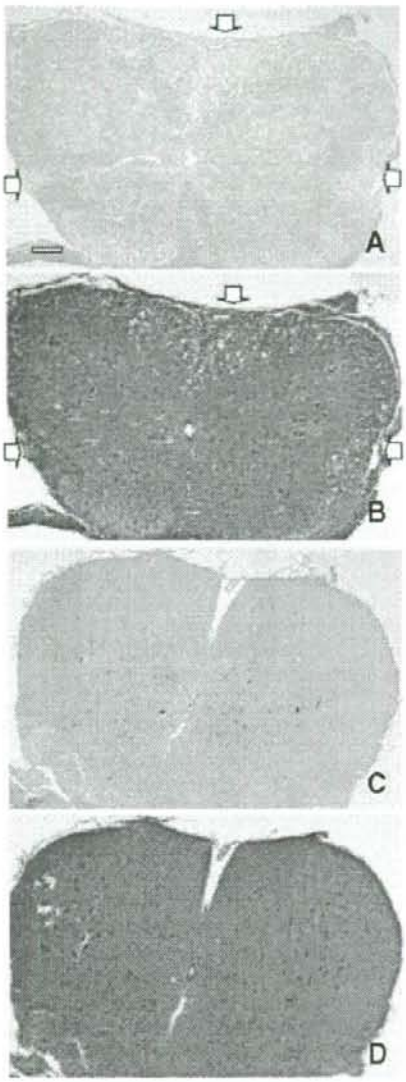

Fig. 2 Histopathological assessment of the CNS region in EAE-induced mice. Brains and spinal cords from EAE mice were removed on Day 14 after immunization as described in Material and methods. Thinly sliced (10 μm) frozen sections of the brains obtained from vehicle-treated mice (**A** and **B**) or celecoxib-treated mice (**C** and **D**) were stained with haematoxylin and eosin (**B** and **D**), or Luxol fast blue (**A** and **C**).

post-EAE-induction. Although indomethacin suppressed EAE to some extent, all mice died around Day 30 after immunization owing to intestinal ulcer. In contrast, oral administration of nimesulid, another COX-2 inhibitor, did not suppress either the incidence or the severity of EAE (Fig. 1B). Composite data from experiments is shown in Tables 1 and 2.

Celecoxib inhibits MOG-specific Th1 response

To determine the mechanisms by which celecoxib inhibits EAE, we examined the level of MOG-specific IgG1 and IgG2a in the serum samples collected from individual EAE-induced mice on Day 30. It is generally accepted that elevation of antigen-specific IgG2a antibody results from augmentation of a Th1 immune response to the antigen, whereas a higher level of IgG1 antibody would reflect a stronger Th2 response to the antigen. There was a significant elevation of the level of MOG_{35-55}-specific IgG1 and a slight reduction in the level of MOG-specific IgG2a in celecoxib-treated group compared with vehicle-treated group (Fig. 3A). In contrast, there was no significant difference in the level of either IgG1 or IgG2a in nimesulid-treated mice compared with vehicle-treated group (Fig. 3B).

To further investigate the response of T cells to MOG_{35-55} in celecoxib-treated mice, we examined the proliferative response and cytokine production of draining LN cells *in vitro*. Mice were immunized with MOG_{35-55} and were administered celecoxib or vehicle on the day of immunization. Ten days after immunization, draining LN cells were collected and cultured with MOG_{35-55} peptide. As shown in Fig. 4A, there was no significant difference in a proliferative response of MOG-reactive T cells between celecoxib-treated and vehicle-treated groups. We next examined the levels of cytokines in the culture supernatant by ELISA. The level of IFN-γ was reduced in the culture supernatants of LN cells obtained from mice treated with celecoxib compared with that from control mice (Fig. 4B). IL-4 and IL-10 were not detected in either culture supernatant. These results indicate that celecoxib reduces Th1 cytokine production from MOG-reactive T cells.

Celecoxib prevents EAE even in COX-2-deficient mice

Since another COX-2 inhibitor, nimesulid, did not have the inhibitory effect on EAE, we examined whether celecoxib could inhibit EAE in COX-2-deficient mice. As shown in Fig. 5A, the maximum EAE score, the day of onset and the severity of EAE were not significantly different between COX-$2^{-/-}$ and wild-type mice. Administration of celecoxib prevented the development of EAE in COX-$2^{-/-}$ mice as well as in wild-type mice. Consistent with the severity of EAE, the levels of MOG-specific IgG1 and IgG2a in COX-$2^{-/-}$ mice were not different compared with wild-type B6 mice (Fig. 5B). Moreover, celecoxib treatment increased the level of MOG-specific IgG1 even in COX-$2^{-/-}$ mice, resulting in the elevation of IgG1 : IgG2a ratio similar to that in wild-type mice (CMC = 0.29, celecoxib = 3.00) and COX-$2^{-/-}$ mice (CMC = 0.42, celecoxib = 2.52). These results indicate that the effect on the inhibition of EAE

Table 2 Clinical scores of EAE treated with celecoxib or other non-steroidal anti-inflammatory drugs

	Max. score	Day of onset	Incidence (%)	Cumulative score	Death (%)
Control (CMC)	3.05 ± 0.20	13.10 ± 1.16	100 (10/10)	26.47 ± 5.13	10 (1/10)
Celecoxib	1.02 ± 0.53*	14.30 ± 1.77	90 (9/10)	7.58 ± 6.72*	0 (0/10)
Nimesulid	2.54 ± 0.68	13.50 ± 1.56	100 (10/10)	22.15 ± 4.75	0 (0/10)
Indomethacin	1.70 ± 0.83	13.90 ± 1.93	100 (10/10)	15.21 ± 3.89	100 (10/10)*

Each mouse was immunized with MOG_{35-55} peptide for induction of EAE. The control CMC solution, or 5 μg/g of drugs diluted in CMC, was orally administered via a cannula every other day. Mean ± SEM of the following parameters are shown: maximum score of EAE (Max. score), the days of EAE onset, incidence of paralysed mice among sensitized rats (Incidence), summation of the clinical scores from Day 0 to 30 (Cumulative score) and the incidence of death during EAE (Death). *$P < 0.05$ versus control.

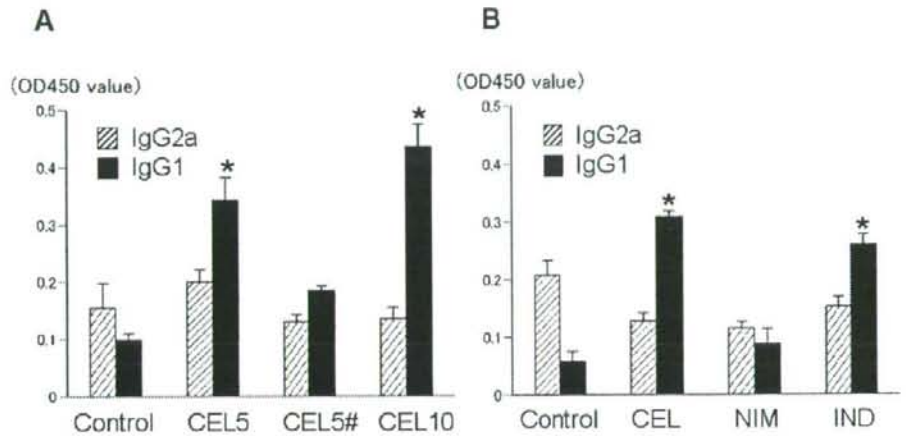

Fig. 3 Analysis of MOG_{35-55} IgG1 and IgG2a in EAE-induced mice. The relative titers of anti-MOG IgG1 and IgG2a in serum samples from individual mice ($n = 10$) on Day 30 after immunization were analysed as indicated in Methods. Data represent mean ± SEM. *$P < 0.05$ versus control. (**a**) Control = vehicle alone, CEL5 = 5 μg/g of celecoxib, CEL5# = 5 μg/g of celecoxib from Day 8 after the immunization, CEL10 = 10 μg/g of celecoxib. (**b**) Control = vehicle alone, CEL = celecoxib, NIM = nimesulid, IND = indomethacin.

and Th1 response by celecoxib is mediated by a COX-2-independent pathway (Table 3)

Celecoxib inhibits an infiltration of immune cells into CNS

To characterize the infiltrated cells into CNS, we isolated mononuclear cells from CNS obtained from celecoxib-treated or vehicle-treated mice. Mononuclear cells isolated from the CNS of vehicle-treated mice include $CD3^+$ T cells that comprised >80% of $CD4^+$ cells. In mice treated with celecoxib, the number of infiltrated cells was less than one-seventh compared with vehicle-treated mice (Table 4). In addition, we analysed apoptotic cells from CNS, spleen and draining LNs using annexin-5 staining. There was no difference in the frequency of apoptotic cells in all organs examined from celecoxib-treated and vehicle-treated mice (data not shown). These results suggest that celecoxib inhibits an infiltration of inflammatory cells into the CNS rather than induction of apoptosis of autoreactive T cells.

Celecoxib suppresses the expression of adhesion molecules and a chemokine related to cell infiltration into CNS

For the recruitment of autoreactive T cells into the brain through the blood–brain barrier (BBB), some adhesion molecules such as ICAM-1, VCAM-1 and P-selectin, and chemokines such as MCP-1 are required (Engelhardt *et al.*, 1997; Hofmann *et al.*, 2002). We performed an immunohistostaining of sliced brain sections from mice with EAE using antibodies against adhesion molecules. ICAM-1, VCAM-1 and P-selectin (Fig. 6A, C and E) were expressed on choroid plexus in the brain obtained from EAE-induced mice. In contrast, in brains obtained from celecoxib-treated mice, the expression level of P-selectin and ICAM-1 was lower compared with the control (Fig. 6B, D and F). In addition, we examined the level of MCP-1, which is an important chemokine involved in recruiting autoreactive T cells into the brain. As shown in Table 5, the level of MCP-1 in the serum obtained from

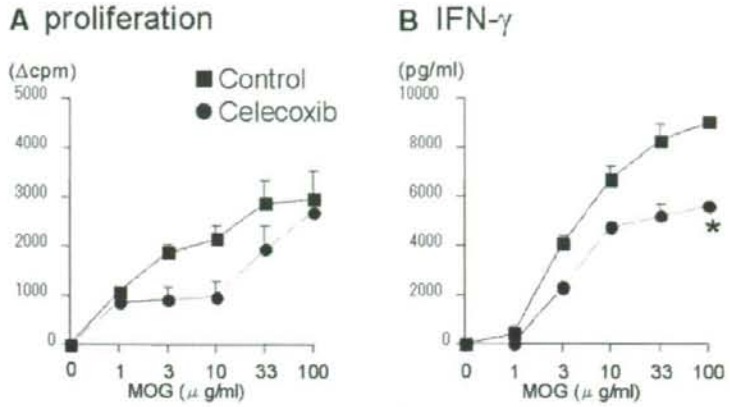

Fig. 4 Comparison of MOG_{35-55}-specific T-cell response after treatment with celecoxib. Popliteal and inguinal LN cells from treated and control animals were incubated in the presence of MOG_{35-55} for 48 h. Proliferative response was determined by the uptake of [^{3}H] thymidine (**A**), and IFN-γ was detected by ELISA (**B**). Representative data of two independent experiments are shown (n = 5 for each group). Error bars represent SEM. *P < 0.05 versus control.

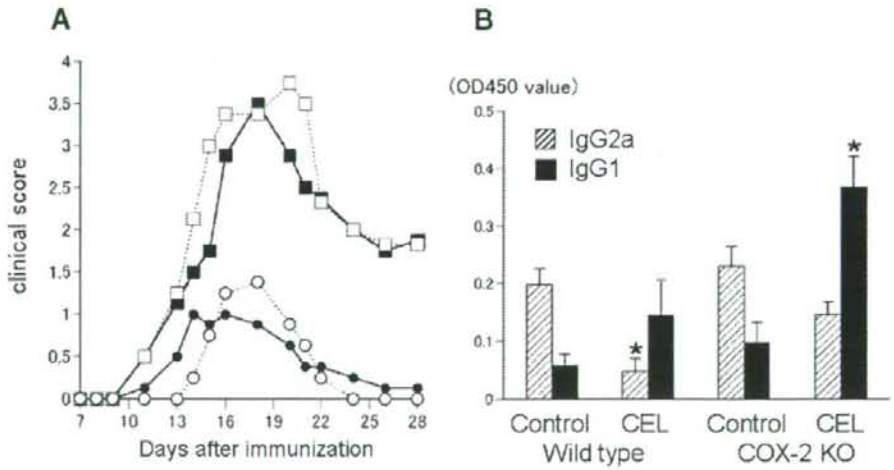

Fig. 5 Effect of celecoxib on actively induced EAE in COX-2-deficient mice. B6 mice and COX-2-deficient mice were immunized with MOG_{35-55} in CFA as described in Material and methods. (**A**) Mice were orally administered celecoxib (5 μg/g) every 2 days starting from the day of the immunization. Statistical analysis is shown in Table 3. Closed squares = vehicle alone for wild-type mice; closed circles = 5 μg/g of celecoxib for wild-type mice, open squares = vehicle alone for COX-2-deficient mice, open circles = 5 μg/g of celecoxib for COX-2-deficient mice. (**B**) The relative titres of anti-MOG IgG1 and IgG2a in serum samples from individual mice on Day 30 after immunization were analysed as indicated in Material and methods. Data represent mean ± SEM. *P < 0.05 versus control. Control = vehicle alone, CEL = celecoxib. One representative experiment of two independent experiments is expressed as mean ± SEM.

celecoxib-treated mice was significantly lower compared with that obtained from vehicle-treated mice. These findings suggested that celecoxib inhibits an infiltration of immune-mediated cells into CNS through the BBB by suppression of P-selectin, ICAM-1 and MCP-1.

