Conventional, Naive CD4⁺ T Cells Provide an Initial Source of IL-4 During Th2 Differentiation

Nancy Noben-Trauth, Jane Hu-Li and William E. Paul

*J Immunol* 2000; 165:3620-3625; doi: 10.4049/jimmunol.165.7.3620

http://www.jimmunol.org/content/165/7/3620

References

This article cites 45 articles, 34 of which you can access for free at:
http://www.jimmunol.org/content/165/7/3620.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Conventional, Naive CD4$^+$ T Cells Provide an Initial Source of IL-4 During Th2 Differentiation

Nancy Noben-Trauth, Jane Hu-Li, and William E. Paul

IL-4 is known to promote the differentiation of CD4$^+$ T cells into IL-4-secreting Th2 cells. However, the cellular source of the early burst of IL-4 that drives Th2 responses in vivo has not been conclusively identified. Mice deficient for the IL-4 receptor α-chain (IL-4Rα$^{-/-}$) retain the capacity to secrete IL-4 and can be used to identify those cell types that produce IL-4 without a requirement for prior IL-4-mediated stimulation. To address whether naive, conventional CD4$^+$ T cells may act as initial producers of IL-4 in Ag-specific responses, we crossed the BALB/c IL-4Rα$^{-/-}$ mice to DO11.10/scid TCR transgenic mice. Lymph node cells from wild-type and IL-4Rα$^{-/-}$ DO11.10/scid mice secreted ~50 pg of IL-4 per10$^6$ cells within 48 h after peptide stimulation. This small amount of IL-4 was sufficient to cause the differentiation of wild-type CD4$^+$ T cells into Th2 cells, particularly if IFN-γ and IL-12 were neutralized during the priming cultures. CD4$^+$ cells from the IL-4Rα$^{-/-}$ mice gave rise to a minor proportion (~2%) of IL-4-producing cells upon stimulation in the presence of anti-IFN-γ and anti-IL-12. These data show that conventional, naive CD4$^+$ T cells may be considered as initial sources of IL-4 and, in the absence of IFN-γ and IL-12, this IL-4 can induce Th2 polarization. *The Journal of Immunology*, 2000, 165: 3620–3625.

L-4 is a major inducer and mediator of allergic and parasitic immune responses. One major mechanism through which it mediates this function is in promoting the differentiation of naive CD4$^+$ T cells into Th2 cells (1–5). The identification of the initial cellular source of the IL-4 that mediates Th2 cell induction is key to designing therapies to intervene in the development of Th2 responses. Based on prior studies, cells that have been identified to secrete IL-4 include cells of the mast cell/basophil lineage (6–9), eosinophils (10, 11), NK1.1$^+$, CD4$^+$ T cells (NK T cells)$^2$ (12–14), γ/δ T cells (15), and conventional CD4$^+$ T cells (16–22).

Conventional T cells, either those that have been previously primed or naive cells, are attractive candidates as a source of the “early” IL-4 because they are in the “right place at the right time.” Whether such cells could be prime movers has been uncertain because their acquisition of the ability to produce IL-4 has generally been regarded as an IL-4-dependent event. However, it has been suggested that naive T cells may make small amounts of IL-4 (or rare naive T cells may make large amounts) simply in response to TCR/CD28-mediated stimuli. To establish this, one needs to use highly purified populations of naive, conventional CD4$^+$ T cells and be assured that these cells have not previously received an IL-4 receptor-mediated signal.

Previous groups have made efforts to establish the capacity of naive T cells to produce the early IL-4 needed for priming. In general, they have used cells with high buoyant density that were sorted for expression of high levels of CD62L or CD45RB and/or low levels of CD44. In some cases, this has been done in mice that were TCR transgenic. These studies generally measured the capacity of these cells to become IL-4 producers in the absence of added IL-4 and concluded that, depending on the concentration of Ag and the degree of costimulation, Th2 priming could be achieved (18, 19, 22). In some experiments, it was shown that such priming was blocked by anti-IL-4.

