of CD40L and following production of inflammatory cytokines by bystander cells in vivo. Int Immunol 2005;17:1619–29.

24. Mizuno M, Masumura M, Tomi C, Chiba A, Oki S, Yamamura T, et al. Synthetic glycolipid OCH prevents insulitis and diabetes in NOD mice. J Autoimmun 2004;23:293–300.

25. Chiba A, Oki S, Miyamoto K, Hashimoto H, Yamamura T, Miyake S. Suppression of collagen-induced arthritis by natural killer T cell activation with OCH, a sphingosine-truncated analog of α-galactosylceramide. Arthritis Rheum 2004;50:305–13.

26. Chiba A, Kaieda S, Oki S, Yamamura T, Miyake S. The involvement of $V_\alpha 14$ natural killer T cells in the pathogenesis of arthritis in murine models. Arthritis Rheum 2005;52:1941–8.

27. Kim HY, Kim HJ, Min HS, Park ES, Park SH, Chung DH. NKT cells promote antibody-induced arthritis by suppressing transforming growth factor β1 production. J Exp Med 2005;201:41–7.

28. Cui J, Shin T, Kawano T, Sato H, Kondo E, Toura I, et al. Requirement for Vα14 NKT cells in IL-12-mediated rejection of tumors. Science 1997;278:1623–6.

29. Murata K, Toba T, Nakanishi K, Takahashi B, Yamamura T, Miyake S, et al. Total synthesis of an immunosuppressive glycolipid, (2S,3S,4R)-1-O-(α-d-galactosyl)-2-tetracosanoylamino-1,3,4-nonanetriol. J Org Chem 2005;70:2398–401.

30. Williams RO, Feldmann M, Maini RN. Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. Proc Natl Acad Sci U S A 1992;89:9784–8.

31. Watts GM, Beurskens FJ, Martin-Padura I, Ballantyne CM, Klickstein LB, Brenner MB, et al. Manifestations of inflammatory arthritis are critically dependent on LFA-1. J Immunol 2005;174:3668–75.

32. Binstadt BA, Patel PR, Alencar H, Nigrovic PA, Lee DM, Mahmood U, et al. Particularities of the vasculature can promote the organ specificity of autoimmune attack. Nat Immunol 2006;7:284–92.

33. Boissier MC, Chiocchia G, Bessis N, Hajnal J, Gianni G, Nicolett F, et al. Biphasic effect of interferon-γ in murine collagen-induced arthritis. Eur J Immunol 2006;25:1184–90.

34. Jacob CO, Holoshitz J, van der Meide PH, Strober S, McDevitt HO. Heterogeneous effects of IFN-γ in adjuvant arthritis. J Immunol 1989;142:1500–5.

35. Wiesenberg I, van der Meide PH, Schellekens H, Alkan SS. Suppression and augmentation of rat adjuvant arthritis with monoclonal anti-interferon-γ antibody. Clin Exp Immunol 1989;78:245–9.

36. Puliti M, von Hunolstein C, Bistoni F, Mosci P, Orefici G, Tissi L. Influence of interferon-γ administration on the severity of experimental group B streptococcal arthritis. Arthritis Rheum 2000;43:2678–86.

37. Manoury-Schwartz B, Chiocchia G, Bessis N, Abehsira-Amar O,

38. Batteux F, Muller S, et al. High susceptibility to collagen-induced arthritis in mice lacking IFN-γ receptors. J Immunol 1997;158:5501–6.

38. Vermeire K, Heremans H, Vandeputte M, Huang S, Billiau A, Matthys P. Accelerated collagen-induced arthritis in IFN-γ receptor-deficient mice. J Immunol 1997;158:5507–13.

39. Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-γ. Annu Rev Immunol 1997;15:749–95.

40. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-γ: an overview of signals, mechanisms and functions. J Leukoc Biol 2004;75:163–89.

41. Takayanagi H, Ogasawara K, Hida S, Chiba T, Murata S, Sato K, et al. T-cell-mediated regulation of osteoclastogenesis by signaling cross-talk between RANKL and IFN-γ. Nature 2000;408:600–5.

42. Cuo CO, Wittmer S, Dalton DK. Failure to suppress the expansion of the activated CD4 T cell population in interferon γ-deficient mice leads to exacerbation of experimental autoimmune encephalomyelitis. J Exp Med 2000;192:123–8.

43. Furlan R, Brambilla E, Ruffini F, Poliani PL, Bergamik A, Marconi PC, et al. Intrathecal delivery of IFN-γ protects C57BL/6 mice from chronic-progressive experimental autoimmune encephalomyelitis by increasing apoptosis of central nervous system-infiltrating lymphocytes. J Immunol 2001;167:1821–9.

44. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Sockinger B. TGFβ in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity 2006;24:179–89.

45. Chen Y, Langrish CL, McKenzie B, Joyce-Shaikh B, Stumhofer JS, McClanahan T, et al. Anti-IL-23 therapy inhibits multiple inflammatory pathways and ameliorates autoimmune encephalomyelitis. J Clin Invest 2006;116:1317–26.

46. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, et al. Transforming growth factor-β induces development of the Th17 lineage. Nature 2006;441:231–4.

47. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector Th17 and regulatory T cells. Nature 2006;441:235–8.

48. Flaishon L, Topilski I, Shoseyov D, Hershkoviz R, Fireman E, Levo Y, et al. Anti-inflammatory properties of low levels of IFN-γ. J Immunol 2002;168:3707–11.

49. Flaishon L, Hershkoviz R, Lantner F, Lider O, Alon R, Levo Y, et al. Autocrine secretion of interferon γ negatively regulates homing of immature B cells. J Exp Med 2000;192:1381–8.

50. Flaishon L, Lantner F, Hershkovi R, Levo Y, Shachar I. Low levels of IFN-γ down-regulate the integrin-dependent adhesion of B cells by activating a pathway that interferes with cytoskeleton rearrangement. J Biol Chem 2001;276:46701–6.

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.

Department of Immunology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Ogawahigashi, Kodaira, Tokyo, Japan

[2] Address correspondence and reprint requests to Dr. Takashi Yamamura, Department of Immunology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan. E-mail address: yamamura@ncnp.go.jp.

[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. 1A, 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
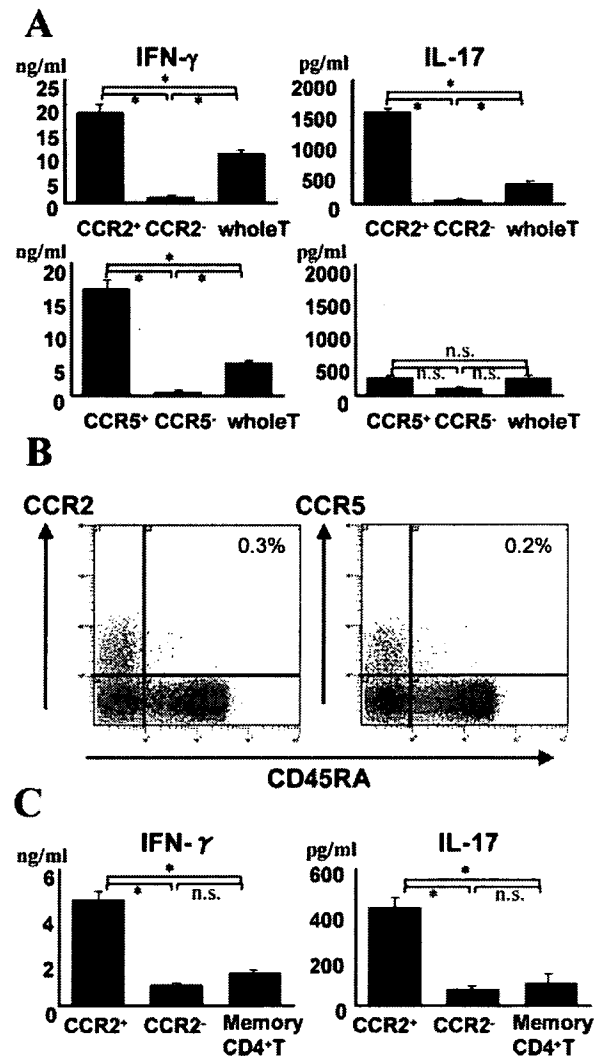

