressing lymphocytes were treated with infliximab. After an induction regimen of 3 mg/kg at weeks 0, 2, and 6, all patients received maintenance therapy every 8 weeks. PBMCs from these 11 RA patients were isolated before infliximab infusion and at weeks 0 and 2. During the first 2 weeks, corticosteroids, methotrexate (MTX) and other DMARDs dosages were kept unchanged.

Statistical analysis

Results are expressed as mean (SD). Student t test was used to compare data of two groups. One way ANOVA followed by Fisher PLSD for multicomparison was used to compare data among three or more groups. Correlations between two variables were examined using Pearson correlation analysis. In the figures, the linear regression line is shown together with the Pearson correlation coefficient (r) and the respective correlation p value. p Values less than 0.05 were considered statistically significant.

Table 1 Characteristics of the study subjects

	RA patients (n = 100)
Age (years)	57 (24–85)*
Sex (females/males)	91/9
Disease duration (years)	8.8 (0.4–32)*
DAS28-3	5.5 (2.3–7.6)*
Steinbrocker stage (I/II/III/IV)	6/35/19/40
ACR functional class (1/2/3/4)	5/81/11/3

*Data are median (range).
ACR, American College of Rheumatology; DAS28-3, Disease Activity Score 28-3; RA, rheumatoid arthritis.

RESULTS

Expression of cell surface P-glycoprotein on peripheral lymphocytes of RA patients

We examined the expression of P-gp using mAb against the MRK-16 epitope of P-gp on peripheral blood lymphocytes from

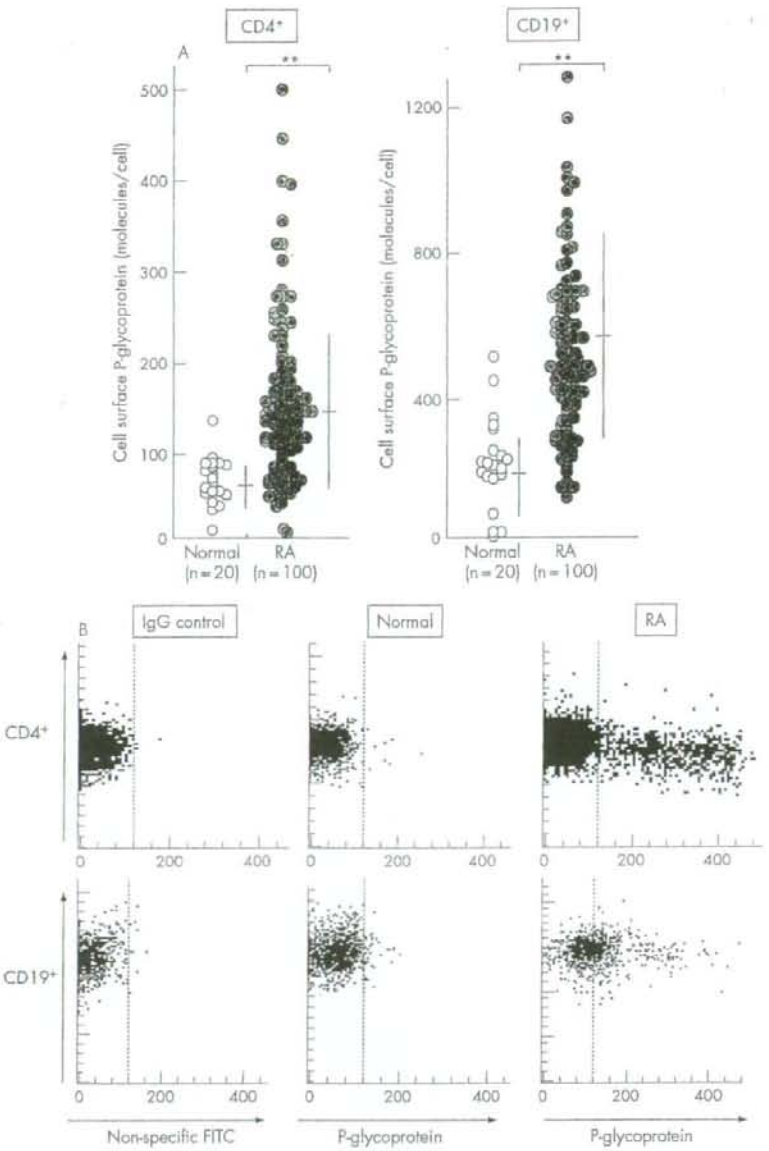

Figure 1 Expression of P-glycoprotein (P-gp) on lymphocytes from patients with rheumatoid arthritis (RA), as determined by flow cytometry. A. P-gp expression on peripheral CD4+ and CD19+ peripheral blood lymphocytes from 20 normal volunteers (open circles) and 100 RA patients (dotted circles). Results were calculated with the use of standard QIFIKIT beads. Values are the mean and SD of independent experiments. **p<0.01, by unpaired t test. B. Typical P-gp expression on CD4+ and CD19+ peripheral blood lymphocytes from a normal volunteer and an RA patient with high P-gp expression. The dotted line represents the gate set to discriminate negative from positive stained cells as determined by control FITC-conjugated anti-mouse IgG Ab.