Discussion

In the present study, we have demonstrated that a new-generation selective COX-2 inhibitor, celecoxib, strongly inhibited the development of EAE as compared with vehicle treatment or a traditional COX-2 inhibitor, nimesulid. The

Table 3 Clinical scores of EAE in COX-2-deficient mice

Mouse	Treatment	Max. score	Day of onset	Incidence (%)	Cumulative score
Wild-type	CMC	3.54 ± 0.28	12.60 ± 1.15	100 (10/10)	24.85 ± 6.37
	Celecoxib	1.13 ± 0.39*	13.20 ± 1.80	80 (8/10)	6.29 ± 4.02*
COX-2$^{-/-}$	CMC	3.75 ± 0.44	12.78 ± 1.57	100 (8/8)	29.88 ± 5.62
	Celecoxib	1.46 ± 0.51*	14.13 ± 1.96	87.5 (7/8)	5.39 ± 3.36*

Wild-type and COX-2$^{-/-}$ mice were immunized with MOG_{35-55} peptide to induce EAE. The control CMC solution, or 5 μg/g of celecoxib diluted in CMC, was administered every other day. Mean ± SEM of the following parameters are shown: maximum score of EAE (Max. score), the days of EAE onset, incidence of paralysed mice among sensitized mice (Incidence) and summation of the clinical scores from Day 0 to 30 (Cumulative score). *$P < 0.05$ versus control.

Table 4 Cell infiltration into the CNS of EAE-induced mice

	Mononuclear cell	$CD3^+$ cell	$CD4^+$ cell	$CD19^+$ cell
EAE mice				
Control (CMC)	667 ± 176	203 ± 69	158 ± 50	6 ± 1
Celecoxib	90 ± 57*	12 ± 8*	9 ± 5*	0 ± 0
Naive mice	20 ± 6	5 ± 2	3 ± 2	1 ± 0

CNS tissues from each group mouse were homogenized on Day 18 after immunization with MOG_{35-55} peptide. Mononuclear cells were isolated by Percoll solution. The cells were stained with cell markers and analysed by flow cytometer. Mean ± SEM of cell number (10^3 cells/mouse) is shown. Representative data of two independent experiments are shown ($n = 5$ for each group). *$P < 0.05$ versus control.

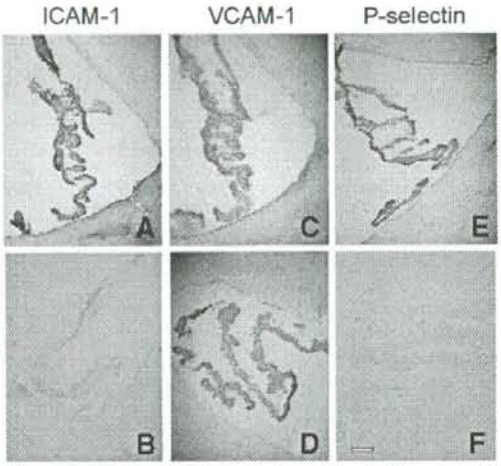

Fig. 6 Immunohistochemical staining with ICAM-1, VCAM-1 and P-selectin of the brain in EAE-induced mice. Brains from EAE mice were removed on Day 14 after immunization as described in Material and methods. Thinly sliced (10 μm) frozen sections of the brain were immunostained with anti-ICAM-1 antibody (**A** and **B**), anti-VCAM-1 antibody (**C** and **D**) and anti-P-selectin antibody (**E** and **F**). Figure shows choroid plexus region. Bar = 100 μm.

inhibitory effect on EAE by celecoxib was also evident in COX-2-deficient mice, indicating that celexoxib suppressed EAE in a COX-2-independent mechanism. In celecoxib-treated mice, MOG-specific Th1 responses were reduced and infiltration of immune cells was significantly inhibited compared with vehicle-treated mice, which were associated with lower expression of ICAM-1 and P-selectin on the choroid plexus in the brain.

Since EAE is an autoimmune inflammatory disease, administering COX-2 inhibitor was expected to inhibit disease as well as other COX inhibitors. Recently, Muthian *et al.* (2006) showed that some COX-2 inhibitors such as NS398 and LM01 suppressed EAE, when administered intraperitoneally every other day. In our study, we could not observe the inhibitory effect of nimesulid on EAE when orally administered every 2 days using the same conditions in which celecoxib exhibited a strong inhibitory effect. The route and timing of administration might be critical to modulate diseases. The inhibitory effect mediated by celecoxib was stronger compared with other COX inhibitors, suggesting that different mechanisms may be occurring in addition to the suppression of production of prostanoids that occurred at sites of disease and inflammation. In fact, COX-2 was not required for the celecoxib-mediated inhibitory effect on EAE. Recent studies have suggested that COX-2-independent pathways may contribute to celecoxib-mediated anti-tumour or anti-arthritic effect through enhanced apoptosis of tumour cells or synovial cells (Kusunoki *et al.*, 2002; Shishodia *et al.*, 2004). In our study, enhancing apoptosis of immune cells was not detected, indicating that different COX-2-independent mechanisms might be important for celecoxib-mediated inhibition of EAE. We observed that celecoxib treatment inhibited Th1 responses of MOG-reactive T cells. In the regulation of Th1/Th2 responses, prostaglandin E2 synthesized by COX has been reported to suppress IL-2 and IFN-γ production by a Th1 clone (Snijdewint *et al.*, 1993). In addition, Meyer *et al.* (2003) reported that administration of COX-2 inhibitor, NS398, increased *Helicobacter*-stimulated IL-12 and IFN-γ production, suggesting that COX-2 inhibition resulted in enhanced Th1 responses. In contrast, celecoxib inhibited Th1 responses of autoreactive T cells. Therefore, this COX-2-independent effect on immune system may be a mechanism to explain why celecoxib suppresses EAE to a greater degree compared with that of other COX-2 inhibitors. Allonza *et al.* (2006) reported that

Table 5 Serum level of MCP-1 in EAE mice after treatment with celecoxib

	Day 0	Day 7	Day 10	Day 14
EAE mice				
Control (CMC) (n = 18)	ND	60.0 ± 21.0	42.6 ± 17.0	ND
Celecoxib (n = 16)	ND	8.5 ± 5.0*	12.9 ± 8.5	ND
Naive mice (n = 10)	ND	ND	ND	ND

B6 mice were immunized with MOG_{35-55} peptide as described in Material and methods. Serum samples from individual mice were collected on Day 0, 7, 10 and 14 after immunization. Serum concentration of MCP-1 was measured by ELISA. Data represent mean ± SEM (pg/ml). ND = not detectable. *$P < 0.05$ versus control.

celecoxib inhibits IL-12 αβ and β2 folding and secretion in association with the increased interaction of IL-12 with calreticulin, an endoplasmic reticulum-resident chaperone in retention of misfolded cargo proteins, while blocking interaction with Erp44. They also demonstrated that an analogue of celecoxib lacking the COX-2 inhibitor activity showed identical effects to that of celecoxib on folding and secretion of IL-12, indicating that the effect is COX-2-independent. Since IL-12 is a key cytokine to provoke Th1 immune response, reduction in MOG-specific Th1 response is consistent with these previous findings.

The infiltration of immune cells in the CNS was significantly inhibited in celecoxib-treated mice. Celecoxib has been reported to reduce expression of P-selectin and ICAM-1 in experimental inflammatory models such as experimental colitis (Cuzzocrea *et al.*, 2001, 2002). In our study, we observed that celecoxib suppressed expression of P-selectin and ICAM-1 in the brain of EAE mice. Since P-selectin and ICAM-1 are the adhesion molecules involved in the recruitment of inflammatory cells into CNS (Engelhardt *et al.*, 1997; Dietrich, 2002; Scott *et al.*, 2004), inhibition of cellular infiltration by celecoxib might be mediated by the downregulation of the expression of adhesion molecules.

Chemokines are also required for recruitment of immune cells into the CNS. MCP-1 is reported to be an essential chemokine in EAE (Hofmann *et al.*, 2002). In the mouse model of atherosclerosis, Wang *et al.* (2005) reported that celecoxib decreased the inflammatory response and hyperplasia following vascular injury through inhibition of MCP-1 induction. We detected a decreased level of MCP-1 in the serum in celecoxib-treated mice on EAE. The suppression of MCP-1 by celecoxib might also contribute to the reduction of infiltrating cells into the CNS.

In conclusion, celecoxib has a potent therapeutic potential for EAE by inducing a Th2 bias and suppressing infiltration of inflammatory cells into the CNS through a COX-2-independent mechanism. Further analysis of celecoxib-mediated suppression of EAE will help drug development for multiple sclerosis. Celecoxib is hoped to be a new choice of the treatment of multiple sclerosis.

Acknowledgement

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THE JOURNAL OF IMMUNOLOGY

CUTTING EDGE

Cutting Edge: Human Th17 Cells Are Identified as Bearing CCR2$^+$CCR5$^-$ Phenotype[1]

Wakiro Sato, Toshimasa Aranami, and Takashi Yamamura[2]

Recent reports have shown that IL-17-producing CD4$^+$ T cells (Th17 cells) belong to a distinct helper T cell lineage and are critically involved in the pathogenesis of autoimmune diseases and allergies. However, the chemokine receptor profile of Th17 cells remains to be clarified. In this study, we report that human Th17 cells are identified as CCR2$^+$CCR5$^-$ memory CD4$^+$ T cells. Analysis of PBMC from healthy donors showed that CCR2$^+$ cells produce much larger amounts of IL-17 than CCR2$^-$ cells, indicating the preferential expression of CCR2 on Th17 cells. Notably, CCR2$^+$CCR5$^-$ memory CD4$^+$ T cells produced a large amount of IL-17 and little IFN-γ, whereas CCR2$^-$CCR5$^+$ cells reciprocally produced an enormous amount of IFN-γ but little IL-17. Moreover, a higher expression of T-bet was seen in the CCR5$^+$ memory T cells. These results indicate that absence of CCR5 distinguishes human Th17 cells from Th1 cells. The Journal of Immunology, 2007, 178: 7525–7529.

CD4$^+$ Th cells are essential regulators of adaptive immune responses. Th cells have been classified as either Th1 or Th2 according to the cytokine production profile and functional properties. However, recent studies have demonstrated that IL-17-producing T cells, rather than Th1 cells, play a pivotal role in the pathogenesis of autoimmune disease models, including experimental autoimmune encephalomyelitis (EAE)[3] (1–4). IL-17 is a cytokine mainly produced by activated memory T cells and could recruit and expand neutrophils through induction of various chemokines and GM-CSF (5–7).

Numerous studies have provided evidence that IL-17-producing T cells belong to a distinct lineage of Th cells whose development is severely hampered in IL-23 knockout but not in IL-12 knockout mice (8, 9). Although IL-23 was initially thought to induce differentiation of the IL-17-producing cells, it now seems that IL-23 is not involved in differentiation but propagation of Th17 cells (10). In fact, recent studies have shown that a combination of TGF-β1 plus IL-6 promotes the differentiation of Th17 cells in vitro (11–13). Differentiation of Th17 cells is prohibited by IFN-γ or IL-4 (11–13), further supporting the concept that Th17 cells comprise a distinct population cross-regulated by Th1 or Th2 cells. Notably, the independent nature of Th17 cells has been further highlighted by the recent discovery that the transcription factor RORγt is critically involved in the development of Th17 cells (14).

During the critical process whereby naive CD4$^+$ T cells differentiate, they acquire reciprocal sets of chemokine receptors (15), which would endow them a unique character of homing or migration to corresponding ligand chemokines. Namely, Th1 cells preferentially express CCR5 and CXCR3 and migrate to inflammatory milieu expressing the corresponding ligand chemokines, whereas Th2 cells express CCR4, CCR8, and CRTh2 indicative of a distinctive homing property (16–19). It is conceivable that Th17 cells may also possess unique chemotactic and migratory property. However, chemokine receptor expression by Th17 cells has not been characterized yet, at least to our knowledge.