FIGURE 1. Th17 cells are enriched in CCR2^{+}CD4^{+}CD45RA^{−} cells. A, IL-17-producing cells are selectively enriched in the CCR2^{+}, but not in a CCR5^{+} population. From PBMC of healthy donors, whole T cells were purified magnetically by negative selection using a Pan T cell isolation kit II with 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 $\gamma\delta$T cells, capable of producing IL-17 (23–25), it remained possible that the major source of IL-17 could be CD8$^+$ T or $\gamma\delta$T 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$^+$, $\gamma\delta$, 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
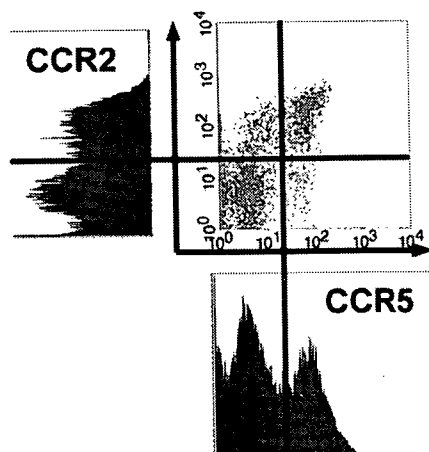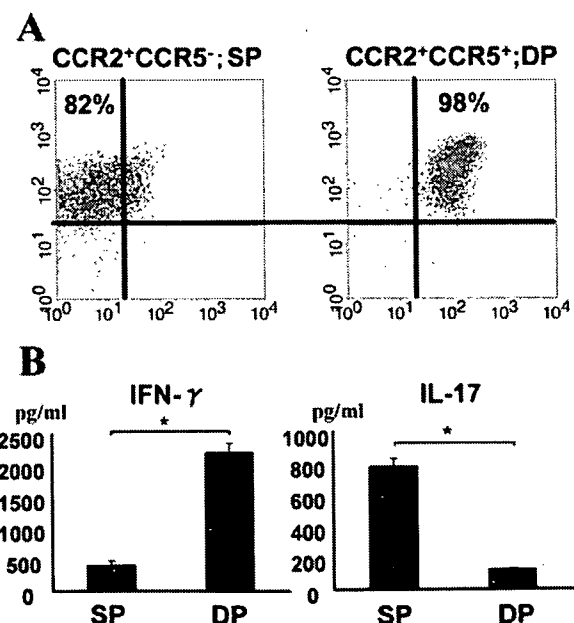
with the cytokine profile ex vivo, we decided to first isolate SP and DP cells from memory CCR2$^+$ T cells by using a flow cytometric cell sorter and stimulate them with PMA and ionomycin. We then measured IFN-γ and IL-17 in the supernatant (Fig. 3*A*). Remarkably, the sorted T cell subpopulations exhibited 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-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. *, $p < 0.01$

A

B

C

D
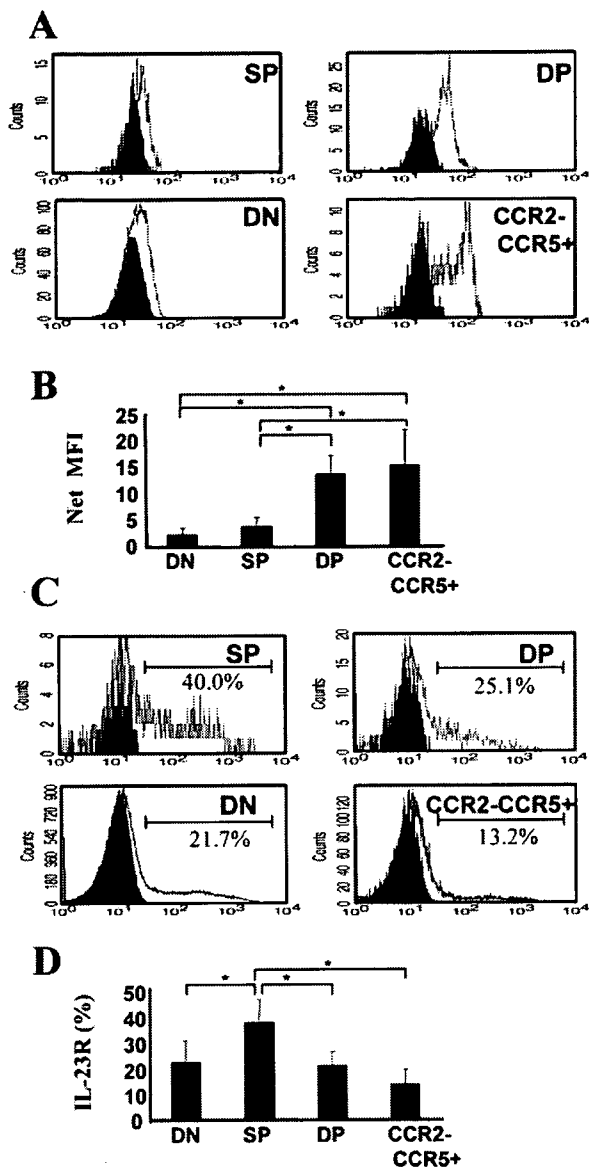
FIGURE 4. T-bet expression of memory CD4$^+$ T cells. *A*, Histogram plots of T-bet expression within memory CD4$^+$ T cells. SP, DP, DN (CCR2$^-$CCR5$^-$), and CCR2$^-$CCR5$^+$ T cells were stained with anti-T-bet or isotype control Ab. Shown is a representative of five individual data sets. *B*, The 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.

Acknowledgments

Disclosures

References
1. Cua, D. J., J. Sherlock, Y. Chen, C. A. Murphy, B. Joyce, B. Seymour, L. Lucian, W. To, S. Kwan, T. Churakova, et al. 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 421: 744–748.
2. Langrish, C. L., Y. Chen, W. M. Blumenschein, J. Mattson, B. Basham, J. D. Sedgwick, T. McClanahan, R. A. Kastelein, and D. J. Cua. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* 201: 233–240.
3. Chen, Y., C. L. Langrish, B. McKenzie, B. Joyce Shaikh, J. S. Stumhofer, T. McClanahan, W. Blumenschein, T. Churakovsa, J. Low, L. Presta, et al. 2006. Anti-IL-23 therapy inhibits multiple inflammatory pathways and ameliorates autoimmune encephalomyelitis. *J. Clin. Invest.* 116: 1317–1326.
4. Yen, D., J. Cheung, H. Scheerens, F. Poulet, T. McClanahan, B. McKenzie, M. A. Kleinschek, A. Owyang, J. Mattson, W. Blumenschein, et al. 2006. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J. Clin. Invest.* 116: 1310–1316.
5. Yao, Z., S. L. Painter, W. C. Fanslow, D. Ulrich, B. M. Macduff, M. K. Spriggs, and R. J. Armitage. 1995. Human IL-17: a novel cytokine derived from T cells. *J. Immunol.* 155: 5483–5486.
6. Kolls, J. K., and A. Linden. 2004. Interleukin-17 family members and inflammation. *Immunity* 21: 467–476.
7. Stark, M. A., Y. Huo, T. L. Burcin, M. A. Morris, T. S. Olson, and K. Ley. 2005. Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. *Immunity* 22: 285–294.
8. Harrington, L. E., R. D. Hatton, P. R. Mangan, H. Turner, T. L. Murphy, K. M. Murphy, and C. T. Weaver. 2005. Interleukin 17-producing CD4$^+$ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* 6: 1123–1132.
9. Park, H., Z. Li, X. O. Yang, S. H. Chang, R. Nurieva, Y. H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat. Immunol.* 6: 1133–1141.
10. Aggarwal, S., N. Ghilardi, M. H. Xie, F. J. de Sauvage, and A. L. Gurney. 2003. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J. Biol. Chem.* 278: 1910–1914.
11. Veldhoen, M., R. J. Hocking, C. J. Atkins, R. M. Locksley, and B. Stockinger. 2006. TGFβ in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24: 179–189.
12. Bettelli, E., Y. Carrier, W. Gao, T. Korn, T. B. Strom, M. Oukka, H. L. Weiner, and V. K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441: 235–238.
13. Mangan, P. R., L. E. Harrington, D. B. O'Quinn, W. S. Helms, D. C. Bullard, C. O. Elson, R. D. Hatton, S. M. Wahl, T. R. Schoeb, and C. T. Weaver. 2006.

Transforming growth factor-β induces development of the T_H17 lineage. *Nature* 441: 231–234.

14. Ivanov, I. I., B. S. McKenzie, L. Zhou, C. E. Tadokoro, A. Lepelley, J. J. Lafaille, D. J. Cua, and D. R. Littman. 2006. The orphan nuclear receptor RORγt directs the differentiation program of proinflammatory IL-17$^+$ T helper cells. *Cell* 126: 1121–1133.

15. Lanzavecchia, A., and F. Sallusto. 2000. Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. *Science* 290: 92–97.

16. Weber, C., K. S. Weber, C. Klier, S. Gu, R. Wank, R. Horuk, and P. J. Nelson. 2001. Specialized roles of the chemokine receptors CCR1 and CCR5 in the recruitment of monocytes and T_H1-like/CD45RO$^+$ T cells. *Blood* 97: 1144–1146.

17. Bonecchi, R., G. Bianchi, P. P. Bordignon, D. D'Ambrosio, R. Lang, A. Borsatti, S. Sozzani, P. Allavena, P. A. Gray, A. Mantovani, and F. Sinigaglia. 1998. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J. Exp. Med.* 187: 129–134.

18. Campbell, J. D., and K. T. HayGlass. 2000. T cell chemokine receptor expression in human Th1- and Th2-associated diseases. *Arch. Immunol. Ther. Exp.* 48: 451–456.

19. Nagata, K., K. Tanaka, K. Ogawa, K. Kemmotsu, T. Imai, O. Yoshie, H. Abe, K. Tada, M. Nakamura, K. Sugamura, and S. Takano. 1999. Selective expression of a novel surface molecule by human Th2 cells in vivo. *J. Immunol.* 162: 1278–1286.

20. Qin, S., J. B. Rottman, P. Myers, N. Kassam, M. Weinblatt, M. Loetscher, A. E. Koch, B. Moser, and C. R. Mackay. 1998. The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *J. Clin. Invest.* 101: 746–754.

21. Izikson, L., R. S. Klein, I. F. Charo, H. L. Weiner, and A. D. Luster. 2000. Resistance to experimental autoimmune encephalomyelitis in mice lacking the CC chemokine receptor (CCR) 2. *J. Exp. Med.* 192: 1075–1080.