100 RA patients and 20 normal volunteers. The expression of P-gp was significantly high on most of peripheral CD4+ and CD19+ lymphocytes of RA patients compared with normal lymphocytes. The level of P-gp expression on RA lymphocytes ranged from moderate to high, whereas the expression on normal lymphocytes ranged from marginal to moderate, with most expressing marginal levels (fig 1A). Figure 1B shows typical pattern of P-gp expression on lymphocytes of RA patients and normal volunteers. In RA patients, CD4+ lymphocytes were divided into P-gp high or low expressing subgroups, and most CD19+ lymphocytes expressed P-gp at moderate or high levels.

Relationship between drug resistance and expression of P-gp

Clinically, we sometimes encounter RA patients with high disease activity who do not respond to treatment with DMARDs or oral corticosteroids, although the mechanism of this drug resistance is not clear. To analyse the relationship between clinical responsiveness to treatment and the level of expression of P-gp on lymphocytes, we defined partial responders as those: (1) with DAS28-3 of >5.1, (2) previously treated with at least two DMARDs or one DMARDs and corticosteroids, and (3) treated for at least 2 years. The P-gp expression levels on CD4+ and CD19+ lymphocytes were significantly increased in the partial responders (table 2).

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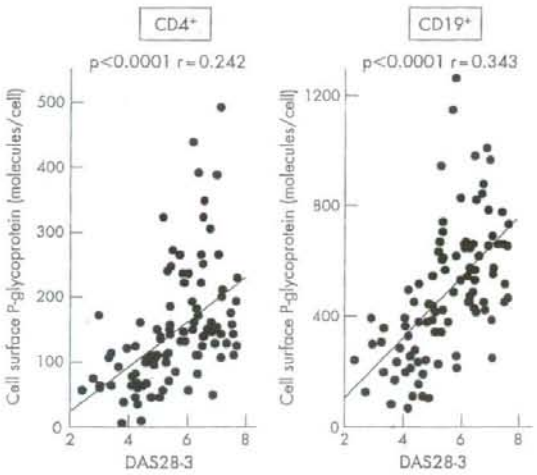

Figure 2 Correlation between P-glycoprotein (P-gp) expression on lymphocytes and scores on the Disease Activity Score (DAS) 28-3 in 100 patients with rheumatoid arthritis (RA). Levels of P-gp expression on RA lymphocytes correlated closely with disease activity of each patient, as estimated by the DAS28-3 score. Vertical axis: numbers of P-gp molecules per cell were calculated with the use of standard QIFIKIT beads. Horizontal axis: the disease activity of each RA patient, as estimated by DAS28-3. Pearson correlation analysis was used to determine statistical significance. n = 100.

Other investigators have reported that transcription of MDR-1 is induced in response to various drugs, including corticosteroids[22] in various malignancies.We then analysed the relationship between prior received drugs (DMARDs and corticosteroids) and the level of expression of P-gp on lymphocytes. However, P-gp expression on RA lymphocytes was not correlated with either the number of previously received DMARDs or prior corticosteroid therapy (table 2).

Relationship between disease activity and expression of P-gp

We investigated the relationship between DAS28-3 scores and P-gp expression on peripheral blood CD4+ and CD19+ lymphocytes from patients with RA. The levels of P-gp expression on RA lymphocytes correlated closely with the disease activity in each patient, as estimated by the DAS28-3 score (fig 2). Several RA patients with high disease activity (DAS28-3>5.1) had extremely high expression of P-gp on lymphocytes.

The level of P-gp on CD4+ and CD19+ lymphocytes was markedly increased in RA patients with high disease activity (DAS28-3 >5.1) than mild and moderate active RA patients (DAS28-3<5.1) (fig 3A). In the next step, we investigated the association between P-gp expression and drug exclusion through P-gp. For this purpose, dexamethasone accumulation analysis of PBMCs was conducted using radioisotope labelled dexamethasone. Intracellular dexamethasone levels in PBMCs of these highly active RA patients (DAS28-3>5.1) with high expression of P-gp on lymphocytes was significantly decreased compared with those in mild to moderate active RA patients (DAS28-3<5.1) and normal volunteers (fig 3B). To confirm the functional involvement of P-gp in the low levels of intracellular dexamethasone, we added tacrolimus, a competitive inhibitor of P-gp, to PBMCs from RA patients with high disease activity. Tacrolimus inhibited the secretion of dexamethasone from PBMCs of highly active RA patients (fig 3C).