In this study, we attempted to identify chemokine receptor expression by human Th17 cells by examining cytokine production profiles of T cell subpopulation-bearing chemokine receptor(s) of interest (16, 20). We started by comparing CCR2$^+$ and CCR2$^-$ memory CD4$^+$ T cells, because CCR2 and its ligand CCL2 were shown to be essential for development of EAE (21, 22). We found that only the CCR2$^+$ subpopulation would produce IL-17. Further analysis has demonstrated that CCR5$^-$ cells among the CCR2$^+$CD4$^+$ memory T cells produce IL-17, whereas a CCR5$^+$ subpopulation produces IFN-γ. Thus, human Th17 cells are identified as uniquely bearing the CCR2$^+$CCR5$^-$ phenotype.

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[3] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; DN, double negative; DP, double positive; SP, single positive; MFI, mean fluorescence intensity.

Materials and Methods

Reagents

Anti-CCR2-biotin mAb, anti-CCR5-FITC mAb, and goat anti-IL-23R polyclonal Abs were purchased from R&D Systems. Streptavidin-PE, anti-CD4-PC5 mAb, and anti-CD45RA-energy-coupled dye (ECD) mAb were obtained from Beckman Coulter, anti-CCR5-allophycocyanin mAb from BD Pharmingen, and donkey anti-goat IgG-FITC from Jackson ImmunoResearch Laboratories. Anti-T-bet mAb and isotype control Ab (mouse IgG1a) purchased from Santa Cruz Biotechnology were used for intracellular staining. RPMI 1640 medium (Invitrogen Life Technologies) was supplemented with 0.05 mM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 10% FBS.

Cell preparation

Peripheral blood was obtained from healthy human volunteers (24–42 years of age) from whom informed consent was obtained. The Ethics Committee of the National Center of Neurology and Psychiatry approved the study. PBMC were freshly isolated by density gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences). To purify whole T cells or memory $CD4^+$ T cells from PBMC, we used a Pan T cell isolation kit II or Memory $CD4^+$ T cell isolation kit (Miltenyi Biotec), respectively. Briefly, PBMC were labeled with a mixture of biotin-conjugated mAbs directed against either non-T or non-memory $CD4^+$ T cells and then reacted with magnetic microbead-conjugated anti-biotin mAbs. The magnetically labeled non-T or non-memory $CD4^+$ T cells were depleted with autoMACS (Miltenyi Biotec), which yielded >95% purity of whole T cells or memory $CD4^+$ T cells as assessed by flow cytometry for the proportion of $CD3^+$ cells or $CD4^+CD45RA^-$ cells.

To further separate the purified cells according to CCR2 or CCR5 expression, they were labeled with anti-CCR2-biotin, streptavidin-PE, and anti-PE microbeads (Miltenyi Biotec) or anti-CCR5-FITC and anti-FITC microbeads (Miltenyi Biotec). The magnetically labeled cells were separated into positive ($CCR2^+$ and $CCR5^+$) and negative ($CCR2^-$ and $CCR5^-$) fractions with autoMACS (>99% purity of $CCR2^-$ or $CCR5^-$ cells and >90% purity of $CCR2^+$ or $CCR5^+$ cells). To obtain $CCR2^+CCR5^-$ and $CCR2^-CCR5^+$ memory $CD4^+$ T cells, $CCR2^+$ memory $CD4^+$ T cells were labeled with anti-CCR5-allophycocyanin and separated into $CCR2^+CCR5^-$ (>80% purity) and $CCR2^+CCR5^+$ cells (>95% purity) by flow cytometric cell sorter Epics Altra (Beckman Coulter).

Cell culture and cytokine measurement by ELISA

Purified T cell populations were resuspended at 5×10^5/ml and stimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) in 96-well U-bottom plates for 24 h. The concentrations of IFN-γ and IL-17 in the supernatants were measured by using a Human IFN-γ ELISA Set (BD Pharmingen) and a Human IL-17 DuoSet (R&D Systems).

Flow cytometric analysis of chemokine receptors

To evaluate the expression of chemokine receptors, purified memory $CD4^+$ T cells were stained with anti-CD4-PC5, anti-CD45RA-ECD, anti-CCR5-FITC and PE-conjugated mAbs against anti-CCR2-biotin were analyzed with Epics flow cytometry (Beckman Coulter). To examine the expression of IL-23R, memory $CD4^+$ T cells were stained with goat anti-IL-23R and anti-goat-IgG-FITC and were analyzed with a FACSCalibur (BD Pharmingen).

Intracellular staining of T-bet

Purified memory $CD4^+$ T cells were first stained with biotin-conjugated anti-CCR2, streptavidin-PE, and allophycocyanin-CCR5, then fixed in PBS containing 2% paraformaldehyde and permeabilized with 0.1% saponin solution. Subsequently, the cells were stained with FITC-anti-T-bet. Mouse IgG1a was used as an isotype control.

Statistics

An unpaired Student's t test or one-way ANOVA was used for statistical analysis. We considered $p < 0.01$ as significant.

Results and Discussion

Both Th17 cells and Th1 cells are enriched in $CCR2^+$ $CD4^+$ memory T cells

Previous reports on the CCR2 requirement for development of EAE (21, 22) prompted us to compare the cytokine-producing ability of $CCR2^+$ and $CCR2^-$ cells isolated from whole T cells. The results showed that $CCR2^+$ cells produced a larger amount of IFN-γ and IL-17 as compared with $CCR2^-$ cells, whereas unseparated whole T cells showed intermediate values (Fig. 1*A*, *upper panels*). This indicates that $CCR2^+$ cells contain the vast majority of Th1 and Th17 cells. We next separated the whole T cells into $CCR5^+$ and $CCR5^-$ populations to compare the cytokine profile. Although $CCR5^+$ cells produced a larger amount of IFN-γ as compared with $CCR5^-$ or the whole T

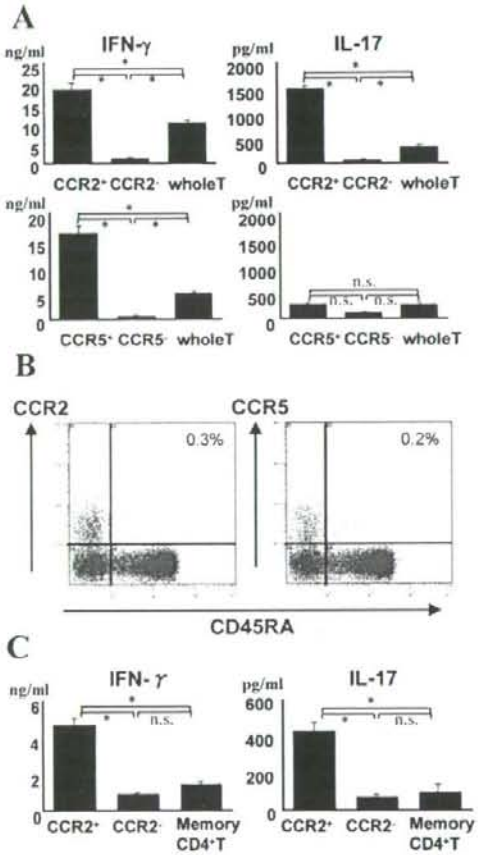

FIGURE 1. Th17 cells are enriched in $CCR2^+CD4^+CD45RA^-$ cells. *A*, IL-17-producing cells are selectively enriched in the $CCR2^+$, but not in a $CCR5^+$ population. From PBMC of healthy donors, whole T cells were purified magnetically by negative selection using a Pan T cell isolation kit II with autoMACS. The purity of the cells was generally >98%, as determined by FACS analysis. Purified $CCR2^+$ and $CCR2^-$ T cells (or $CCR5^-$ and $CCR5^+$ T cells) were stimulated with PMA and ionomycin for 24 h before the supernatants were collected. The IFN-γ and IL-17 protein in each supernatant was measured using ELISA. Results are expressed as mean ± SD of a representative of five independent experiments. *B*, Chemokine receptor (CCR2 or CCR5) expressing T cells are largely confined to $CD45RA^-$ memory T cells. PBMC from healthy subjects were stained with anti-CCR2 (PE), anti-CCR5 (FITC), anti-CD4 (PC5), and anti-CD45RA (ECD) and analyzed after being gated for CD4. Shown is a representative of five individual data sets. *C*, Th17 cells are enriched in $CCR2^+CD4^+CD45RA^-$ cells. Memory $CD4^+$ T cells were purified by a memory $CD4^+$ T cell isolation kit with autoMACS. $CCR2^+$ and $CCR2^-$ T cells were further isolated by anti-CCR2-biotin, streptavidin-PE, anti-PE microbeads, and autoMACS. Purified cells were stimulated with PMA and ionomycin for 24 h before collecting supernatants. Results are expressed as mean ± SD of a representative of five independent experiments. *, $p < 0.01$.

cells (Fig. 1*A, lower panels*), production of IL-17 did not increase after enrichment for $CCR5^+$ cells. These results suggest that Th17 cells may be selectively enriched in $CCR2^+$, but not in $CCR5^+$ populations. However, because the $CCR2^+$ T cell preparation also contains $CD8^+$ T cells and γδT cells, capable of producing IL-17 (23–25), it remained possible that the major source of IL-17 could be $CD8^+$ T or γδT cells. Therefore, we next needed to assess the production of IFN-γ and IL-17 from purified $CD4^+$ T cells. Preparatory experiments showed that $CCR2^+$ or $CCR5^+CD4^+$ populations are mainly confined to the $CD45RA^-$ memory T cell population (Fig. 1*B*). Consequently, we decided to use memory $CD4^+$ T cells that could be obtained after deleting $CD8^+$, γδ, and naive $CD4^+$ T cells for further analysis. Analysis of the purified memory $CD4^+$ T cells has also demonstrated that the $CCR2^+$ population produced a significantly larger amount of both IFN-γ and IL-17 compared to the $CCR2^-$ population, with the values of unseparated cells being intermediate (Fig. 1*C*). These results strongly indicate that Th17 cells as well as Th1 cells are enriched in $CCR2^+CD4^+$ memory T cells. However, since Th1 and Th17 cells are thought to belong to distinct T cell lineages, we speculated that they might be further divided into two subpopulations based on expression of chemokine receptors.

$CCR2^+CCR5^-$ memory $CD4^+$ T cells predominantly produce IL-17 but not IFN-γ

Simultaneous staining of CCR2 and CCR5 showed that the $CCR2^+$ memory T cell population could be divided into $CCR5^-$ (CCR2 single positive (SP)) and $CCR5^+$ (CCR2 and CCR5 double positive (DP)) subpopulations (Fig. 2). Since CCR5 is reported to be expressed predominantly on Th1 cells (16–18), we hypothesized that SP and DP cells might correspond to Th17 and Th1 cells, respectively. To correlate cytokine production profile and chemokine receptor expression in T cell populations, we first thought of staining total unseparated T cells to detect intracellular cytokines as well as surface CCR; however, the cell activation process required for intracellular cytokine staining was found to down-regulate CCR2 and CCR5 significantly (data not shown), as reported previously (26). To accurately correlate the expression of CCR2 or CCR5 with the cytokine profile ex vivo, we decided to first isolate SP and DP cells from memory $CCR2^+$ T cells by using a flow cytometric cell sorter and stimulate them with PMA and ionomycin. We then measured IFN-γ and IL-17 in the supernatant (Fig. 3*A*). Remarkably, the sorted T cell subpopulations exhibited different cytokine production patterns: SP cells produced a large amount of IL-17 and a small amount of IFN-γ, whereas DP cells produced a small amount of IL-17 and a large amount of IFN-γ (Fig. 3*B*). These results suggest that Th17 cells are largely confined to SP cells, whereas DP cells contain a majority of Th1 cells.

T-bet and IL-23R expression in memory $CD4^+$ T cells

Finally, we assessed whether SP and DP cells are distinctive in expression of transcription factor T-bet and IL-23R. T-bet is an essential transcription factor for Th1 differentiation (27), whereas it was reported to be redundant for Th17 cells (3, 8, 9, 11, 14). IL-23 has been shown to play a pivotal role in the survival and expansion of Th17 cells (2, 10). Magnetically purified memory $CD4^+$ T cells were first stained with biotin-conjugated CCR2, streptavidin-PE, and allophycocyanin-CCR5, and then were intracellularly stained with FITC-anti-T-bet or were stained with goat anti-IL-23R Ab and anti-goat IgG-FITC. We compared T-bet expression in SP vs DP cells by evaluating the mean fluorescence intensity (MFI) (Fig. 4, *A* and *B*). T-bet was significantly expressed by SP as well as $CCR2^-CCR5^-$ double-negative (DN) cells, but its expression was much higher in DP cells and $CCR2^-CCR5^+$ cells, suggesting that Th1 cells may be confined to $CCR5^+$ populations. On the other hand, the frequency of $IL\text{-}23R^+$ cells was highest in the SP fraction, compared with the others (Fig. 4, *C* and *D*).