22. Fife, B. T., G. B. Huffnagle, W. A. Kuziel, and W. J. Karpus. 2000. CC chemokine receptor 2 is critical for induction of experimental autoimmune encephalomyelitis. *J. Exp. Med.* 192: 899–905.

23. Vanden Eijnden, S., S. Goriely, D. De Wit, F. Willems, and M. Goldman. 2005. IL-23 up-regulates IL-10 and induces IL-17 synthesis by polyclonally activated naive T cells in human. *Eur. J. Immunol.* 35: 469–475.

24. Umemura, M., T. Kawabe, K. Shudo, H. Kidoya, M. Fukui, M. Asano, Y. Iwakura, G. Matsuzaki, R. Imamura, and T. Suda. 2004. Involvement of IL-17 in Fas ligand-induced inflammation. *Int. Immunol.* 16: 1099–1108.

25. Lockhart, E., A. M. Green, and J. L. Flynn. 2006. IL-17 production is dominated by $\gamma\delta$T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. *J. Immunol.* 177: 4662–4669.

26. Loetscher, P., M. Seitz, M. Baggiolini, and B. Moser. 1996. Interleukin-2 regulates CC chemokine receptor expression and chemotactic responsiveness in T lymphocytes. *J. Exp. Med.* 184: 569–577.

27. Szabo, S. J., S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman, and L. H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100: 655–669.

28. Gocke, A. R., P. D. Cravens, L. H. Ben, R. Z. Hussain, S. C. Northrop, M. K. Racke, and A. E. Lovett Racke. 2007. T-bet regulates the fate of Th1 and Th17 lymphocytes in autoimmunity. *J. Immunol.* 178: 1341–1348.

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

Kaori Sakuishi,[*][†] Shinji Oki,[*] Manabu Araki,[*] Steven A. Porcelli,[‡] Sachiko Miyake,[*] and Takashi Yamamura[2][*]

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.

I nvariant 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,

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.
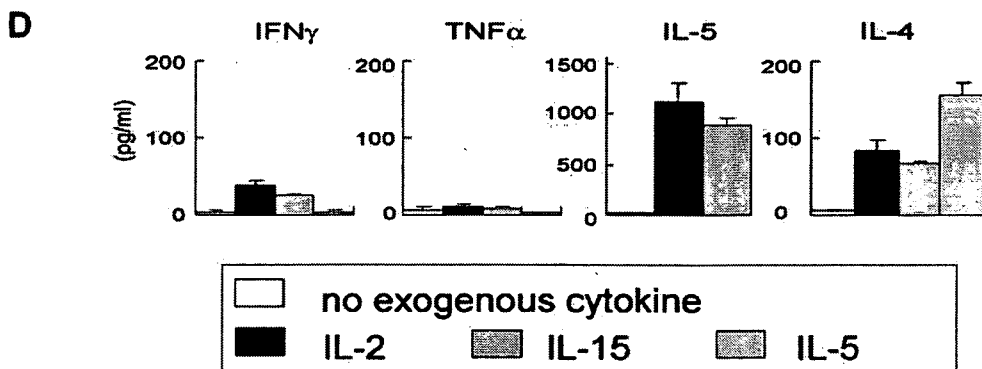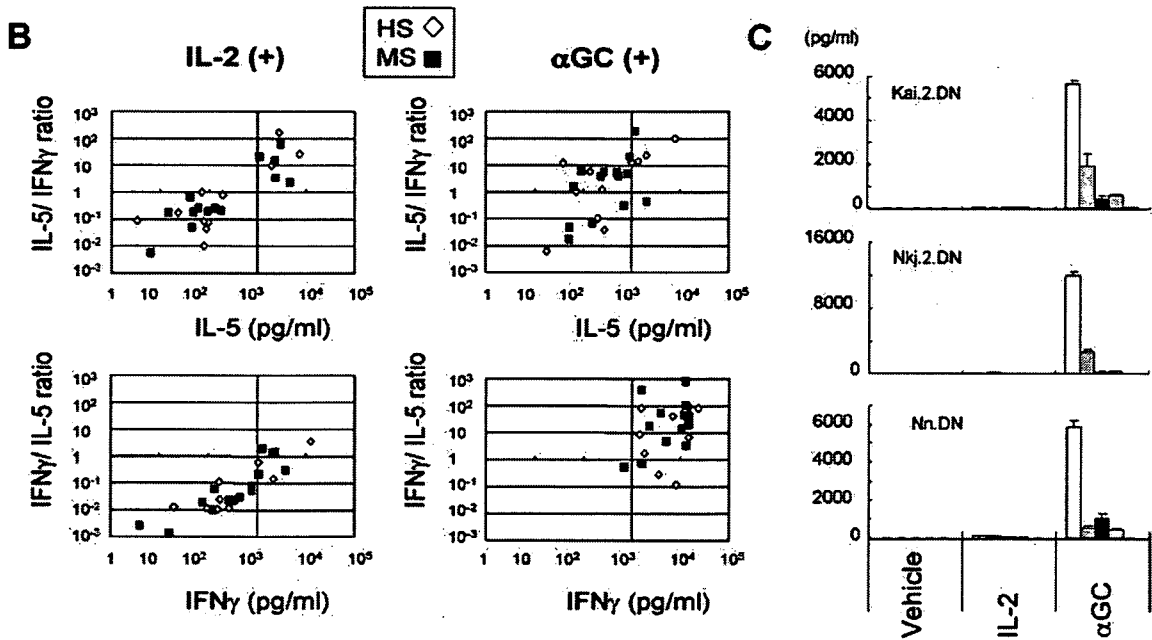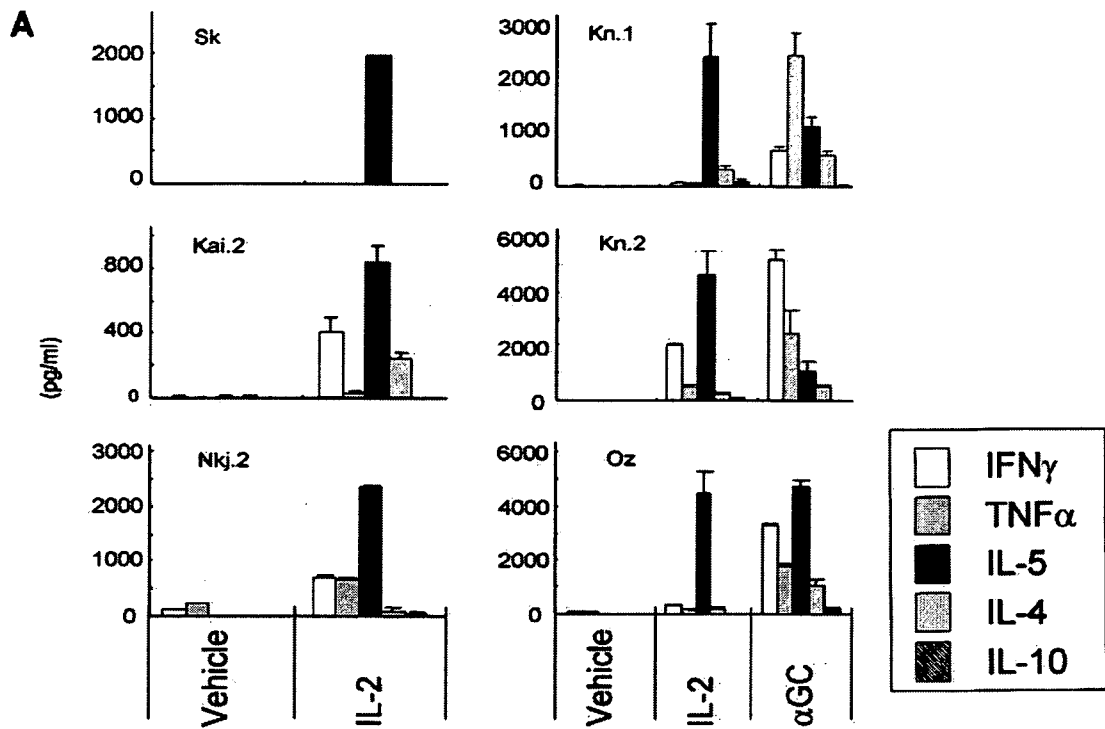
Human iNKT clones

PBMCs were isolated by density gradient centrifugation and suspended at 1 × 10⁶/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 × 10⁴ 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 TCRβ⁺ 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 × 10⁴ 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 × 10⁴ 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. 1A; Table I). Remarkably, the level of IL-5 induced by IL-2 equaled or exceeded the amount that was induced by αGC (Fig. 1A, *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. 1B). 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. 1B, *lower right panel*). A much wider range of IL-5 in quantity was produced after stimulation with IL-2 or αGC (Fig. 1B, *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. 1B, *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. 1C).

When we evaluated the profile of IL-5 and IFN-γ secretion (Fig. 1B), 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

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.