Effects of MTX and corticosteroids on P-gp expression in highly active RA patients

The level of P-gp on lymphocytes in highly active RA patients (DAS28-3>5.1) varied extensively among individuals despite almost similar disease activity (figs 2 and 3A). MTX is the preferred first anchor drug in the treatment of RA. Recent evidence indicates that MTX efflux via P-gp is uncommon.[23] We found that immunosuppressants, not substrates of P-gp, induced reduction of P-gp expression on lymphocytes.[11] Therefore, in highly active RA patients, we analysed the relationship between P-gp expression level on lymphocytes and prior treatment with non-P-gp substrate drugs (eg, MTX), or corticosteroids (which are inducers and substrates of P-gp).[23] The disease activity was not significantly different between MTX users and MTX non-users or between corticosteroid users and corticosteroid non-users. However, the expression level of P-gp on CD19+ lymphocytes was significantly higher in corticosteroids users or MTX non-users compared with MTX users (table 3). We also analysed interaction between MTX and corticosteroids at the level of P-gp expression on lymphocytes.

Table 2 Expression of P-glycoprotein (P-gp) on lymphocytes of rheumatoid arthritis (RA) patients with resistance to treatment and those previously treated with disease-modifying antirheumatic drugs (DMARDs)

	P-glycoprotein, molecules/cell	
	CD4+ lymphocytes	CD19+ lymphocytes
Responders (n = 54)	123.5 (94.4)	365.0 (210.3)
Partial responders (n = 46)	167.1 (85.0)*	526.8 (209.0)*
Number of DMARDs used previously for treatment:		
0–1 (n = 20)	161.2 (111.0)	449.7 (219.8)
2 (n = 24)	116.5 (83.6)	358.6 (214.9)
3 (n = 24)	150.1 (73.1)	447.8 (243.6)
4 (n = 14)	141.5 (120.8)	433.2 (176.9)
5 (n = 18)	153.0 (80.8)	529.5 (230.1)
Corticosteroids		
User (n = 63)	145.5 (95.7)	469.5 (231.0)
Non-user (n = 37)	140.3 (87.7)	388.2 (204.0)

Values are mean (SD). *p<0.01, compared with responders. There were no differences in P-gp expression on each CD type based on the number of DMARDs or the use of corticosteroids.