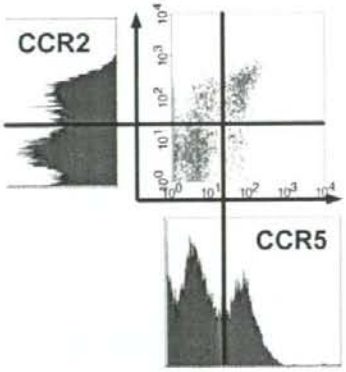

FIGURE 2. $CCR2^-$ memory $CD4^+$ T cells consist of $CCR5^+$ and $CCR5^-$ subsets. Purified memory $CD4^+$ T cells were stained with anti-CCR2-biotin and streptavidin-PE as well as anti-CCR5-FITC. The separation of positive and negative populations was determined by histogram plots.

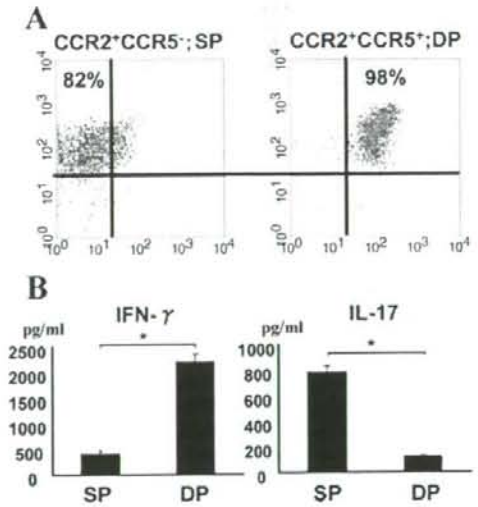

FIGURE 3. $CCR2^+CCR5^-$ (SP) and $CCR2^+CCR5^+$ (DP) cells correspond to Th17 and Th1 cells, respectively. SP and DP subsets were sorted from memory $CD4^+$ T cells by flow cytometry and stimulated with PMA/ionomycin. *A*, A representative of five individual data sets showing the purity of the sorted cells. *B*, SP and DP cells were stimulated with PMA/ionomycin for 24 h. Then the amounts of IFN-γ and IL-17 in the supernatant were measured using ELISA. Results are expressed as mean ± SD of a representative of five independent experiments. *, $p < 0.01$

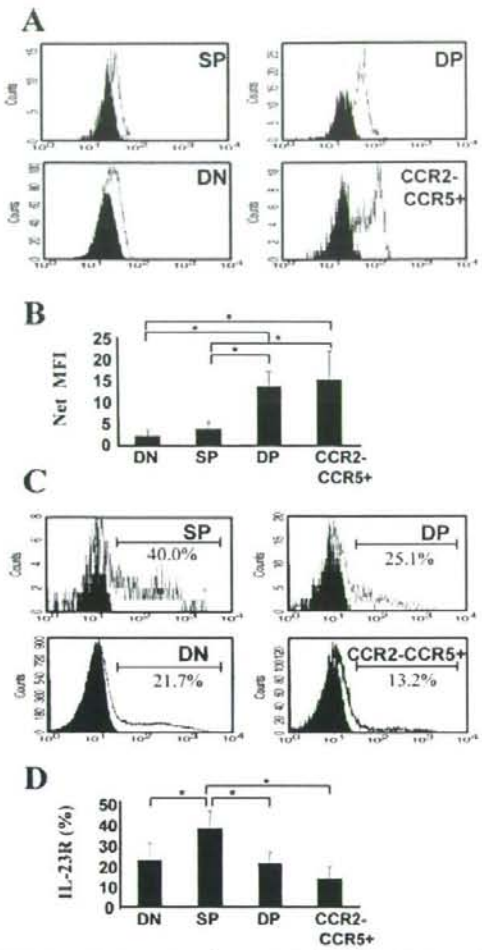

FIGURE 4. T-bet expression of memory $CD4^+$ T cells. *A*, Histogram plots of T-bet expression within memory $CD4^+$ T cells. SP, DP, DN ($CCR2^-CCR5^-$), and $CCR2^-CCR5^+$ T cells were stained with anti-T-bet or isotype control Ab. Shown is a representative of five individual data sets. *B*, The MFI of each histogram plot. Data for the MFI of T-bet expression subtracted by that of control Ab are calculated and shown as bar graphs with error bars showing the SD of four individual data sets. *C*, Histogram plots of IL-23R expression within memory $CD4^+$ T cells. The cells were stained with goat anti-IL-23R polyclonal Ab and anti-goat-FITC Ab. A representative of seven individual data sets is shown. *, $p < 0.01$. *D*, The frequency of IL-23R-positive cells of each histogram plot. Data are shown as bar graphs with error bars showing the SD of seven individual data sets. *, $p < 0.01$.

Given the distinguished ability to produce IL-17 as well as higher IL-23R and lower T-bet expression, we propose that Th17 cells are confined to SP cells, whereas Th1 cells are either DP or $CCR2^-CCR5^+$. It has recently been reported that T-bet directly regulates the transcription of IL-23R in mice (28). It is possible that weak expression of IL-23R by non-Th17 cells (DP, DN, and $CCR2^-CCR5^+$) may result from baseline activation of T-bet.

Additional remarks

Using freshly isolated healthy human lymphocytes, we showed here that $CCR2^+CCR5^-$ memory T cells would produce a large amount of IL-17 but not IFN-γ, whereas $CCR2^-$ memory T cells produced IFN-γ, but not IL-17. Although we presented the data obtained after stimulation with PMA/ionomycin, polyclonal stimulation by anti-CD3/CD28 also gave similar results (data not shown). Moreover, when we stimulated $CCR2^+CD4^+$ memory T cells by IFN-γ, IL-4, IL-2, or IL-23 in addition to PMA/ionomycin, IL-17 production was not changed (data not shown).

The frequency of Th17 cells among this subset is an important issue to be investigated. By using the ELISPOT assay, we found that ~200 spots of IL-17-producing cells could be detected among 1×10^5 memory $CCR2^+CD4^+$ T cells (~0.2%), whereas the numbers of IFN-γ-producing cells were about 5-fold higher (~1.0%). Although this needs to be systematically verified, the lower frequency of IL-17-producing cells is consistent with the lower value of IL-17 than IFN-γ in supernatants detected by ELISA.

The unique chemokine receptor expression pattern of Th17 cells provides a basis for their recruitment to specialized inflammatory conditions in vivo, which should be relevant for understanding the pathogenesis of autoimmune diseases.

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Disclosures

The authors have no financial conflict of interest.

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Invariant NKT Cells Biased for IL-5 Production Act as Crucial Regulators of Inflammation[1]

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Although invariant NKT (iNKT) cells play a regulatory role in the pathogenesis of autoimmune diseases and allergy, an initial trigger for their regulatory responses remains elusive. In this study, we report that a proportion of human CD4$^+$ iNKT cell clones produce enormous amounts of IL-5 and IL-13 when cocultured with CD1d$^+$ APC in the presence of IL-2. Such IL-5 bias was never observed when we stimulated the same clones with α-galactosylceramide or anti-CD3 Ab. Suboptimal TCR stimulation by plate-bound anti-CD3 Ab was found to mimic the effect of CD1d$^+$ APC, indicating the role of TCR signaling for selective induction of IL-5. Interestingly, DNA microarray analysis identified *IL-5* and *IL-13* as the most highly up-regulated genes, whereas other cytokines produced by iNKT cells, such as IL-4 and IL-10, were not significantly induced. Moreover, iNKT cells from BALB/c mice showed similar IL-5 responses after stimulation with IL-2 ex vivo or in vivo. The iNKT cell subset producing IL-5 and IL-13 could play a major role in the development of allergic disease or asthma and also in the immune regulation of Th1 inflammation. *The Journal of Immunology*, 2007, 179: 3452–3462.

Invariant NKT (iNKT)[3] cells are a nonconventional population of T cells, expressing a canonical invariant TCR α-chain (Vα14-Jα18 for mice and Vα24-Jα18 for human) and TCR β-chains using limited Vβ segments (Vβ8.2, 2, and 7 in mice and Vβ11 in humans) (1–4). They are selected and restricted by CD1d, a nonclassical MHC class I-like molecule, and proliferate vigorously in response to α-galactosylceramide (αGC), a prototypical iNKT cell ligand, originally isolated from marine sponge (5). Although most iNKT cells express NK cell markers such as CD161, they also contain a small population of cells that are negative for NK cell markers (6). Importantly, CD1d-restricted T cells also contain T cells that neither express the canonical TCR α-chain nor respond to αGC (7, 8). To avoid confusion, it has recently been recommended that iNKT cells should be defined by their reactivity to αGC loaded onto CD1d multimers, instead of expression of NK cell markers (6). iNKT cells comprise CD4$^+$ and CD4$^-$ cells, which show differential expression of regulatory cytokines. In humans, studies have shown that the former produce both Th1 and Th2 cytokines, whereas the latter predominantly produce proinflammatory cytokines such as IFN-γ and TNF-α (9, 10). Accordingly, the CD4$^+$ cells are thought to be the major source of Th2 cytokines for controlling Th1 cell-mediated inflammation or promoting Th2-dependent pathologies.

Although earlier studies have tended to focus on the ability of iNKT cells to down-modulate inflammatory responses, more recent works have shown that they could promote joint inflammation in models of arthritis (11–13) or mediate airway inflammation in bronchial asthma (14, 15). The divergent effects of iNKT cells in inflammatory pathologies are thought to reflect a broad spectrum of their functions in vivo. In fact, iNKT cells explosively produce a number of pro- and anti-inflammatory cytokines after nonphysiological stimulation with αGC (2, 5, 16) or anti-CD3 mAb (17), although stimulation with alternative ligands such as αGC analogues may lead to selective Th1 (18) or Th2 cytokine production (19, 20). Regarding the molecular mechanism for iNKT cell-mediated immune regulation, previous studies have suggested the role of iNKT cell-derived IL-4 or IL-10 in controlling Th1-mediated inflammation (16, 19, 20), whereas the role of IL-13 secreted by iNKT cells has recently been highlighted in the pathogenesis of asthma (14, 15) and ulcerative colitis (21). The published results, however, do not exclude the possible role of other cytokines secreted by iNKT cells. In fact, it is not clear whether iNKT cells could produce specific cytokines that are truly needed to exert regulatory functions or whether they produce cytokines in a redundant way. Another important question is what would trigger the regulatory iNKT cells to promote a cytokine response in vivo during the natural course of disease. Although TCR and/or costimulatory molecule signaling are likely to be the triggers involved, direct evidence for this postulate so far has not been provided.

Based on the observation of neonatal iNKT cells expressing memory-activated phenotype (CD45RO$^+$CD62L$^-$CD25$^+$) (22, 23) and resting adult iNKT cells containing preformed transcripts of IFN-γ and IL-4 (24), it has been suggested that iNKT cells are preactivated by endogenous ligands. If endogenous ligands for iNKT cells are to exist in vivo, we speculate that they transmit a relatively weak signal through TCR (25). Supportive of this idea,

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[3] Abbreviations used in this paper: iNKT, invariant NKT; αGC, α-galactosylceramide; iGb3, isoglobotrihexosylceramide; HS, healthy subject; MS, multiple sclerosis; DN, double negative; DC, dendritic cell; iDC, immature DC; CBA, cytometric bead array.

Table I. *IL-5 versus IFN-γ secretion profile of CD4⁺ iNKT cell clones generated from HS and MS*[a]

	Clone	Age	Primary Stimulation	IL-5	IL-5/IFN-γ Ratio	Medication
HS						
1	Kai.1	32	αGC	7336.5	26.29	
2	Sk	32	αGC	1950.0	9.81	
3	Ot.1	34	αGC	80.0	0.001	
4	Ok.1	39	αGC	3.3	0.09	
5	Kai.2	32	OCH	2796.1	160.70	
6	Ar	35	OCH	191.7	0.78	
7	Ot.2	34	OCH	97.7	0.07	
8	Nn	28	OCH	87.3	0.04	
9	Ln	35	OCH	79.6	0.08	
10	Yk	31	OCH	70.1	0.98	
11	Ok.2	39	OCH	23.1	0.17	
MS						
12	Kn.1	22	αGC	2998.3	59.53	PSL[b]
13	Oz	31	αGC	2252.3	14.62	IFN-β
15	Kk	31	αGC	1095.6	20.67	PSL
14	Og	32	αGC	176.9	0.21	IFN-β
16	Sd	37	αGC	95.0	0.19	None
17	Ich	37	αGC	59.7	0.26	None
18	Nkj.1	61	αGC	48.4	0.18	None
19	Tj	31	αGC	44.0	0.05	None
20	Mtz.1	47	αGC	41.2	0.65	None
21	Yta	35	αGC	15.1	0.17	None
22	Kn.2	22	OCH	4636.0	2.26	PSL
23	Nkj.2	61	OCH	2353.4	3.32	None
24	Mtz.2	47	OCH	133.7	0.25	None
25	Ag	34	OCH	6.3	0.01	None
26	Ko	35	OCH	0.0	0.0000052	None

[a] The clone cells were cocultured with iDCs in the presence of exogenous IL-2. The amount of IL-5 and IFN-γ from day 2 supernatant was measured by CBA.
[b] PSL, Prednisolone.