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
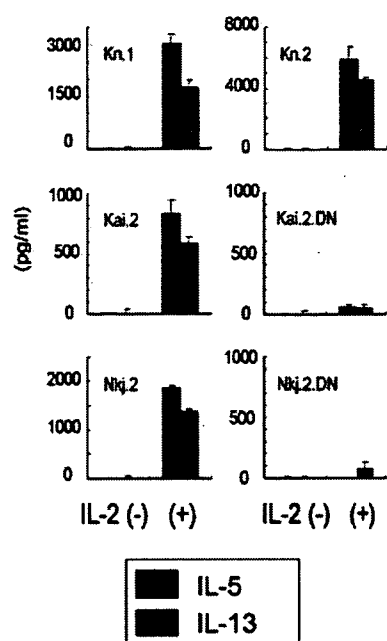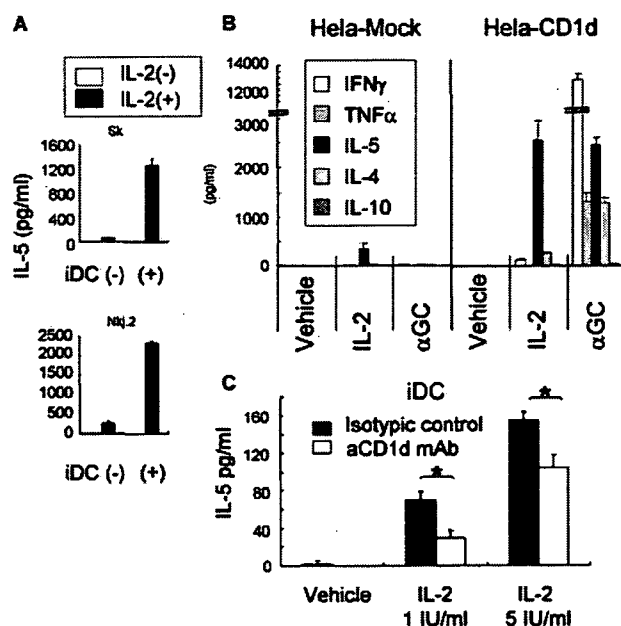
FIGURE 2. Exogenous IL-2 also triggered IL-13 production. CD4$^+$ iNKT cell clones Kn.1, Kai.2, Nkj.2, and Kn.2 as well as DN clones Kai.2. DN and Nkj.2. DN were stimulated with iDCs in the presence of IL-2. Supernatants were collected 48 h later and used for measurement of IL-5 (CBA) and -13 (ELISA). 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. 1D). 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.
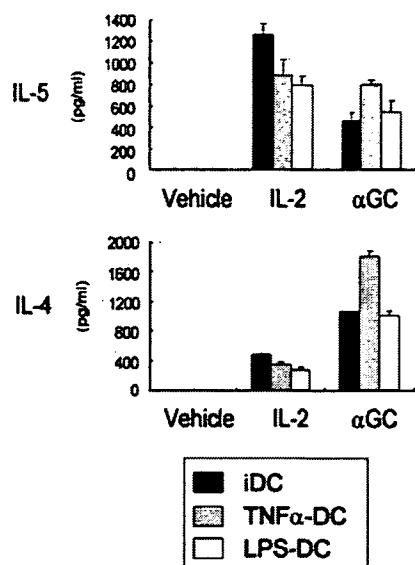
FIGURE 4. Comparison of the ability to induce IL-5 between iDC and mature DCs. The ability of iDCs and mature DCs to induce IL-5 from CD4$^+$ iNKT cells in the presence of IL-2 was compared. To obtain mature DCs, iDCs were further stimulated with TNF-α (10 ng/ml) or LPS (2 ng/ml) for 12 h (designated 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.

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. 3A), 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. 3B), 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 (aCD1d59) on IL-5 production from the iNKT-iDC coculture. As shown in Fig. 3C, 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,
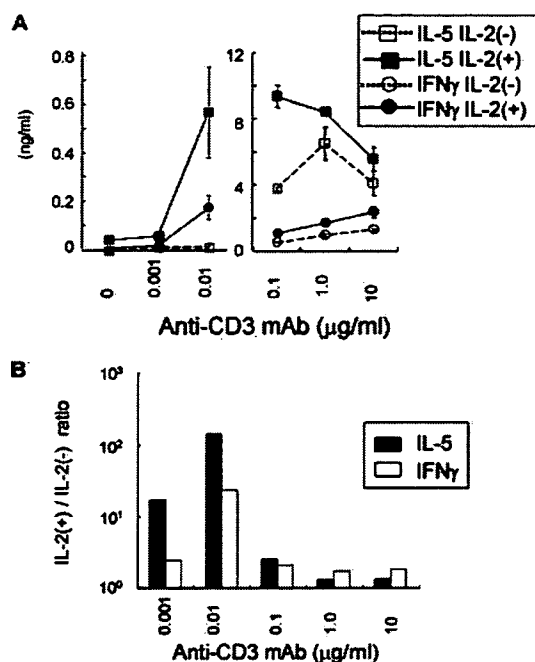
**FIGURE 5.** Suboptimal anti-CD3 stimulation mimics the effect of CD1d$^+$ APCs for IL-5 induction from CD4$^+$ iNKT clones in the presence of IL-2. CD4$^+$ iNKT cell clones Kn.2 and Kai.2 were stimulated by plate-bound anti-CD3 mAb (0.001–10 μg/ml) in the presence or absence of IL-2 (10 IU/ml). IL-5 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-2$^+$-IL-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).

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. 3B) 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)
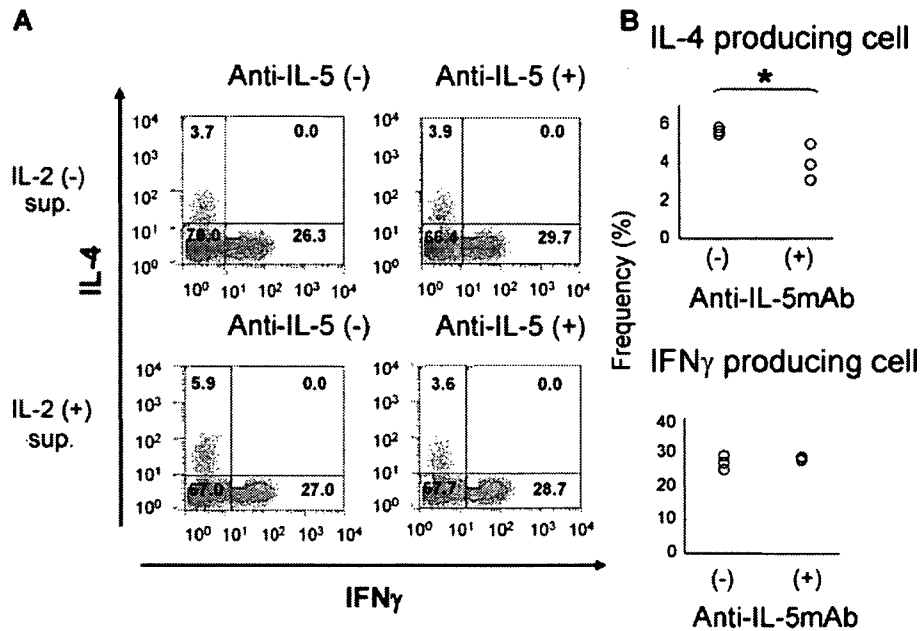
FIGURE 6. iNKT/iDC coculture supernatant induced Th2 CD4 T cell differentiation in an IL-5-dependent way. Here, we stimulated naive CD4 T cells with plate bound-anti-CD3 mAb (10 μg/ml) and soluble anti-CD28 mAb (2 μg/ml) and evaluated the effect of adding IL-2-induced IL-5-enriched supernatant from iNKT/iDC 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

A

B
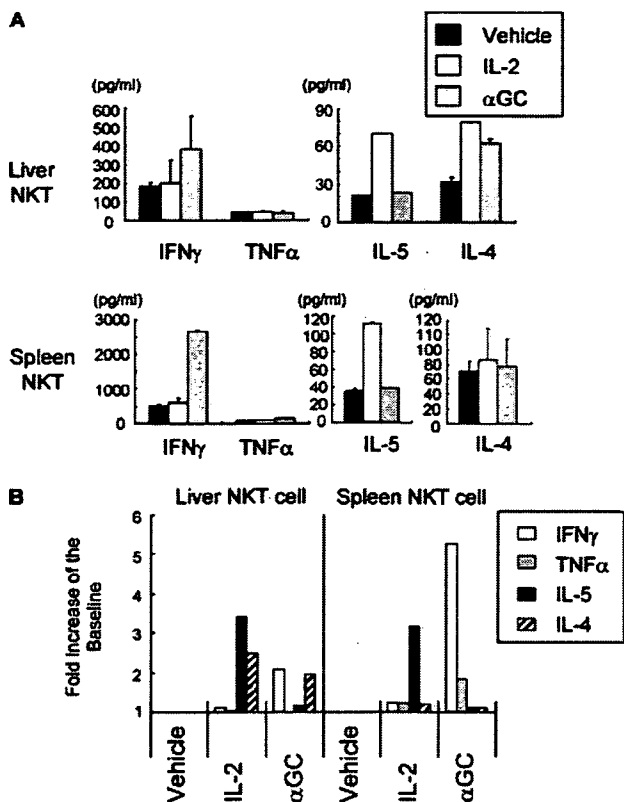
FIGURE 7. Freshly isolated iNKT cells from BALB/c mice produced IL-5 in the presence of IL-2. iNKT cells were isolated from livers and spleens pooled from six BALB/c mice of the same age by using αGC loaded-CD1d dimer X. iNKT cells were cocultured with magnetically isolated CD11c$^+$ splenocytes and 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.
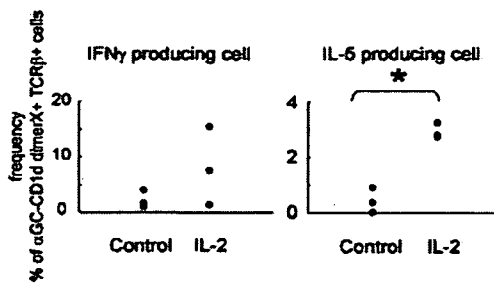
FIGURE 8. Increase of IL-5-producing iNKT cell by in vivo administration of IL-2. Liver lymphocyte were isolated after i.v. IL-2 administration and stained for intracellular IL-5 or IFN-γ production. The frequency of αGC-loaded CD1d dimer X$^+$ TCRβ$^+$ iNKT cells was plotted for IL-5- and IFN-γ-producing 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

in the presence of IL-2 as a readout. We assume that such a weak self-reactivity of the cells has hampered identifying this potentially important iNKT cell subset in prior studies.