These studies, although arguing that naive cells can be the source of IL-4, do not conclusively establish this point. Most importantly, the cells were drawn from mice that can both make and respond to IL-4 so that such cells could have received an IL-4 signal in vivo and differentiated into Th2 cells and then returned to the resting state. Such cells would then be able to make IL-4 upon restimulation. Indeed, because the TCR transgenic mice used were not on scid or Rag backgrounds, their expression of alternative TCR α-chains makes environmental immunization possible. Furthermore, it is now clear that CD62L is not a reliable marker of the naive state because memory cells can revert to being CD62L bright (23).

We have redressed this important issue using mice in which the IL-4Rα$^{-}$ chain has been disrupted, crossed to TCR transgenic mice on a scid background, and have further used IL-4$^{-}$×IL-4Rα double-knockout (KO) cells as APC. The possibility of γ/δ or NK T cells as a source of IL-4 is eliminated in this system because neither cell type is present in these mice. Neither basophils/mast cells nor eosinophils are likely to be involved because few, if any, of these cells are found in lymph nodes (LNs), which were our source of T cells. Finally, because we use APCs from IL-4$^{-}$×IL-4Rα donors, the APC can neither produce nor consume IL-4. This makes a source of IL-4 other than the naive T cells most unlikely and also allows us to quantitate IL-4 production because depletion of IL-4 from culture medium by receptor-mediated endocytosis should be largely eliminated. This system allows an unambiguous measure of the early production of IL-4 by conventional, naive T cells and a determination of whether this IL-4 is sufficient to prime naive IL-4Rα$^{-}$ cells to become Th2 cells.

Materials and Methods

**Mice**

BALB/c IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ mice were generated as described (24, 25) and bred under specific pathogen-free conditions in the National Institute of Allergy and Infectious Diseases Animal Care Unit. DO11.10/scid 8BALB/c mice were TCR transgenic. These studies generally measured the capacity of these cells to become IL-4 producers in the absence of added IL-4 and concluded that, depending on the concentration of Ag and the degree of costimulation, Th2 priming could be achieved (18, 19, 22). In some experiments, it was shown that such priming was blocked by anti-IL-4.

These studies, although arguing that naive cells can be the source of IL-4, do not conclusively establish this point. Most importantly, the cells were drawn from mice that can both make and respond to IL-4 so that such cells could have received an IL-4 signal in vivo and differentiated into Th2 cells and then returned to the resting state. Such cells would then be able to make IL-4 upon restimulation. Indeed, because the TCR transgenic mice used were not on scid or Rag backgrounds, their expression of alternative TCR α-chains makes environmental immunization possible. Furthermore, it is now clear that CD62L is not a reliable marker of the naive state because memory cells can revert to being CD62L bright (23).

We have redressed this important issue using mice in which the IL-4Rα$^{-}$ chain has been disrupted, crossed to TCR transgenic mice on a scid background, and have further used IL-4$^{-}$×IL-4Rα double-knockout (KO) cells as APC. The possibility of γ/δ or NK T cells as a source of IL-4 is eliminated in this system because neither cell type is present in these mice. Neither basophils/mast cells nor eosinophils are likely to be involved because few, if any, of these cells are found in lymph nodes (LNs), which were our source of T cells. Finally, because we use APCs from IL-4$^{-}$×IL-4Rα donors, the APC can neither produce nor consume IL-4. This makes a source of IL-4 other than the naive T cells most unlikely and also allows us to quantitate IL-4 production because depletion of IL-4 from culture medium by receptor-mediated endocytosis should be largely eliminated. This system allows an unambiguous measure of the early production of IL-4 by conventional, naive T cells and a determination of whether this IL-4 is sufficient to prime naive IL-4Rα$^{-}$ cells to become Th2 cells.

**Laboratory of Immunology, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892**

Received for publication February 23, 2000. Accepted for publication July 12, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Address correspondence and reprint requests to Dr. Nancy Noben-Trauth, Laboratory of Immunology, National Institutes of Health, Twnbrook II, 12441 Parklawn Drive, Rockville, MD 20852. E-mail address: mmoben@niaid.nih.gov
2 Abbreviations used in this paper: NK T cells, NK1.1$^+$, CD4$^+$ T cells, WT, wild-type; LN, lymph node; cRPMI, complete RPMI; KO, knockout; L, ligand.
TCR mice (26) were crossed to the IL-4Ra−/− mice and screened by FACS for the TCR cloneotype KJ1-26 (Caltag, South San Francisco, CA) and CD45R/B220 (PharMingen, San Diego, CA) expression on PBLs and for the presence of the mutated IL-4Ra locus by PCR. 5C7×RAR2 TCR (27) mice and IL-4−/− mice on a B10.A background were bred at Taconic Farms (Germantown, NY). The BALB/c nude mouse strain (nu/nu) was purchased from the Division of Cancer Treatment, National Cancer Institute (Frederick, MD).