Brigl et al. (26) have recently shown that human iNKT cell clones as well as freshly separated rodent iNKT cells could exert an enormous IFN-γ response, when they react to an endogenous ligand in the presence of costimulatory IL-12 (26). As such, a very weak autoreactive iNKT cell response to CD1d-positive cells could be remarkably augmented by various additional signals such as cytokines and costimulatory molecules. Several candidates for endogenous ligands have been previously reported (27–29). More recent studies have demonstrated that lysosomal glycosphingolipid isoglobotrihexosylceramide (iGb3) is a possible endogenous ligand naturally presented to iNKT cells in the context of CD1d (30, 31). Notably, Mattner et al. (31) has shown that iNKT cell activation following bacterial infection could be elicited either by stimulation with bacterial glycolipids or by endogenous iGb3 bound to CD1d, depending on the strain of bacteria. This indicates that recognition of endogenous ligand may be critical in triggering at least certain iNKT cell responses in vivo. However, it is unclear whether recognition of endogenous ligand by iNKT cells may lead to production of Th2 cytokines required for iNKT cell-mediated Th2 immune deviation. Taking these into consideration, we have attempted to re-examine the functional properties of human CD4⁺ iNKT cell clones by exploring the effects of cytokines on the autoreactive iNKT cell responses to CD1d⁺ DCs.

We report here that although none of the iNKT cell clones responded to CD1d⁺ DCs after coculture, addition of exogenous IL-2 could trigger the production of enormous amounts of IL-5 and IL-13 from some of the clones. Comprehensive analysis using DNA microarray has shown that *IL-5* and *IL-13* Th2 cytokine genes are almost exclusively and robustly induced from the clones in response to CD1d⁺ DCs and IL-2. IL-2 alone did not induce IL-5 production, but IL-2 together with suboptimal TCR stimulation by anti-CD3 Ab could provoke a striking IL-5 response. Because a similar Th2 bias was reproducibly demonstrated by using iNKT cells freshly isolated from BALB/c mice, we propose that the combination of IL-2 and a weak TCR stimulus by endogenous ligand/CD1d could be a mechanism by which CD4⁺ iNKT cells could start producing Th2 cytokines IL-5 and IL-13 in autoimmune diseases and allergy.

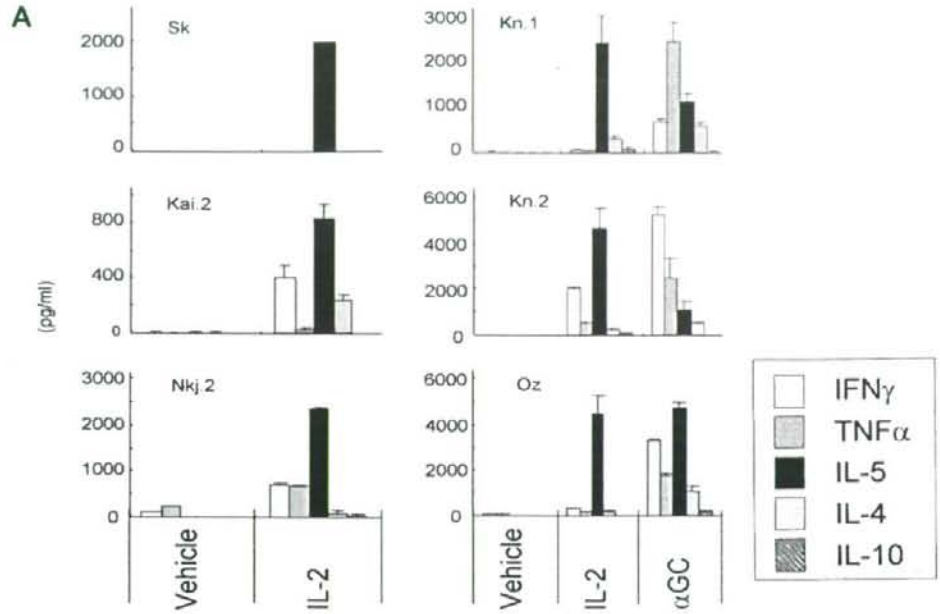
Materials and Methods

Subjects

Venous samples of nine healthy subjects (HS) and 13 multiple sclerosis patients (MS) were used for study (Table I). All the patients had conventional MS, fulfilled standard criteria for the diagnosis of relapsing-remitting MS, and were in remission at examination based on clinical and magnetic resonance imaging assessment. Four patients were on medication for >3 mo: two on low-dose corticosteroids and the other two on IFN-β. HS (33.9 ± 2.2 years old) and MS (37.53 ± 11.8 years old) were age matched. Written informed consent was obtained from all subjects and the Ethics Committee of the National Center of Neurology and Psychiatry approved this study.

Abs and reagents

PE-labeled anti-Vα24, FITC-anti-Vβ11, phycoerythrin-Texas Red X-anti-CD4, PC5-anti-CD8, PE-anti-CD206, and anti-mouse IgM were purchased from Immunotech and PE-anti-iNKT cells (specific for invariant Vα24-Jα18 TCR: 6B11) (32), PE-anti-human IL-4, FITC-anti-human IFN-γ, mouse CD1d dimer (dimer X), FITC-anti-mouse TCRβ, PE-anti-mouse NK1.1, and PE-rat anti-mouse IgG1 were purchased from BD Biosciences/BD Pharmingen. All human recombinant cytokines were obtained from PeproTech and microbeads coated with anti-CD14, anti-CD45RO, or anti-PE and the CD4 T cell isolation kit were obtained from Miltenyi Biotec. Flow cytometry was performed on an Epics XL and analyzed with EXPO 32 software (Coulter). Cell sorting was conducted on an Epics Altra (Coulter) or autoMACS cell sorter (Miltenyi Biotec). αGC and OCH (19) were solubilized in DMSO (100 μg/ml). Anti-CD1d mAb (aCD1d 59; IgM) was prepared in the laboratory of S. A. Porcelli.


A
Sk
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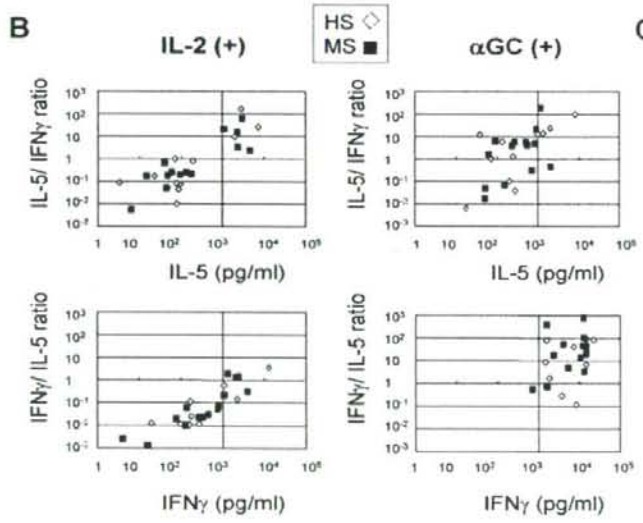
Kai.2
Kn.2
Nkj.2
Oz
(pg/ml)
Vehicle
IL-2
αGC
IFNγ
TNFα
IL-5
IL-4
IL-10


B
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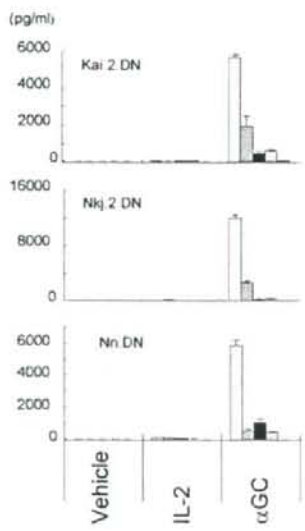
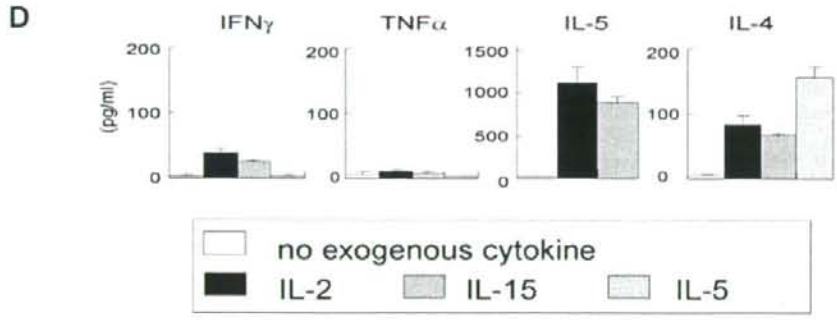
HS ◇
MS ■
αGC (+)
IL-5/ IFNγ ratio
IL-5 (pg/ml)
IFNγ/ IL-5 ratio
IFNγ (pg/ml)


C
(pg/ml)
Kai.2 DN
Nkj.2 DN
Nn.DN
Vehicle
IL-2
αGC


D
IFNγ
TNFα
IL-5
IL-4
(pg/ml)
no exogenous cytokine
IL-2
IL-15
IL-5

Human iNKT clones

PBMCs were isolated by density gradient centrifugation and suspended at 1×10^6/ml in AIM-V medium (Invitrogen Life Technologies), supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (hereafter referred to as "basic medium"). Cells were stimulated with αGC or OCH (100 ng/ml) in the presence of IL-2 (50 IU/ml) and IL-7 (10 ng/ml). After 7 days, half of the medium was changed every 3–5 days with basic medium containing IL-2 (10 IU/ml) and IL-7 (5 ng/ml). Fourteen to 18 days after stimulation, $CD4^+$ or double-negative (DN) iNKT cells were sorted after staining with the fluorescence-labeled anti-Vβ11, anti-iNKT, anti-CD4, and anti-CD8 Abs. The sorted cells were cultured with fresh allogenic X-irradiated (100 Gy) PBMC at a cell ratio of 1:3, stimulated with 1.0 μg/ml PHA-P (PHA; Sigma-Aldrich), IL-2 (50 IU/ml), and IL-7 (10 ng/ml) for 3 days and then maintained by basic medium supplemented with IL-2 (10 IU/ml) and IL-7 (5 ng/ml). iNKT cell sorting and PHA stimulation was repeated every 4–5 wk. Two to 3 wk after the most recent stimulation, the clones were used for assays, before which they were cultured in cytokine-free medium for at least 4 days.

Coculture experiments

Immature dendritic cells (iDCs) as APCs were derived from $CD14^+$ monocytes (33). The iDCs were X-irradiated (55 Gy) and seeded at 3×10^4 cells/well with or without αGC (100 ng/ml) in U-bottom 96-multiwell plates. Six hours later, they were washed and added with iNKT cells at a 1:1 ratio, with or without IL-2 (10 IU/ml). Cytokines in the day 2 supernatant were measured by the Cytometric Beads Array (CBA) kit from BD Biosciences/BD Pharmingen as previously described (34). CD1d-transfected (CD1d HeLa) and mock-transfected HeLa (mock HeLa) cells were also used for coculture after mitomycin C treatment (50 μg/ml, 30 min). To block the CD1d molecule, anti-CD1d mAb (aCD1d 59) was added to iDC and cultured for an hour. After washing out nonbinding mAb, iNKT cells were added at a 1:1 ratio and incubated with or without IL-2 (1 or 5 IU/ml) for 48 h. IL-5 in the supernatant was measured by CBA.

Microarray analysis

After 24 h of culture with iDCs, iNKT cells were negatively separated from the cell mixture with 95% purity. The iDCs were stained with PE-anti-CD206 and depleted using secondary anti-PE microbeads. mRNA was purified from the iNKT cells and then pooled at −80°C. The mRNA was labeled with biotin by using the Ovation Biotin System (Nugen Technologies). The targets containing fragmented and biotin-labeled cDNA were hybridized and analyzed on GeneChip Human Genome U133A arrays (Affymetrix). The array probes were scanned and gene transcript levels were determined using algorithms in the GeneChip Analysis Suite software. Gene transcriptions of IL-2-stimulated (IL-2 sample) and vehicle-stimulated iNKT cells (negative control) was separately compared for each clone, and those significantly elevated by IL-2 stimulation were selected by paired *t* test. All of the genes elevated in any of the clones were analyzed by two-factor ANOVA to investigate a statistical significance.

Intracellular cytokine analysis

We isolated naive $CD4^+$ T cells from PBMC of HS by positive (CD4 T cell isolation kit) followed by negative selection (CD45RO microbeads). The isolated cells were stimulated by plate-bound anti-CD3 mAb (incubated at 10 μg/ml overnight) with soluble anti-CD28 mAb (2 μg/ml) in AIM-V in the presence of iNKT/iDC supernatant, with or without neutralizing anti-IL-5 mAb (10 μg/ml). Three days later, the cells were transferred onto a new plate. Half of the medium was changed every second day. On day 7, the intracellular IFN-γ and IL-4 were stained after restimulating the cells with PMA (10 ng/ml) and ionomycin (500 ng/ml) for 6 h in the presence of monensin (1 μg/ml). Appropriate control Abs were used to define the background immunofluorescence.