Although we added rIL-2 and IL-15 exogenously to stimulate iNKT cells, these are the cytokines commonly produced in the inflammatory milieu. This allows us to speculate that the IL-5-producing iNKT cells might have a chance to encounter CD1d$^+$ APCs in the presence of either of the cytokines, thereby playing an important role in the local control of inflammation. Interestingly, IL-15 blockade has recently been shown to prevent the induction of allergic airway inflammation (37), implicating indirect evidence for the presence of IL-15 in the site of airway inflammation. Although this study has not identified iNKT cells as a target of IL-15, the critical role of iNKT cells shown in other rodent studies (14, 15) and human asthma (38) supports the idea that local IL-15 may stimulate iNKT cells to produce IL-5 and IL-13, which then leads to augmentation of allergic inflammation involving activation of eosinophils (39, 40). Another point of interest is that the role of IL-2 has been indicated in Th2 polarization processes involving iNKT cells. Although this is most elegantly shown in the case of eradication of certain parasites (41, 42), it is possible that iNKT cells are the target of IL-2 for inducing Th2 polarization. It is also likely that iNKT cells in the inflammatory lesions of MS may produce IL-5 or IL-13 after being triggered by IL-2 or IL-15 in the inflammatory lesions. The IL-5 produced by iNKT cells may directly promote Th2 cell differentiation, thereby deviating Th1/Th2 balance toward Th2. Alternatively, Th2 polarization could be mediated by other cytokines that were triggered by IL-5 in an autocrine or paracrine fashion. In fact, we showed that IL-4 was induced by the IL-5-producing iNKT cells in the presence of IL-5 (Fig. 1D) and overproduction of IL-4 from CD4$^+$ iNKT cells could be demonstrated in the remission state of MS (33).

Recent reports have shown that striking Th1 responses against exogenous pathogens could be triggered by iNKT cells after recognizing an endogenous ligand in the presence of IL-12 (26, 31). Given its remarkable homology to the IL-5 response triggered by IL-2 reported here in this article, we speculate that stimulation with an endogenous ligand and locally produced cytokines is a fundamental mechanism that would lead iNKT cells to provoke a decisive response for dealing with infection, allergy, and autoimmunity. In infection models, iNKT cell recognition of iGb3 has been identified as an important trigger for inducing Th1 response by iNKT cells (31). However, iNKT cells may recognize different endogenous Ags (43) and therefore the microenvironment of different tissues or types of inflammation (e.g., Th1 vs Th2) encountered may be instrumental in determining the phenotype of iNKT cytokine production. In this regard, endogenous ligand(s) involved in IL-5 production by IL-2 is an area to be further investigated in the future.

It is important to realize that as much as 8 of the 26 CD4$^+$ iNKT clones (Table I) have demonstrated a bias for IL-5 production following IL-2 stimulation. We speculate that this could arise from the heterogeneity of β-chain CDR3 sequence, although this point is another area that needs to be formally verified. Supportive of this speculation is the recent study showing that individual TCR β-chain may contribute to the variation of Ag recognition among iNKT cells (36). Another possibility is that these IL-5-producing iNKT cells may comprise a distinct lineage with the unique machinery to overproduce IL-5. It may also be due to differences in the frequency of IL-5-biased iNKT cells among each individual. This idea is supported by the observation that IL-5-biased clones tended to be generated from the same individuals (Table I). Furthermore, our preliminary data has demonstrated that IL-2-dependent production from iNKT cells might be sub-

ject to mouse strain differences: namely, the production of IL-5 from iNKT cells found in BALB/c could not be demonstrated in C57BL/6 mice (data not shown). This discrepancy between Th1 (C57BL/6) and Th2 (BALB/c) polarized mice may give us an important clue to further analyze this issue.

In summary, the present study has identified presence of human CD4$^+$ iNKT cells that produce IL-5 and IL-13 in response to suboptimal TCR stimulation together with IL-2 or IL-15. Previous studies using αGC or anti-CD3 Ab for stimulation of iNKT cells was unsuccessful in revealing the identity and the unique property of these iNKT cells, reflecting the nonphysiological nature of the methods used for stimulating iNKT cells. Analysis of our data and studies from other groups suggest that this iNKT cell population produce IL-5 and IL-13 in vivo by recognizing an endogenous ligand. In the pathogenesis of allergy, autoimmune diseases or parasite infection, CD4$^+$ iNKT may play a key role in deviating immune responses toward Th2 and thus provide a suitable target for immune intervention. Our results also imply that cytokines could play a major role in instructing the iNKT cell populations to respond differentially in vivo, whether it is beneficial or hazardous. Taking all these into consideration, we propose that sensing the presence of cytokines is probably one of the most fundamental abilities for the iNKT cells that are to be given only a weak TCR signal in vivo.

Acknowledgments

Disclosures

References
1. Bendelac, A., M. Bonneville, and J. F. Kearney. 2001. Autoreactivity by design: innate B and T lymphocytes. Nat. Rev. Immunol. 1: 177–186.
2. Taniguchi, M., M. Harada, S. Kojo, T. Nakayama, and H. Wakao. 2003. The regulatory role of Va14 NKT cells in innate and acquired immune response. Annu. Rev. Immunol. 21: 483–513.
3. Wilson, B. S., and T. L. Delovitch. 2003. Janus-like role of regulatory iNKT cells in autoimmune disease and tumour immunity. Nat. Rev. Immunol. 3: 211–222.
4. Kronenberg, M. 2005. Toward an understanding of NKT cell biology: progress and paradoxes. Annu. Rev. Immunol. 23: 877–900.
5. Kawano, T., J. Cui, Y. Koezuka, Y. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, et al. 1997. CD1d-restricted and TCR-mediated activation of Va14 NKT cells by glycosylceramides. Science 278: 1626–1629.
6. Godfrey, D. I., H. R. MacDonald, M. Kronenberg, M. J. Smyth, and L. van Kaer. 2004. NKT cells: what's in a name? Nat. Rev. Immunol. 4: 231–237.
7. Duarte, N., M. Stenstrom, S. Campino, M. L. Bergman, M. Lundholm, D. Holmberg, and S. L. Cardell. 2004. Prevention of diabetes in nonobese diabetic mice mediated by CD1d-restricted nonclassical NKT cells. J. Immunol. 173: 3112–3118.
8. Terabe, M., J. Swann, E. Ambrosino, P. Sinha, S. Takaku, Y. Hayakawa, D. I. Godfrey, S. Ostrand-Rosenberg, M. J. Smyth, and J. A. Berzofsky. 2005. A nonclassical non-Va14-Ja18 CD1d-restricted (type II) NKT cell is sufficient for down-regulation of tumor immunosurveillance. J. Exp. Med. 202: 1627–1633.
9. Gumperz, J. E., S. Miyake, T. Yamamura, and M. B. Brenner. 2002. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. J. Exp. Med. 195: 625–636.
10. Lee, P. T., K. Benlagha, L. Teyton, and A. Bendelac. 2002. Distinct functional lineages of human Va24 natural killer T cells. J. Exp. Med. 195: 637–641.
11. Kim, H. Y., H. J. Kim, H. S. Min, S. Kim, W. S. Park, S. H. Park, and D. H. Chung. 2005. NKT cells promote antibody-induced joint inflammation by suppressing transforming growth factor β1 production. J. Exp. Med. 201: 41–47.
12. Chiba, A., S. Kaieda, S. Oki, T. Yamamura, S. Miyake, and 2005. The involvement of Va14 NKT cells in the pathogenesis of arthritis in murine models. Arthritis Rheum. 52: 1941–1948.
13. Ohnishi, Y., A. Tsutsumi, D. Goto, S. Itoh, I. Matsumoto, M. Taniguchi, and T. Sumida. 2005. TCR Va14 natural killer T cells function as effector T cells in mice with collagen-induced arthritis. Clin. Exp. Immunol. 141: 47–53.
14. Akbari, O., P. Stock, E. Meyer, M. Kronenberg, S. Sidobre, T. Nakayama, M. Taniguchi, M. J. Grusby, R. H. DeKruyff, and D. T. Umetsu. 2003. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. Nat. Med. 9: 582–588.
15. Lisbonne, M., S. Diem, A. de Castro Keller, J. Lefort, L. M. Araujo, P. Hachem, J.-M. Fourneau, S. Sidobre, M. Kronenberg, M. Taniguchi, et al. 2003. Cutting

edge: invariant Vα14 NKT cells are required for allergen-induced airway inflammation and hyperreactivity in an experimental asthma model. *J. Immunol.* 171: 1637–1641.

16. Godfrey, D. I., and M. Kronenberg. 2004. Going both ways: immune regulation via CD1d-dependent NKT cells. *J. Clin. Invest.* 114: 1379–1388.

17. Chen, H., and W. E. Paul. 1997. Cultured NK1.1$^+$CD4$^+$ T cells produce large amounts of IL-4 and IFN-γ upon activation by anti-CD3 or CD1. *J. Immunol.* 159: 2240–2249.