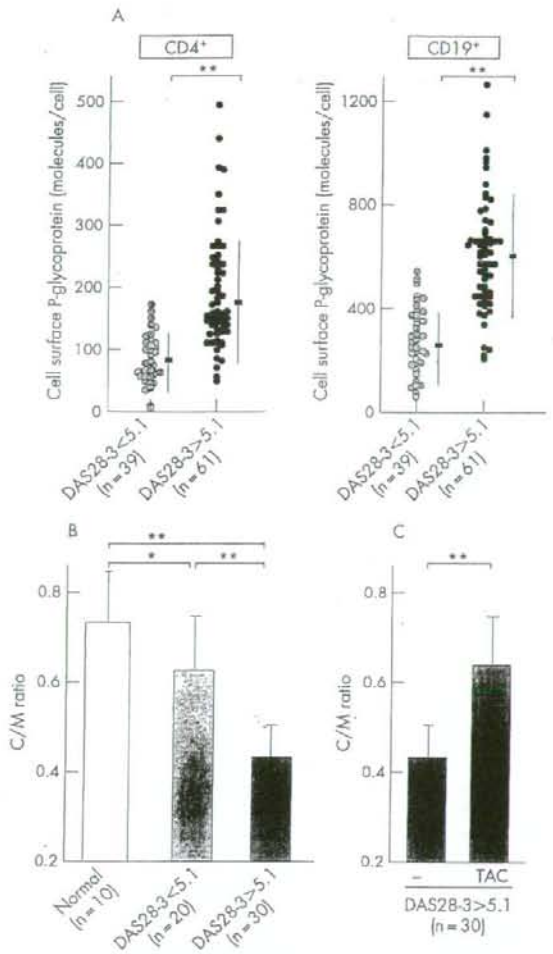

Figure 3 Decrease in intracellular dexamethasone levels in peripheral blood mononuclear cells (PBMCs) from rheumatoid arthritis (RA) patients based on levels of P-glycoprotein (P-gp) expression on lymphocytes and inhibition of dexamethasone excretion through P-gp by tacrolimus. A. Flow cytometric analysis showed P-gp expression on peripheral CD4+ and CD19+ lymphocytes in 39 RA patients whose DAS28 were less than 5.1 points (hatched circles) and 61 highly active RA patients whose DAS28 were more than 5.1 points (solid circles). Data represent the number of molecules expressed per cell, calculated using standard QIFIKIT beads. Data are mean (SD). **p<0.01, by non-paired t test. B. Intracellular dexamethasone was evaluated by the C/M ratio of PBMCs in 10 normal volunteers (open bar), 20 RA patients whose DAS28 were less than 5.1 points (hatched bar) and 30 highly active RA patients whose DAS28 were more than 5.1 points (solid bar). Data are mean (SD). Statistical analysis was performed using the non-paired t test. *p<0.05, **p<0.01. C. C/M ratio of PBMCs in 30 highly active RA patients whose DAS28 were more than 5.1 points was evaluated in the absence or presence of 10 ng/ml of tacrolimus. Values are mean and SD of 30 independent experiments. Statistical analysis was performed using the paired t test. **p<0.01.

The expression of P-gp on CD19+ lymphocytes was significantly high in patients treated with corticosteroids without MTX, compared with those treated with MTX without corticosteroids, despite the same disease activity level (table 4, fig 4). These results indicate that the expression of P-gp on lymphocytes in highly active RA is enhanced with increased disease activity, in association with lymphocyte activation, and is modified by medication.

Effects of infliximab on P-gp expression

The expression of P-gp is induced by extracellular stimuli such as lymphocyte-activating cytokines, including IL-2, and adhesion to extracellular matrix proteins such as hyaluronan.[12 13] In fact, cytokines and other immune-stimuli activated lymphocytes in RA patients with high disease activity apparently acquired P-gp overexpression. Based on the above experiments, we provided infliximab as an intravenous infusion (3 mg/kg) in 11 patients with refractory RA whose lymphocytes expressed high levels of P-gp despite being treated with MTX (table 5). Then, we evaluated P-glycoprotein expression on lymphocytes and the levels of intracellular dexamethasone before and 2 weeks after infusion.

Surprisingly, a single infusion of infliximab significantly reduced P-gp on CD4+ and CD19+ lymphocytes (fig 5A). Figure 5B demonstrates down-regulation of P-gp expression on lymphocytes from four representative cases. Two weeks after infliximab infusion, the P-gp high-expressing subgroups of CD4+ lymphocytes were eliminated, and most CD19+ lymphocytes expressed P-gp at only low levels. Reduction of P-gp resulted in recovery of intracellular dexamethasone levels in PBMCs of these RA patients (fig 5C), and then acquired improvement of disease activity (fig 5D).

DISCUSSION

In pathogenesis of RA, lymphocytes are the main orchestrators of immune responses. Intercellular adhesion such as antigen presentation, cytokines, and adhesion to the extracellular matrix (ECM) transduce signals via functional molecules on lymphocytes, resulting in activation of lymphocytes in RA.[14] These activated lymphocytes enhance inflammatory and destructive arthropathy.

We found that lymphocyte activating cytokines, such as IL-2[13] and TNF, up-regulated the expression of P-gp on lymphocytes via activation of transcriptional factor YB-1 and that such up-regulation markedly reduced intracellular corticosteroid concentration in vitro.[12] The serum and synovial concentrations of these cytokines are high in active RA patients.[15–17] We also found that fragmented hyaluronan, detected in certain pathological conditions such as inflammation and tumours,[18 34] induced YB-1 activation followed by P-gp expression in accordance with activation of CD4+ T cells. Fragmented hyaluronan is increased in the RA synovium and synovial fluid.[19 20] Although the production of native hyaluronan by RA synovial cells is promoted according to the aggravation of inflammation by inflammatory cytokines including IL-1 and TNF,[35] the activity of hyaluronidase produced by CD14+ monocytes is so high that native hyaluronan is digested to fragmented hyaluronan immediately at the loci of inflammation.[36] Furthermore, the production of fragmented hyaluronan is accelerated due to oxygen-derived free radicals in the inflammation locus, as in synovitis.[19 20]

Therefore, lymphocytes in RA patients with high disease activity are activated by cytokines and ECM adhesion, resulting in acquiring MDR-1-mediated multidrug resistance. Thus, the results of our studies showed overexpression of P-gp on CD4+