Analysis of BALB/c iNKT cells

BALB/c mice in specific pathogen-free conditions were used at 8–13 wk of age. Animal care and use were in accordance with institutional guidelines. Lymphocytes were separated from liver and spleen by gradient centrifugation and stained with FITC-anti-TCRβ and αGC-loaded CD1d dimer (dimer X) with secondary staining by PE-conjugated rat anti-mouse IgG1. Then $TCR\beta^+$ αGC-loaded dimer X^+ cells were sorted by using the Altra cell sorter. DCs were isolated from splenocytes by using CD11c microbeads and were used after being X-irradiated (30 Gy). The iNKT cells and the DCs were cocultured for 72 h in U-bottom 96-plates at a 1:1 ratio (1.5×10^4 cells for each) with or without IL-2. The supernatants were analyzed by CBA. To evaluate in vivo effects of IL-2 on iNKT cells, BALB/c mice were injected i.v. with 5000 IU of IL-2. Two hours later, the mice were sacrificed and their liver lymphocytes were isolated. The isolated cells were carefully stained with αGC-loaded dimer X and TCRβ on ice to avoid direct activation by these reagents. The stained cells were fixed and perforated for staining intracellular IL-5 or IFN-γ according to BD Biosciences protocol, except without any additional in vitro stimulation.

Results

A distinct group of $CD4^+$ iNKT cell clones produce IL-5 in the presence of IL-2

We have used a total of 26 $CD4^+$ iNKT cell clones derived from HS or patients with MS to evaluate their self-reactivity. Because we were initially interested in comparing MS with HS regarding the functions of iNKT cells, we used a panel of iNKT cell clones from HS and MS. In the presence of iDCs as APCs, all of the clones vigorously responded to αGC by producing a large amount of IFN-γ (>1000 pg/ml) and variable amounts of IL-4 and IL-5 (500–2500 pg/ml), confirming that they maintained the essential property of iNKT cells to react with αGC. These iNKT cell clones showed very little background response to the iDCs in the simple coculture. However, to our surprise, when we added IL-2 (10 IU/ml),

FIGURE 1. Production of IL-5 by human iNKT cell clones in the presence of exogenous IL-2. iNKT cell clones were cultured with the same number of allogenic iDCs (3×10^4 of each/well) in the presence or absence of exogenous IL-2 (10 IU/ml). The iDCs were preincubated for 6 h with αGC (100 ng/ml) or DMSO (vehicle) then washed before adding iNKT clones. After 48 h, concentrations of cytokines (IFN-γ, TNF-α, IL-5, IL-4, and IL-10) in the supernatant were determined by CBA. All data represent mean cytokine concentration from triplicate samples with error bars indicating +SD. *A*, Exogenous IL-2 induces IL-5 production from some iNKT cell clones. Shown are the representative experiments using two clones from HS (Sk and Kai.2) and four clones from MS (Kn.1, Kn.2, Oz. and Nkj.2). Clones in the *left panels* were stimulated with IL-2 alone, whereas those in the *right panels* were stimulated with IL-2 or αGC for comparison. *B*, IL-2 or αGC responses of each iNKT clone evaluated by production of IL-5 and IFN-γ. All $CD4^+$ iNKT cell clones from HS (◇) or MS (■) were stimulated with IL-2 (*left panels*) or αGC (*right*), and the content of IL-5 and IFN-γ in the supernatant was measured by CBA. In each panel, results of individual clones are plotted according to the production of IL-5 (picograms per milliliter) vs IL-5-IFN-γ ratio (*upper panels*) or IFN-γ production vs IFN-γ-IL-5 ratio (*lower panels*). By conducting this analysis, we could identify a distinctive group of clones that produced high IL-5 in response to IL-2 and presented with a high IL-5-IFN-γ ratio (*left, upper panel*). *C*, DN iNKT cell clones respond to αGC but not to IL-2. $CD4^-CD8^-$ DN iNKT cells were derived from HS and MS in parallel with $CD4^+$ iNKT clones, and the assay was conducted exactly the same as $CD4^+$ iNKT cell clones. Their cytokine responses to IL-2 and αGC were compared. Shown are the data of two clones from HS (Kai.2. DN, and Nn.DN) and one from MS (Nkj.2.DN). The counterpart $CD4^+$ clones of Kai2.DN and Nkj2.DN produced IL-5 in response to IL-2 (*A*), whereas the Nn.DN counterpart did not (data not shown). Data represent mean cytokine concentration from triplicate samples and error bars indicate +SD. The same legend for cytokine is used as in *A*. *D*, IL-15 also stimulates IL-5 production from the clones responsive to IL-2. iNKT cell clones producing IL-5 in response to IL-2 were cultured with iDCs for 48 h in presence of IL-3, -4, -5, -7, -9, -12, -15, GM-CSF (10 ng/ml), or IL-2 (10 IU/ml). Cytokines in the coculture supernatant were measured by CBA. Experiments using three clones (Kn.1, Kai.1, and Kai.2) gave similar results. Shown here is the cytokine production induced by exogenous IL-2 (10 IU/ml) and IL-15 and IL-5 (10 ng/ml) by clone Kn.1. Note that IL-5 data for exogenous IL-5 (1716.4 pg/ml) is eliminated from the graph. Data represent mean cytokine concentration from triplicate samples with error bars indicating +SD.

instead of αGC, to the coculture. 8 of the 26 clones produced an excessive amount of IL-5 (1500–7500 pg/ml; Fig. 1*A*; Table I). Remarkably, the level of IL-5 induced by IL-2 equaled or exceeded the amount that was induced by αGC (Fig. 1*A*, *right panels*). Although αGC induced large quantities of proinflammatory (IFN-γ, TNF-α) and Th2 cytokines from all the clones, exogenous IL-2 induced only a modest amount of the proinflammatory cytokines (20–700 pg/ml) and various amounts of IL-4 (0 pg/ml in Sk.1, 70–230 pg/ml in six other clones) from the eight clones capable of producing IL-5. To obtain deeper insights into this discrepancy, we plotted the ratios for IL-5 to IFN-γ or IFN-γ to IL-5 (vertical axis) vs quantities of IL-5 or IFN-γ in the supernatant (horizontal axis) (Fig. 1*B*). Regarding the ability to induce production of IFN-γ, αGC stimulation was much more potent than IL-2 and induced uniformly high responses from all the clones tested (Fig. 1*B*, *lower right panel*). A much wider range of IL-5 in quantity was produced after stimulation with IL-2 or αGC (Fig. 1*B*, *upper panels*). Interestingly, IL-2 stimulation revealed the presence of a distinct group of clones capable of producing an outstanding amount of IL-5 (1000 pg/ml<), also showing higher IL-5-IFN-γ ratios (Fig. 1*B*, *left upper panel*). In contrast, αGC stimulation could not elicit such a clear separation (*right upper panel*). The addition of a blocking Ab to IL-2R α-chain (anti-CD25 mAb) completely abolished the cytokine production triggered by IL-2 (data not shown). These results suggest that iNKT cells possess a previously unrecognized property to selectively produce an enormous amount of IL-5, which is probably restricted to a subset of CD4$^+$ iNKT cells. In parallel, we have generated three CD4$^-$CD8$^-$ DN iNKT cell clones and examined their reactivity to αGC or IL-2 in the same assay. These DN clones produced a large amount of IFN-γ and a lesser amount of TNF-α or Th2 cytokines in response to αGC. Although a large majority of CD4$^+$ iNKT clones produced IL-5 and/or IFN-γ in response to IL-2, none of the DN clones showed a significant response to IL-2 as measured by the production of cytokines (Fig. 1*C*).

When we evaluated the profile of IL-5 and IFN-γ secretion (Fig. 1*B*), there was no noticeable difference between iNKT cell clones derived from HS (◇) and MS (■). Furthermore, the clones producing a large amount of IL-5 could be generated at a similar frequency from HS and MS: 3 of 11 clones from HS (27.3%) vs 5 of 15 from MS (33.3%) (Table I). We used αGC or its synthetic analog OCH for primary stimulation to generate iNKT cell clones. OCH has a shorter sphingosine chain compared with αGC and has been shown to induce a selective production of Th2 cytokines from iNKT cells (19). To evaluate whether functional differences exist between αGC-derived and OCH-derived clones, we used both αGC and OCH as primary stimulus on PBMCs from all donors, always expanding every sample separately by each of these two glycolipids. A total of 26 iNKT cell clones were derived from 22 donors; pairs of αGC- and OCH-primed clones could be obtained only from 4 of the 22 donors (Nkj, Kai, Kn, and Ok). Although the sample size is not large enough to make any conclusive remarks, it seems that the choice of αGC or OCH is not a key factor in generating the IL-5-producing iNKT clones. Five of 14 clones generated by αGC stimulation (35.7%) produced a large amount of IL-5 in response to IL-2, and similarly 3 of 12 clones stimulated by OCH (25%) were able to do so. Moreover, when we closely examined the four pairs of αGC- and OCH-primed clones generated from the same donors, we still could not find any constant tendency concerning the ability of IL-5 production within these two types of clones (Table I).

The next important task was to evaluate the actual frequency of IL-5-producing iNKT cells within each individual. For this purpose, we freshly isolated PBMCs, stimulated them with IL-2, and examined the frequency of the IL-5-producing Vα24$^+$Vβ11$^+$ cell population by flow cytometric demonstration of intracellular IL-5. However, for unknown reason, we could not reveal the presence of IL-5-producing iNKT cells by this method. Then, we decided to generate a number of CD4$^+$ iNKT cell clones from same

Table II. *List of all genes significantly up-regulated after iDC and IL-2 stimulation*[a]

No.	Fold	Gene Name
1	18.86	**IL-5** (colony-stimulating factor, eosinophil)
2	13.70	**IL-2R, α**
3	11.40	**IL-13**
4	9.40	**IL-17R B**
5	7.93	**Chemokine (CC motif) ligand 4**
6	6.79	**Granzyme A (CTL-associated serine esterase 3)**
7	6.76	**Matrix metalloproteinase 12 (macrophage elastase)**
8	6.13	HEG homolog
9	6.05	Pim-1 oncogene
10	5.57	**NK cell transcript 4**
11	5.47	Hypothetical protein MAC30
12	5.46	Protein tyrosine phosphatase, receptor type, K
13	5.31	Arginine-rich, mutated in early stage tumors
14	5.00	A disintegrin and metalloproteinase domain 19 (meltrin β)
15	4.91	Cyclin D2
16	4.88	Hepatoma-derived growth factor, related protein 3
17	4.61	β5-tubulin
18	4.47	**Chemokine (CC motif) receptor 2**
19	4.12	Suppressor of var1, 3-like 1 (*Saccharomyces cerevisiae*)
20	3.91	Sideroflexin 1
21	3.89	**CD48 Ag (B cell membrane protein)**
22	3.70	Phosphoglycerate kinase 1
23	3.63	Bromodomain adjacent to zinc finger domain, 1B
24	3.52	Glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2)
25	3.52	Karyopherin (importin) β1
26	3.46	Chromosome 4 open reading frame 9
27	3.45	Emopamil-binding protein (sterol isomerase)
28	3.37	Nucleoporin 50 kDa
29	3.35	Lactate dehydrogenase B
30	3.29	UDP-Gal:βGlcNAc β 1,4-galactosyltransferase, polypeptide 5
31	3.28	Proteasome (prosome, macropain) subunit, α type, 1
32	3.26	RAS guanyl-releasing protein 1 (calcium and DAG regulated)
33	3.20	Proteasome (prosome, macropain) subunit, α type, 1
34	3.17	Proteasome (prosome, macropain) activator subunit 2 (PA28 β)
35	3.17	Heat shock 60-kDa protein 1 (chaperonin)
36	3.00	Proteasome (prosome, macropain) activator subunit 1 (PA28 α)
37	2.94	Chaperonin containing TCP1, subunit 4 (δ)
38	2.86	Synaptotagmin XI
39	2.84	Proteasome (prosome, macropain) subunit, α type, 1
40	2.80	**IL-2R, γ (severe combined immunodeficiency)**
41	2.61	Polypyrimidine tract-binding protein 1
42	2.55	Polypyrimidine tract-binding protein 1
43	2.52	Ribosomal protein S4, X-linked

[a] CD4$^+$ iNKT cell clones were cocultured with allogenic iDCs in the presence or absence of exogenous IL-2. The iNKT cells were separated and examined by using DNA microarray. Listed are all the genes that were significantly up-regulated (paired *t* test, $p < 0.05$) in the iNKT clone cells by the presence of exogenous IL-2. The genes are listed in order by fold increase of control. Immune-related genes are highlighted in bold.