18. Schmieg, J., G. Yang, R. W. Franck, and M. Tsuji. 2003. Superior protection against malaria and melanoma metastases by a c-glycoside analogue of the natural killer T cell ligand α-galactosylceramide. *J. Exp. Med.* 198: 1631–1641.

19. Miyamoto, K., S. Miyake, and T. Yamamura. 2001. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing Th2 bias of natural killer T cells. *Nature* 413: 531–534.

20. Miyake, S., and T. Yamamura. 2005. Therapeutic potential of glycolipid ligands for natural killer (NK) T cells in the suppression of autoimmune disease. *Curr. Drug Targets Immune Endocr. Metabol. Disord.* 5: 315–322.

21. Heller, F., I. J. Fuss, E. E. Nieuenhuis, R. S. Blumberg, and W. Strober. 2002. Oxazolone colitis, a Th2 colitis model resembling ulcerative colitis, is mediated by IL-13-producing NK-T cells. *Immunity* 17: 629–638.

22. D'Andrea, A., D. Goux, C. De Lalla, Y. Koczuka, D. Montagna, A. Moretta, P. Dellabona, G. Casorati, and S. Abrignani. 2000. Neonatal invariant Vα24$^+$ NKT lymphocytes are activated memory cells. *Eur. J. Immunol.* 30: 1544–1550.

23. Park, S. H., K. Benlagha, D. Lee, E. Balish, and A. Bendelac. 2000. Unaltered phenotype of, tissue distribution and function of Vα14$^+$ NKT cells in germ-free mice. *Eur. J. Immunol.* 30: 620–625.

24. Matsuda, J. L., L. Gapin, J. L. Baron, S. Sidobre, D. B. Stetson, M. Mohrs, R. M. Locksley, and M. Kronenberg. 2003. Mouse Vα14i natural killer T cells are resistant to cytokine polarization in vivo. *Proc. Natl. Acad. Sci. USA* 100: 8395–8400.

25. Oki, S., A. Chiba, T. Yamamura, and S. Miyake. 2004. The clinical implication and molecular mechanism of preferential IL-4 production by modified glycolipid-stimulated NKT cells. *J. Clin. Invest.* 113: 1631–1640.

26. Brigl, M., L. Bry, S. C. Kent, J. E. Gumperz, and M. B. Brenner. 2003. Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. *Nat. Immunol.* 4: 1230–1236.

27. Joyce, S., A. S. Woods, J. W. Yewdell, J. R. Bennink, D. De Silva, A. Boesteanu, S. P. Balk, R. J. Cotter, and R. R. Brutkiewicz. 1998. Natural ligand of mouse CD1d1; cellular glycosylphosphatidylinositol. *Science* 279: 1541–1544.

28. Gumperz, J. E., C. Roy, A. Makowska, D. Lum, M. Sugita, T. Podrebarac, Y. Koczuka, S. Porceli, S. Cardell, M. B. Brenner, and S. M. Behar. 2000. Murine CD1d-restricted T cell recognition of cellular lipids. *Immunity* 12: 211–221.

29. Rauch, J., J. E. Gumperz, C. Robinson, M. Skold, C. Roy, D. C. Young, M. Lafler, B. D. Moody, M. B. Brenner, and C. E. Costello. 2003. Structural features of the acyl chain determine self-phospholipid antigen recognition by a CD1d-restricted invariant NKT (iNKT) cell. *J. Biol. Chem.* 278: 47508–47515.

30. Zhou, D., J. Mattner, C. Cantu, 3rd, N. Schrantz, N. Yin, Y. Gao, Y. Sagiv, K. Hudspeth, Y. Wu, T. Yamashita, et al. 2004. Lysosomal glycosphingolipid recognition by NKT cells. *Science* 306: 1786–1789.

31. Mattner, J., K. L. Debord, N. Ismail, R. D. Goff, C. Cantu, 3rd, D. Zhou, P. Saint-Mezard, V. Wang, Y. Gao, N. Yin, et al. 2005. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature* 434: 525–529.

32. Thomas, S. Y., R. Hou, J. E. Boyson, T. K. Means, C. Hess, D. P. Olson, J. L. Strominger, M. B. Brenner, J. E. Gumperz, S. B. Wilson, and A. D. Luster. 2003. CD1d-restricted NKT cells express a chemokine receptor profile indicative of Th1-type inflammatory homing cells. *J. Immunol.* 171: 2571–2580.

33. Araki, M., T. Kondo, J. E. Gumperz, M. B. Brenner, S. Miyake, and T. Yamamura. 2003. Th2 bias of CD4$^+$ NKT cells derived from multiple sclerosis in remission. *Int. Immunol.* 15: 279–288.

34. Takahashi, K., T. Aranami, M. Endoh, S. Miyake, and T. Yamamura. 2004. The regulatory role of natural killer cells in multiple sclerosis. *Brain* 127: 1917–1927.

35. van Der Vliet, H. J., N. Nishi, T. D. de Gruijl, B. M. von Blomberg, A. J. van den Eertwegh, H. M. Pinedo, G. Giaccone, and R. J. Scheper. 2000. Human natural killer T cells acquire a memory-active phenotype before birth. *Blood* 95: 2440–2442.

36. Brigl, M., P. van den Elzen, X. Chen, J. H. Meyers, D. Wu, C.-H. Wong, F. Reddington, P. A. Illarianov, G. S. Besra, M. B. Brenner, and J. E. Gumperz. 2006. Conserved and heterogeneous lipid antigen specificities of CD1d-restricted NKT cell receptors. *J. Immunol.* 176: 3625–3634.

37. Ruckert, R., K. Brandt, A. Braun, H.-G. Hoymann, U. Herz, V. Budagian, H. Durkop, H. Renz, and S. Bulfone-Paus. 2005. Blocking IL-15 prevents the induction of allergen-specific T cells and allergic inflammation in vivo. *J. Immunol.* 174: 5507–5515.

38. Akbari, O., J. L. Falu, J. L. Hoyte, G. J. Berry, J. Wahlstrom, M. Kronenberg, R. H. DeKruyff, and D. T. Umetsu. 2006. CD4$^+$ invariant T-cell-receptor$^+$ natural killer T cells in bronchial asthma. *N. Engl. J. Med.* 354: 1117–1129.

39. Yamaguchi, Y., T. Suda, J. Suda, M. Eguchi, Y. Miura, N. Harada, A. Tominaga, and K. Takatsu. 1988. Purified interleukin 5 supports the terminal differentiation and proliferation of murine eosinophilic precursors. *J. Exp. Med.* 167: 43–56.

40. Yamaguchi, Y., Y. Hayashi, Y. Sugama, Y. Miura, T. Kasahara, S. Kitamura, M. Torisu, S. Mita, A. Tominaga, and K. Takatsu. 1988. Highly purified murine interleukin (IL-5) stimulates eosinophil function and prolongs in vitro survival: IL-5 as eosinophil chemotactic factor. *J. Exp. Med.* 167: 1737–1742.

41. Cui, J., N. Watanabe, T. Kawano, M. Yamashita, T. Kamata, C. Shimizu, M. Kimura, E. Shimizu, J. Koike, H. Koseki, et al 1999. Inhibition of T helper cell type 2 cell differentiation and immunoglobulin E response by ligand-activated Vα14 natural killer T cells. *J. Exp. Med.* 190: 783–792.

42. Mallevaey, T., J. P. Zanetta, C. Faveeuw, J. Fontain, E. Maes, F. Platt, M. Capron, M. L. de-Moraes, and F. Trottein. 2006. Activation of invariant NKT cells by the helminth parasite *Schistosoma mansoni*. *J. Immunol.* 176: 2476–2485.

43. Poruhsky, S., A. O. Speak, B. Luckow, V. Cerundolo, F. M. Platt, and H.-J. Grone. 2007. Normal development and function of invariant natural killer T cells in mice with isoglobosylceramide (iGb3) deficiency. *Proc. Natl. Acad. Sci. USA* 104: 5977–5982.

Cross-Regulation between Type I and Type II NKT Cells in Regulating Tumor Immunity: A New Immunoregulatory Axis[1]

Elena Ambrosino,* Masaki Terabe,[2]* Ramesh C. Halder,[†] Judy Peng,* Shun Takaku,* Sachiko Miyake,[‡] Takashi Yamamura,[‡] Vipin Kumar,[†] and Jay A. Berzofsky*

Negative immunoregulation is a major barrier to successful cancer immunotherapy. The NKT cell is known to be one such regulator. In this study we explored the roles of and interaction between the classical type I NKT cell and the poorly understood type II NKT cell in the regulation of tumor immunity. Selective stimulation of type II NKT cells suppressed immunosurveillance, whereas stimulation of type I NKT cells protected against tumor growth even when responses were relatively skewed toward Th2 cytokines. When both were stimulated simultaneously, type II NKT cells appeared to suppress the activation in vitro and protective effect in vivo of type I NKT cells. In the absence of type I, suppression by type II NKT cells increased, suggesting that type I cells reduce the suppressive effect of type II NKT cells. Thus, in tumor immunity type I and type II NKT cells have opposite and counteractive roles and define a new immunoregulatory axis. Alteration of the balance between the protective type I and the suppressive type II NKT cell may be exploited for therapeutic intervention in cancer. *The Journal of Immunology*, 2007, 179: 5126–5136.