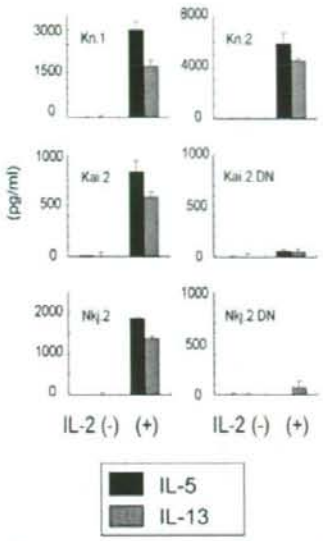


FIGURE 2. Exogenous IL-2 also triggered IL-13 production. $CD4^+$ iNKT cell clones Kn.1, Kai.2, Nkj.2, and Kn.2 as well as DN clones Kai.2. DN and Nkj.2. DN were stimulated with iDCs in the presence of IL-2. Supernatants were collected 48 h later and used for measurement of IL-5 (CBA) and -13 (ELISA). All of the data represent mean cytokine concentration from triplicate samples with error bars indicating +SD.

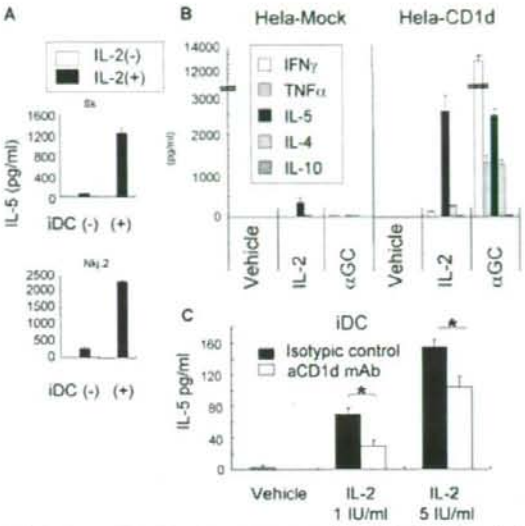


FIGURE 3. TCR-CD1d interaction was required for IL-2-mediated IL-5 production from iNKT clones. *A*, Presence of iDCs is critical for IL-5 production. $CD4^+$ iNKT clone cells Sk and Nkj.2 were cultured with or without iDCs in the presence or absence of IL-2 (10 IU/ml). Cytokines in the supernatant were measured by CBA after 48 h coculture. *B*, Comparison of CD1d- and Mock-transfected HeLa cells for IL-5 induction. The cytokine production of $CD4^+$ iNKT clone cells (Kn.1 and Kn.2) in response to IL-2 (10 IU/ml) and αGC (100 ng/ml) was evaluated by culturing them with CD1d-transfected or mock-transfected HeLa cells. For αGC stimulation, HeLa cells were loaded with αGC or DMSO (vehicle) for 12 h before incubation with iNKT cells. *C*, Suppression of IL-5 production by anti-CD1d blocking Ab. IL-5 production of $CD4^+$ iNKT clone cells (Kai.1 and Kk) in response to IL-2 stimuli (1 IU/ml, 5 IU/ml) was evaluated in the coculture with iDCs. To evaluate the involvement of CD1d, we added anti-CD1d mAb (aCD1d 59; 10 μg/ml) or isotype control Ab from the start of culture. Data represent mean cytokine concentration from triplicate samples with error bars indicating +SD. Although not indicated here, this blocking Ab significantly suppressed IFN-γ production induced by αGC stimulation by the same clones (*, $p < 0.01$, one-factor ANOVA).

donors by the single-cell sorting method, and estimate the frequency of IL-5-producing cells. Although the method is feasible, because it is laborious and time consuming, we selected two donors from whom we could reproducibly generate IL-5-producing clones. We found that two of five single cell-sorted clones derived from one donor efficiently produced IL-5 in response to IL-2. In another donor, the number was one of four (data not shown). This data implies that the frequency of IL-5-producing iNKT cell clones may reach 25–40% of total $CD4^+$ iNKT cells in individuals who have a higher number of IL-5-producing iNKT cells.

IL-15 could replace IL-2 in mediating the IL-5 production

To determine whether any cytokines other than IL-2 could also induce IL-5 production, representative IL-5-producing clones Kn.1, Kai.1, and Kai.2 were stimulated with IL-4, IL-7, IL-9, IL-15, IL-12, IL-3, or GM-CSF in the presence of iDCs. Among these cytokines examined, only IL-15 showed an IL-2-like potential to provoke the production of IL-5 from the iNKT cells (Fig. 1*D*). Of note, receptors for IL-4, IL-7, IL-9, and IL-15 share γ-chain of IL-2R referred to as the common γ-chain, whereas IL-15R also shares β-chain with IL-2R. This implies that the intermediate affinity IL-2R complex composed of the β- and γ-chains would mediate signals needed for IL-5 production. Of further interest, an addition of exogenous IL-5 induced a low but significant amount of IL-4 production. This raises a possibility that, at least in some clones, IL-5 produced by iNKT cells in response to IL-2 may subsequently trigger IL-4 production from the same cells in an autocrine fashion or from other iNKT cells in the close vicinity, thereby augmenting the ability of iNKT cells to polarize the Th cell toward Th2.

Gene expression profile of iNKT cells responding to IL-2

To further confirm that our observations represent a previously overlooked property of iNKT cells, we conducted a comprehensive gene expression analysis. An Affymetrix DNA microarray was applied to characterize the mRNA expression of four IL-5-producing clones Kn.1, Kn.2, Kai.2, and Nkj.2. The results showed that 43 genes were significantly up-regulated following IL-2 stimulation in the presence of iDCs (Table II). Most notably, *IL5* was identified as the gene with the highest increase of expression after simulation (fold increase = 18.86). As the direct consequence of IL-2 stimulation, IL-2Rα (*IL2RA*) was ranked as the second (fold increase = 13.70) and IL-2Rγ (*IL2RG*) as the 40th (fold increase = 2.8). Furthermore, *IL13* was ranked as the third (fold increase = 11.4), whereas neither *IL4* nor *IFNG* was among the genes significantly up-regulated in the examined culture condition. The increased expression of *IL13* prompted us to measure the content of the encoded protein in the supernatant by using ELISA. Consistent with the microarray data, the IL-5-producing clones were found to secrete a large amount of IL-13 as well (Fig. 2). These results indicate that the selective production of IL-5 and IL-13 in response to IL-2 could be a significant property of a subset of $CD4^+$ iNKT cells.

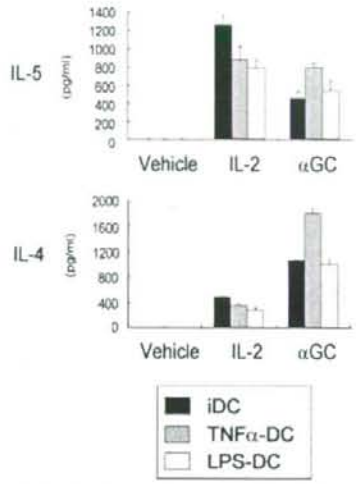


FIGURE 4. Comparison of the ability to induce IL-5 between iDC and mature DCs. The ability of iDCs and mature DCs to induce IL-5 from $CD4^+$ iNKT cells in the presence of IL-2 was compared. To obtain mature DCs, iDCs were further stimulated with TNF-α (10 ng/ml) or LPS (2 ng/ml) for 12 h (designated as TNF-α-DC and LPS-DC, respectively). Representative IL-5 producing clones, Sk.1 and Oz, were stimulated with IL-2 (10 IU/ml) or αGC (100 ng/ml) using these DC populations. Similar results were obtained by both clones. Shown here is the representative data of Sk.1. Supernatants were collected after 48 h and IL-5/IL-4 production was measured by CBA.

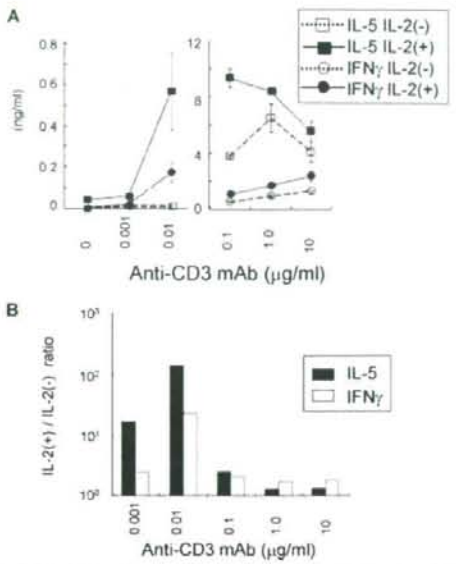


FIGURE 5. Suboptimal anti-CD3 stimulation mimics the effect of $CD1d^+$ APCs for IL-5 induction from $CD4^+$ iNKT clones in the presence of IL-2. $CD4^+$ iNKT cell clones Kn.2 and Kai.2 were stimulated by plate-bound anti-CD3 mAb (0.001–10 μg/ml) in the presence or absence of IL-2 (10 IU/ml). IL-5 and IFN-γ in the supernatant were measured by using ELISA. Clones Kn.2 and Kai.2 gave similar results. Shown are representative data obtained from Kn.2. Data represent mean cytokine concentration from triplicate samples and error bars indicate + SD. *A*, Production of IL-5 and IFN-γ when stimulated with various concentrations of plate bound anti-CD3 Ab. Horizontal axis indicates concentration of anti-CD3 mAb used (micrograms per milliliter), whereas amount of the cytokines (nanograms per milliliter) are shown in vertical axis. *B*, The effect of exogenous IL-2 on the cytokine values. To evaluate the augmenting effect of exogenous IL-2 on cytokine production, the amounts of cytokine produced at the presence of IL-2 were divided by those obtained at the absence of IL-2. This $IL\text{-}2^+/IL\text{-}2^-$ ratio was obtained using the data shown in *A*. Exogenous IL-2 induced IL-5 most efficiently and selectively when iNKT clones were stimulated at a suboptimal dose of anti-CD3 (0.01 μg/ml).

Requirement of TCR-CD1d interaction for the selective IL-5 production by iNKT cells

We have further addressed whether the IL-5 production from the iNKT cell clones may be induced in the absence of iDCs. When we stimulated iNKT cells with exogenous IL-2 in the absence of iDCs, only a trace amount of IL-5 was detected (Fig. 3*A*), elucidating the requirement of iDCs. To determine whether iNKT cells would interact with iDCs via TCR or accessory molecules, we next cultured the iNKT cell clones with CD1d-transfected or mock-transfected HeLa cells and again examined the effect of IL-2. The results showed that CD1d-transfected cells could serve as efficient APCs for the IL-5 production induced by IL-2 (Fig. 3*B*), whereas mock-treated cells could not. To clarify whether IL-5 production after iNKT cell interaction with iDC also depends on CD1d, we examined the effect of the CD1d-blocking Ab (αCD1d59) on IL-5 production from the iNKT-iDC coculture. As shown in Fig. 3*C*, addition of the anti-CD1d Ab significantly reduced IL-5 production in response to IL-2. This indicates that TCR-CD1d interaction is critical for iNKT cell clones to produce IL-5, supporting the involvement of TCR signaling. Autoreactive iNKT cells are generally thought to recognize endogenous ligands loaded onto CD1d molecules. Therefore, we tried to stimulate iNKT cells with CD1d dimer (dimer X) loaded with iGb3, a recently identified endogenous ligand for iNKT cells (30, 31). However, loading iGb3 to dimer X was not successful in inducing IL-5 production. When cultured on a plastic plate precoated simply with unloaded dimer X, iNKT cells did not respond to IL-2 (data not shown). We also used TNF-α- or LPS-induced mature DCs as APCs for comparison with iDCs, assuming that up-regulated costimulatory molecules in mature DCs may help further promote IL-5 production (Fig. 4). Regarding αGC-induced IL-5 or IL-4 production, TNF-α-induced mature DCs appeared to be more potent than iDCs. In contrast, iDCs seemed as potent as mature DCs in the induction of IL-5 production by iNKT cells in response to IL-2, indicating that the IL-2-induced IL-5 response is not heavily influenced by the maturation state of DCs.