To prevent diseases induced by autoimmune attack or to control collateral damage during an immune response, the immune system has developed many mechanisms of negative regulation. In the context of tumor immunity, the strict regulation of immune responses to maintain self-tolerance and prevent autoimmunity can represent a barrier to successful anti-tumor therapy. Because cancer vaccines to date have been able to induce robust T cell responses but only little clinical benefit (1), a greater understanding of these barriers and the discovery of ways to circumvent them may be necessary to finally realize the promise of cancer vaccines and immunotherapy. Recently, several cell types with suppressive function have been described such as the widely studied $CD4^+CD25^+$ T regulatory cell (2), M2 or tumor-associated macrophages (3–5), myeloid-derived suppressor cells (6–8), and the CD1d-restricted NKT cell (9–16). However, there is only limited information on the interaction between different regulatory cells (12, 17–19).

In this report we will propose a novel axis between regulatory subsets of NKT cells with opposite and counter-regulatory functions. In earlier studies, NKT cells appeared to have a paradoxical role in tumor immunity because they were shown to promote (20–23) as well as suppress (10–13, 16) tumor immunosurveillance. NKT cells are specialized T cells that recognize lipid Ags presented by CD1d molecules and also express NK cell receptors. Although the classical (or type I) NKT cell carrying an invariant TCR using the Vα14Jα18 TCR α-chain is the most prevalent and extensively studied, NKT cells are a heterogeneous population

composed of different subsets (24). A less well-studied type of NKT cell, the so-called type II NKT cell with a more diverse TCR repertoire, has been identified in both humans and mice (25–29). Little is yet known about type II NKT cell physiologic function. Although a role for type II NKT cells has been documented in a transgenic model of hepatitis B virus infection (30), *Trypanosoma cruzi* infection (31), and experimental autoimmune encephalomyelitis (9) as well as in humans in the immunopathogenesis of ulcerative colitis (32), their role in tumor immunity is not known. Further, no interaction between the two types of NKT cells has been reported.

Although the range of actions attributed to date to NKT cells is extremely diverse and in some case conflicting, it is important to consider that not all of the studies discriminated between type I and type II NKT cells. We recently found that type II NKT cells were sufficient to suppress tumor immunosurveillance (12), but it was not clear whether type I NKT cells could also suppress as well as protect. In this study, to define the relative roles and potential interactions of these two classes of NKT cells, for the first time we compared their roles in the immune response against the same tumors by selectively stimulating them with specific ligands. We directly showed a role for type II NKT cells in suppressing tumor immunosurveillance, defining an important physiological function for the little-studied type II NKT cell. In contrast, type I NKT cells were directly involved in protection, indicating opposite roles for the two NKT cell subsets. Moreover when the two NKT cell populations were simultaneously stimulated, the effect of type I NKT cell activation was suppressed by type II NKT cell activation both in vitro and in vivo in two different tumor models, suggesting a counter-regulation between the two NKT cell subsets and identifying a potential new immunoregulatory axis.

Materials and Methods
Mice

Female BALB/c mice were purchased from Animal Production Colonies, Frederick Cancer Research Facility (National Institutes of Health, Frederick, MD). BALB/c CD1d-deficient mice (CD1dKO mice; provided by M. Grusby, Harvard University, Boston MA) and BALB/c Jα18-deficient
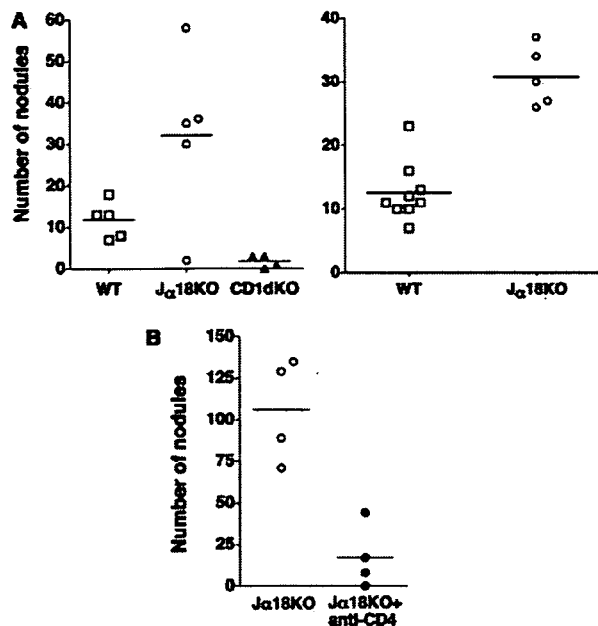
FIGURE 1. The presence of CD4⁺ CD1d-restricted type II NKT cells down-regulates tumor immunosurveillance and the absence of type I NKT cells correlates with a higher susceptibility to tumor growth. *A*, On day 0 in two different experiments, 5 × 10⁵ CT26 tumor cells were injected i.v. into BALB/c WT, Jα18KO, and CD1dKO mice. Eight days after tumor challenge the mice were sacrificed and the number of lung nodules was counted. At an early stage of tumor growth, Jα18KO (○) were more susceptible (*left panel, p* = 0.04; *right panel, p* = 0.001; Mann-Whitney test) and CD1dKO mice (▲) were less susceptible (*p* = 0.02; Mann-Whitney test) than WT mice (□). The experiment was repeated five times with similar results, and two representative experiments are shown. The horizontal bars indicate the means. *B*, On day 0, 5 × 10⁵ CT26 tumor cells were injected i.v. into BALB/c Jα18KO mice. Anti-CD4 (1.5 mg; clone GK1.5) was injected i.p. on days −2. −1, 0, and 7. Mice were sacrificed 11 days after tumor challenge. CD4⁺ T cell depletion (●) significantly decreased the number of tumor nodules in BALB/c Jα18KO mice (*p* = 0.03 against untreated WT mice; Mann-Whitney test). The Ab treatment was confirmed to result in >99% depletion of CD4⁺ T cells by flow cytometry using a staining Ab that is not blocked by GK1.5. The results shown are representative of two similar experiments. The horizontal bars indicate the means.

mice (Jα18KO mice; provided by M. Taniguchi, RIKEN Institute, Yokohama, Japan and by D. Umetsu, Harvard Medical School, Boston, MA) were bred at the National Cancer Institute (Bethesda, MD) under pathogen-free conditions. Female mice >6 wk of age were used for experiments. All experiments were approved by the National Cancer Institute's institutional animal care and use committee.

Tumor cell lines

The colon carcinoma CT26 cell line was maintained in RPMI 1640 supplemented with 10% FCS, L-glutamine, nonessential amino acids, sodium pyruvate, streptomycin and penicillin, and 2-ME (5 × 10⁻⁵ M). The fibrosarcoma line 15-12RM (33) was maintained in RPMI 1640 supplemented with 10% FCS, L-glutamine, nonessential amino acids, sodium pyruvate, streptomycin and penicillin, 2-ME (5 × 10⁻⁵ M), and 200 μg/ml G418.

Reagents

Synthetic α-galactosylceramide (αGalCer)[3] was provided by Kirin Brewery (Tokyo, Japan). For study, the stock solution was further diluted with PBS. Myelin-derived 3'-sulfogalactosylceramide (sulfatide) was purified

---

[3] Abbreviations used in this paper: αGalCer, α-galactosylceramide; WT, wild type.
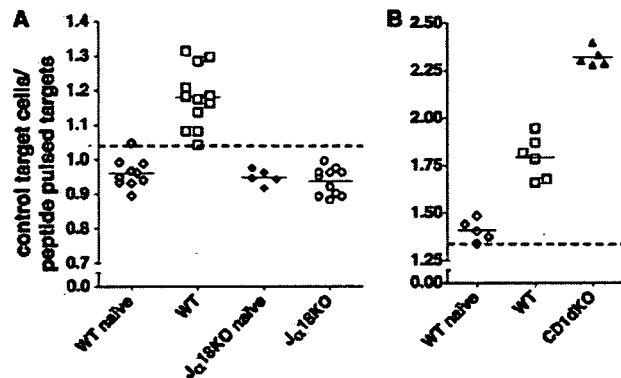
FIGURE 2. The presence of type II NKT cells but the absence of type I NKT cells correlates with a lower tumor-specific CTL response in vivo at an early stage of tumor growth. To examine the anti-tumor CTL response in BALB/c WT and Jα18KO mice at the same tumor stage as in Fig. 1, the in vivo CTL assay was performed. *A*, At this stage of tumor growth, tumor-challenged WT mice (□) were able to weakly but significantly respond against tumor-Ag pulsed target cells (*p* = 0.0001 against naive WT mice; Mann-Whitney test). In contrast, tumor-challenged Jα18KO mice (○), like naive WT (◇) and naive Jα18KO (◆) mice, showed no detectable specific CTL response against the same target. *B*, In a different experiment from that in *A*, to examine the anti-tumor CTL response in BALB/c CD1dKO mice at similar early time point, BALB/c CD1dKO and WT mice were compared by in vivo CTL assay. Tumor-challenged WT mice showed, again, a tumor-specific CTL response (*p* = 0.004 against naive WT mice; Mann-Whitney test) and BALB/c CD1dKO showed a strong tumor-Ag specific response (*p* < 0.0001 against naive and *p* = 0.004 against challenged WT mice; Mann-Whitney test). The horizontal bars indicate the means. The horizontal dashed line indicates the ratio of control target cells to specific target cells in the mixture injected into the mice. The experiments in *A* and *B* were repeated twice.