Suboptimal anti-CD3 stimulation with exogenous IL-2 promotes IL-5 production

As demonstrated above, CD1d expression on APCs (Fig. 3*B*) as well as the presence of exogenous IL-2 is critically required for IL-5 induction from iNKT cells. Our speculation is that IL-5-producing iNKT cells are autoreactive to CD1d ligand, but they cannot mount the detectable IL-5 response unless accessory IL-2 signaling is provided. This is based on the premise that the endogenous ligand expressed by DCs could not provide sufficiently strong TCR signals able to provoke IL-5 production. To test this hypothesis, we explored whether suboptimal cross-linking of TCR in the presence of IL-2 could induce selective IL-5 production. We stimulated iNKT cells with plate-bound anti-CD3 mAb in the presence of IL-2. Using this APC-free system, we found that even without adding IL-2, the iNKT clone cells produce a large amount of IL-5 and somewhat a lesser amount of IFN-γ at higher concentrations of anti-CD3 mAb (0.1, 1.0, and 10 μg/ml)

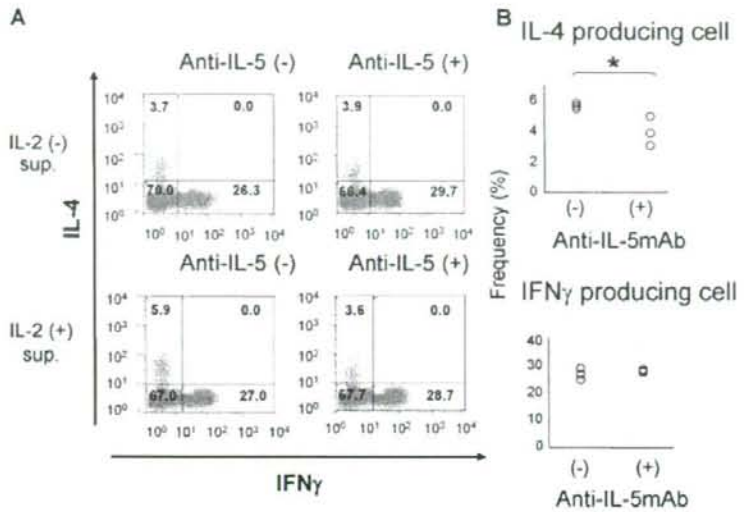


FIGURE 6. iNKT/iDC coculture supernatant induced Th2 CD4 T cell differentiation in an IL-5-dependent way. Here, we stimulated naive CD4 T cells with plate bound-anti-CD3 mAb (10 μg/ml) and soluble anti-CD28 mAb (2 μg/ml) and evaluated the effect of adding IL-2-induced IL-5-enriched supernatant from iNKT/iDC coculture (Kn.2). The presence of IL-5 in IL-2$^+$ sup and its absence in IL-2$^-$ sup was confirmed by CBA before the assay. To evaluate the effect of IL-5 in the iNKT/iDC supernatant, assays were also conducted in the presence of anti-IL-5-neutralizing mAb. Seven days after culture, cells were harvested and intracellular cytokines (IL-4 and IFN-γ) were stained after 6 h PMA/ionomycin stimulation. *A*, Flow cytometry analysis of intracellular IL-4/IFN-γ staining of CD4$^+$ T cells following CD3/CD28 stimulation. The numbers indicate the percentage of cells in the given quadrant. More IL-4-producing cells were generated in the presence of IL-2$^+$ sup compared with IL-2$^-$ sup. The number of IL-4-producing T cell was reduced when anti-IL-5 mAb was given. In contrast, the number of IFN-γ-producing cells remained unaffected. Shown here is a representative data of two separate experiments with consistent results. *B*, Effect of anti-IL-5 mAb on the induction of IL-4$^+$ or IFN-γ$^+$ T cells. The frequency of IL-4- or IFN-γ-producing cells after culture with IL-2$^+$ sup was determined as in *A*. In the presence of anti-IL-5 mAb, IL-4-producing cells were significantly reduced (the mean frequency dropped to 4.3% from 5.76%) (*, $p < 0.05$, one-factor ANOVA), whereas the frequency IFN-γ-producing cells stayed the same (the mean frequency was 27.0% and 28.2%).

(Fig. 5*A*). In this range of high Ab concentration, production of both IL-5 and IFN-γ was almost equally augmented by adding exogenous IL-2 (Fig. 5*B*), as judged by IL-2$^+$-IL-2$^-$ ratios. As expected, the lower concentration of anti-CD3 (0.001 and 0.01 μg/ml) without IL-2 induced only trace amounts of IL-5 or IFN-γ from iNKT cell clones. However, when exogenous IL-2 was added, the production of the cytokines was greatly augmented (Fig. 5*A*). In particular, IL-5 production at 0.01 μg/ml anti-CD3 was greatly and selectively induced. The IL-2$^+$-IL-2$^-$ ratio for IL-5 was 140.2, whereas that for IFN-γ was −23.0 (Fig. 5*B*). As such, a suboptimal TCR stimulation with anti-CD3 could mimic the stimulation with CD1d$^+$ APCs, supporting our hypothesis that the suboptimal TCR and IL-2 signaling would cause IL-5 production from autoreactive iNKT cells.

IL-5 from iNKT cells promotes Th2 differentiation of naive CD4$^+$ T cells

Given that some CD4$^+$ iNKT cells selectively produce IL-5, it is important to know whether the iNKT cells may actively modulate an immune response by producing IL-5. To clarify this point further, we have collected the supernatant from iNKT cells that were stimulated with CD1d$^+$ iDCs and IL-2 (IL-2$^+$ sup), or from those stimulated with the iDCs alone (IL-2$^-$ sup). Then, we examined the effect of adding these supernatants on in vitro differentiation of naive T cells induced with plate-bound anti-CD3 mAb stimulation. After 7 days of culture with the supernatant, the percentage of IL-4- or IFN-γ-producing CD4$^+$ T cells was enumerated by intracellular cytokine staining. The results showed that the addition of IL-2$^+$ sup induced higher frequencies of IL-4$^+$CD4$^+$ T cells, as compared with IL-2$^-$ sup (5.9 vs 3.7% in Fig. 6*A*, *left*). In contrast, IFN-γ$^+$CD4$^+$ T cells did not differ significantly (27.0 vs 26.3%). Similar results were obtained in repeated experiments, indicating that IL-2$^+$ sup possess the biological activity to induce significant Th2 polarization of Th cells. To assess the role of IL-5 in the supernatant, we added neutralizing IL-5 Ab to the IL-2$^+$ sup and then evaluated its effect in the same culture system. The results showed that presence of anti-IL-5 significantly lowered the frequency of the IL-4$^+$CD4$^+$ T cells on day 7 (Fig. 6*B*, *upper*), whereas it did not change the percentage of IFN-γ$^+$ T cells (Fig. 6*B*, *upper*). Collectively, these results suggest that the IL-5 secreted by iNKT cells could contribute to mediating Th2 bias, although other factors such as IL-13 may also play some role.

Th2 cytokine deviation by IL-2 in BALB/c iNKT cells

The present results demonstrate that predominant production of IL-5, which has not been appreciated as an important property of iNKT cells, would characterize a proportion of autoreactive iNKT cell clones. Interestingly, the IL-5 response could be elicited by a weak TCR stimulus together with IL-2 or IL-15, which might occur during in vivo inflammatory reactions. However, one could argue that we might have seen an artifact arising from use of the iNKT cell clones that were repeatedly stimulated and expanded in vitro. To challenge this criticism, we examined the responsiveness of freshly separated mouse iNKT cells to CD1d$^+$ iDCs in the presence of IL-2. At the beginning, we tried to reproduce the results by using fresh human iNKT cells, but the low frequency of iNKT cells (0.01–0.1% of the PBMC) precluded our attempt. Therefore, freshly isolated iNKT cells and CD11c$^+$ DCs from

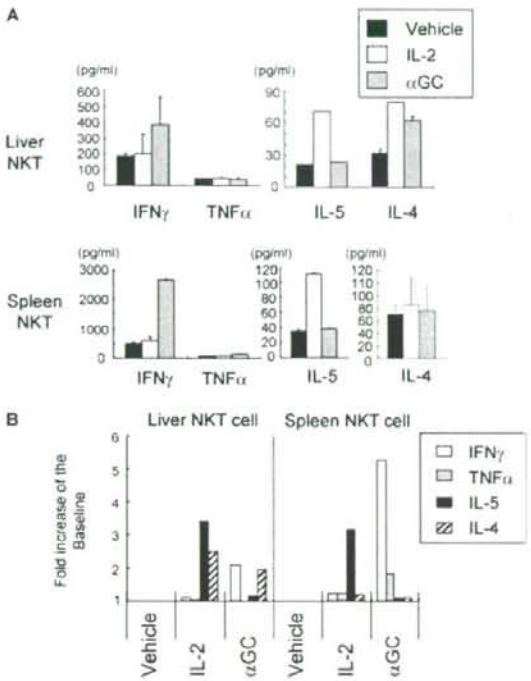


FIGURE 7. Freshly isolated iNKT cells from BALB/c mice produced IL-5 in the presence of IL-2. iNKT cells were isolated from livers and spleens pooled from six BALB/c mice of the same age by using αGC loaded-CD1d dimer X. iNKT cells were cocultured with magnetically isolated $CD11c^+$ splenocytes and stimulated with IL-2 or αGC for 72 h. Cytokines in the supernatant were evaluated by CBA. Data represent mean cytokine concentration from triplicate samples with error bars indicating +SD. Shown here is the representative data from three separate experiments, which gave similar results. *A*, Cytokine values (picograms per milliliter). *B*, Cytokine induction evaluated by fold increase from the baseline.

BALB/c mice were cocultured in the presence of IL-2 or αGC. Compared with human iNKT clone-iDC cocultures, the mouse cocultures showed a relatively high background response in any cytokine measured. However, as seen in humans, exogenous IL-2 apparently augmented the production of IL-5 from liver and spleen iNKT cells (Fig. 7), whereas neither IFN-γ nor TNF-α was altered by IL-2 stimulation. In contrast to IL-2, αGC stimulation induced a massive production of IFN-γ from liver and spleen iNKT cells and some TNF-α from spleen iNKT cells. However, IL-5 triggered by αGC was negligible.

To further convince ourselves of the significance of the physiological effect of IL-2 on iNKT cells, we investigated the effect of IL-2 on iNKT cells in vivo. We injected the cytokine i.v. to BALB/c mice. Two hours later, we separated liver lymphocytes and analyzed the intracellular expression of IL-5 by flow cytometry analysis. Consistent with the ex vivo IL-2 stimulation data, a significant increase in the number of IL-5-producing iNKT cells was observed in the BALB/c mice injected with IL-2, while a similar increase in the number of IFN-γ-producing iNKT cells could not be found (Fig. 8). These data imply that rodent iNKT cells are able to produce IL-5 ex vivo as well as in vivo in response to IL-2 as demonstrated in human iNKT clones. Taken together, we propose that this selective IL-5 production could represent a physiological iNKT cell response.

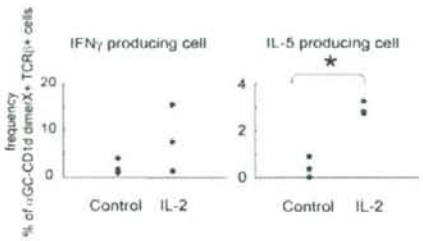


FIGURE 8. Increase of IL-5-producing iNKT cell by in vivo administration of IL-2. Liver lymphocyte were isolated after i.v. IL-2 administration and stained for intracellular IL-5 or IFN-γ production. The frequency of αGC-loaded CD1d dimer X^+ $TCR\beta^+$ iNKT cells was plotted for IL-5- and IFN-γ-producing cells. Each dot represents data obtained from one mouse. Three mice were used for each condition. IL-5-producing iNKT cells were significantly increased in IL-2 i.v. mice ($p < 0.05$, Welch's t test) though no significant difference was observed in IFN-γ-producing iNKT cells.

Discussion

The cardinal property of iNKT cells to produce regulatory cytokines has been well-documented in prior studies. It is, however, of note that the reagents used for inducing cytokines were either αGC, an unnatural ligand for iNKT cells, or anti-CD3 Ab in most cases. These reagents would transmit potent TCR signaling, thereby provoking production of a very wide range of Th1 and Th2 cytokines from iNKT cells. Therefore, it is still unclear as to which cytokines are naturally secreted and which are not involved in physiological immune regulation during the natural course of diseases. In this study, we have reported that some human $CD4^+$ iNKT cell clones could selectively produce enormous amounts of IL-5 and IL-13 when cultured with $CD1d^+$ APCs in the presence of IL-2 or IL-15. Importantly, the amount of IL-5 was even higher than that induced by αGC. Moreover, DNA microarray analysis identified *IL5* and *IL13* as the genes that were most highly upregulated during their response to $CD1d^+$ APCs in the presence of IL-2. Subsequent analysis showed that plate-bound anti-CD3 Ab could replace the $CD1d^+$ APCs, suggesting that iNKT cells may exhibit the IL-5-producing function after recognizing an endogenous self-ligand expressed by APCs. Also, the IL-5-enriched supernatant from the iNKT cells showed a Th2-biasing effect in vitro, which could be blocked by neutralizing anti-IL-5 mAb. Finally, we showed that freshly isolated iNKT cells could also produce IL-5 in response to $CD1d^+$ DCs in the presence of IL-2. These results indicate that a subset of $CD4^-$ iNKT cells may use IL-5 and IL-13 as important mediators to regulate Th cell responses in vivo. It is likely that the iNKT cells producing these cytokines may play a decisive role in the control of Th1-mediated pathogenesis or mediating allergic conditions.

It has been well-recognized freshly isolated iNKT cells express activation markers such as CD69 even shortly after birth (23, 35). Together with the recent discovery that iGb3 could serve as an endogenous ligand for iNKT cells (30, 31), it is generally accepted that iNKT cells are autoreactive cells that are being constantly stimulated by self-ligand(s). Consistent with this concept, studies have shown that a proportion of iNKT cell clones exhibit various degrees of self-reactivity as measured by proliferative responses against $CD1d^+$ APCs (29, 36). However, it has remained unclear what percentage of iNKT cells may exhibit such an autoreactive response as demonstrated by simple proliferation assays. In this regard, it is noteworthy that the self-reactivity of our iNKT cell clones could be demonstrated by using their ability to produce IL-5