(>90%) from a >98% pure sulfatide preparation from bovine brain (Matreya) as described earlier (9), dissolved in vehicle (0.5% polysorbate 20), and diluted with PBS. OCH was prepared as described by Oki et al. (34). Purified rat anti-mouse CD4 (clone GK1.5) was obtained from Harlan Bioproducts for Science. The AH1 peptide (SPSYVYHQF), a CTL epitope from murine leukemia virus gp70 expressed in CT26 tumor cell line and presented by H-2Lᵈ (35), was synthesized by NeoMPS.

In vivo tumor assay

A single cell suspension of 5 × 10⁵ CT26 cells or 1 × 10⁶ 15-12RM cells in 200 μl of PBS was injected i.v. or s.c., respectively, in mice. In the case of the CT26 lung metastases model, the determination of pulmonary metastasis was performed as previously described (13). In the 15-12RM tumor model, tumor size was measured periodically by caliper gauge.

In vivo Ab or glycolipid treatment

Purified rat anti-mouse CD4 was diluted to 1.5 mg in 200 μl of PBS and injected i.p. 2 consecutive days before tumor challenge, the day of challenge, and then 1 wk after the last injection. The glycolipids were diluted from the stock solutions at the desired concentration in PBS and administered i.p. αGalCer was administered at the concentration of 4 μg/mouse, and OCH was administered at the concentration of 2 μg/mouse (because this same concentration was reported to be effective in vivo in an autoimmune disease model (36)). When the sulfatide was titrated in vitro, we observed a very reproducible biphasic dose-response curve, typical for most T cell responses, with the maximum effect at 20–30 μg of sulfatide. In all of our experiments sulfatide showed an effect at a dose range of 20–30 μg/mouse in vivo or 20–30 μg/ml in vitro, so the amount of sulfatide used is therefore indicated as 25 ± 5 μg or μg/ml.

In vivo CTL assay

A single cell suspension of 5 × 10⁷ naive spleen cells/ml was left unpulsed (control target cells) or pulsed with AH1 peptide (specific target cells) for 2 h at 37°C. After washing, spleen cells were then incubated with the fluorescent dye CFSE (Molecular Probes) at either 5 μM (specific target
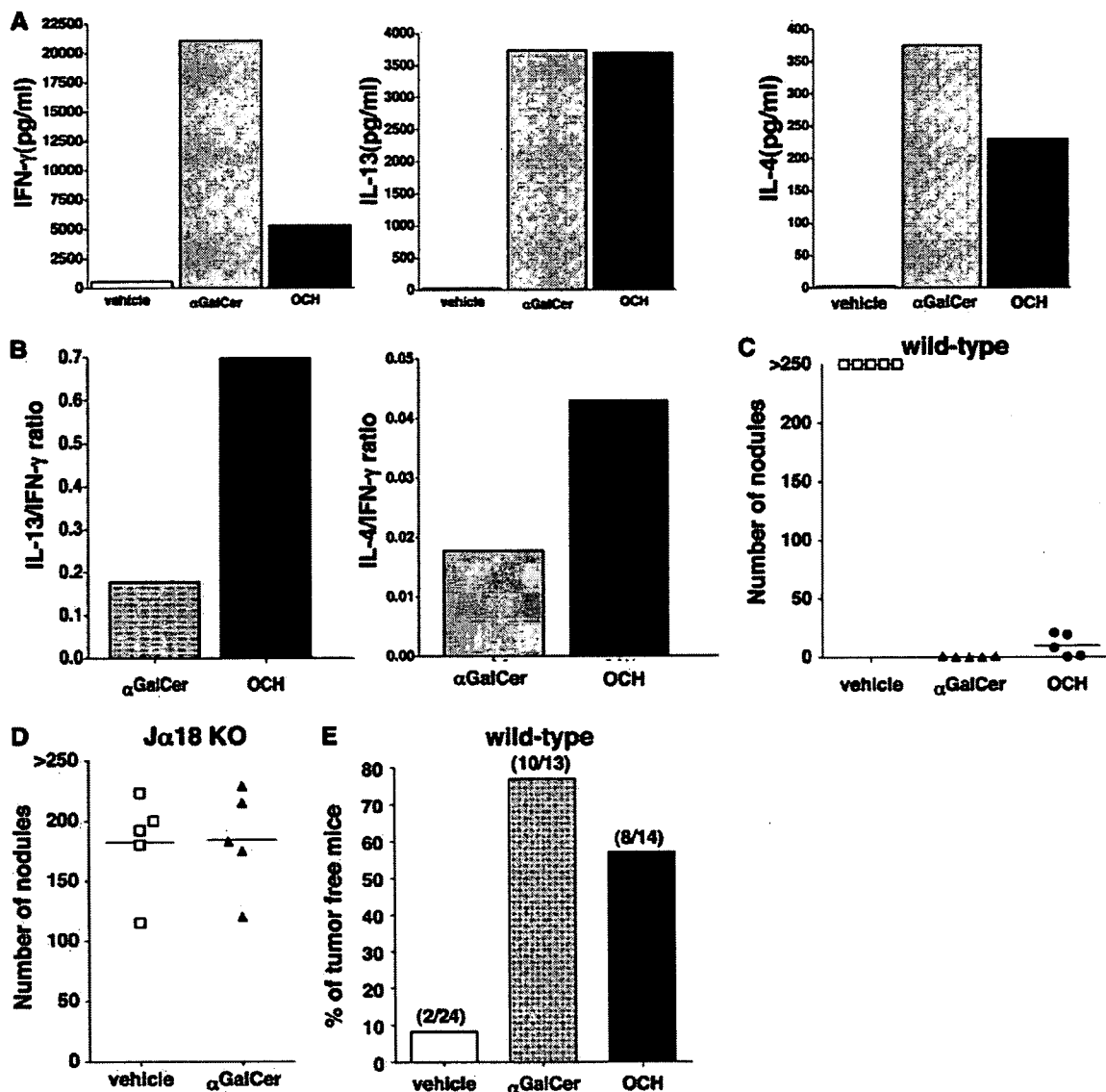
FIGURE 3. Stimulation of type I NKT cells protects mice from tumor growth. *A*, BALB/c spleen cells were stimulated in vitro with either 100 ng/ml αGalCer (gray bar) or 100 ng/ml OCH (filled bar), or vehicle (open bar). *A*, The amount of IFN-γ, IL-13, and IL-4 produced in vitro after 48 h of culture was examined by Luminex assay. *B*, The weak type I NKT cell agonist OCH induced a higher ratio of IL-13/IFN-γ and IL-4/IFN-γ secretion compared with αGalCer. *C* and *D*, The effect of type I NKT cell stimulation by either vehicle (□), αGalCer (▲) or OCH (●) on the CT26 in vivo tumor growth was investigated. In different experiments, BALB/c WT (*C*) and Jα18KO (*D*) mice were challenged with 5 × 10⁵ CT26 cells i.v. and were injected i.p. with 4 μg per mouse of αGalCer or 2 μg per mouse of OCH on the same day. *C*, Two weeks after tumor challenge all of the mice were sacrificed and the number of lung nodules was counted. In WT mice, αGalCer treatment prevented CT26 growth (*p* = 0.008 against vehicle-treated WT mice; Mann-Whitney test), and OCH-treated mice were protected from CT26 tumor growth (*p* = 0.008 against vehicle-treated WT mice; Mann-Whitney test). *D*, CT26-challenged Jα18KO mice did not responded to αGalCer and were not protected from tumor growth. *E*, Protection by either αGalCer or OCH in the 15-12RM s.c. fibrosarcoma model. BALB/c WT mice were challenged s.c. with 1 × 10⁶ 15-12RM cells and, on the same day, were injected i.p. with vehicle (open bar), 4 μg per mouse of αGalCer (gray bar), or 2 μg per mouse of OCH (filled bar). The presence of a recurrent tumor was measured on day 50. WT mice were protected against recurrence of the 15-12RM fibrosarcoma by either αGalCer (*p* < 0.0001 against vehicle-treated WT mice; log rank test) or OCH (*p* = 0.0006 against vehicle-treated WT mice; log rank test). The numbers indicate the number of tumor-free mice in each group. The data presented are in a pool from three independent experiments.

cells) or 0.5 μM (control target cells) for 15 min at room temperature. Spleen cells were washed and then incubated for 30 min at room temperature in RPMI 1640 with 10% FCS and then washed twice more. The two spleen cell populations were finally mixed together at a 1:1 ratio and injected i.v. into experimental mice. After ∼18 h the mice were sacrificed and single-cell suspensions from their spleens were processed individually to evaluate the presence of 5 μM or 0.5 μM CFSE-labeled cells by flow cytometry (FACScalibur, BD Biosciences). The results are reported as ratio of the proportion of control target cells over the proportion of specific target cells left in each mouse (control target cells/peptide-pulsed targets).

In vitro cell activation and proliferation

A single-cell suspension of splenocytes was prepared from naive BALB/c mice. The cells were cultured at a density of 8 × 10⁵/well in a 96-well plate in 200 μl of RPMI 1640 supplemented with 10% FCS, L-glutamine, nonessential amino acids, sodium pyruvate, streptomycin and penicillin and 2-ME (5 × 10⁻⁵ M). In some wells OCH (100 ng/ml), αGalCer (1–100 ng/ml), sulfatide (25 ± 5 μg/ml), sulfatide vehicle, or both αGalCer and sulfatide were added to the culture. Twenty-four, 48, and 72 h after the stimulation 100 μl of culture medium was collected from the wells and