**Culture medium**

Complete RPMI (cRPMI) consisted of RPMI 1640 medium (Biofluids, Rockville, MD) supplemented with 10% FBS (Life Technologies, Rockville, MD), 1 mM sodium pyruvate, 2 mM l-glutamine, 0.05 mM 2-ME, 100 U/ml penicillin, and 100 µg/ml streptomycin.

**Abs and cytokines**

CD4-Chrome, IL-4-PE, IFN-γ-FTTC, Vβ8-FTTC, anti-CD3, and anti-IL-3 were all purchased from PharMingen. Human IL-2 was a gift from Perkin-Elmer/Cetus (Norwalk, CT). Anti-IL-4 (11B11), anti-IL-12 (1C17.8), anti-IFN-γ (XMG 1.2), and anti-mouse Fcγ receptor (2.4G2) mAbs were all purified from ascites by Harlan Biosciences (Madison, WI). Anti-mouse IL-4Ra (M1) was purchased from Genzyme (Cambridge, MA). Goat anti-mouse IgD plasma was a generous gift from Fred Finckelman (University of Cincinnati, OH). Mouse IL-4 was obtained from a recombinant baculovirus prepared by Cynthia Watson (Laboratory of Immunology, National Institute of Allergy and Infectious Diseases). Recombinant mouse IL-3 was purchased from Genzyme. For T cell depletions, spleen cells were incubated with 20% supernatants of anti-Thy-1.2 mAb HO13.4 and rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada).

**ELISAs**

IL-4 (Endogen, Woburn, MA) and IL-2 (PharMingen) ELISAs were performed according to manufacturer directions. For the serum IgE ELISA, 96-well plates were coated with 2 µg/ml each of two monoclonal anti-IgE Abs (0212D from PharMingen and AM2501 from BioSource, Camarillo, CA). After blocking and overnight incubation with serum samples, plates were developed with HRP-conjugated goat anti-IgE Abs (Southern Biotechnology Associates, Birmingham, AL) followed by peroxidase substrate (Bio-Rad, Hercules, CA). The mAb IgE standard (D-8406) was purchased from Sigma BioSciences.

**In Vitro priming**

Mesenteric LN cells (1 × 10⁶/ml) from DO11.10/scid IL-4Ra−/− and IL-4Ra−/− mice were cultured in 24-well plates in cRPMI and IL-2 (10 U/ml) and stimulated with 3 µM OVA peptide and T-depleted irradiated spleen cells (5 × 10⁶/ml) from BALB/c IL-4−/− mice as APC. The peptides for OVA (323–339) and pigeon cytochrome c (88–104) were purchased from the Division of Cancer Treatment, National Cancer Institute (Frederick, MD). Some cultures also contained anti-IFN-γ (10 µg/ml) and anti-IL-12 (10 µg/ml), IL-4 (1000 U/ml), or anti-IL-4 (20 µg/ml). On the second or third day of culture, the cells were diluted 1:5 in cRPMI with IL-2 and expanded into 6-well plates. Five to six days after the initial stimulation, cells were harvested, washed twice, and resuspended in 1 × 10⁶/ml in cRPMI with IL-2 and challenged for an additional 5-day priming under the same conditions. LN cells from 5C7×RAR2 TCR transgenics were primed similarly, but with 3 µM PCC peptide and T-depleted irradiated spleen cells (5 × 10⁶/ml) from B10.A IL-4−/− mice as APC.

**Intracellular cytokine staining**

In vitro primed cells were harvested, washed, and cultured at 1 × 10⁶/ml in cRPMI in 48-well plates with 3 µM OVA peptide, 3 µM monensin (PharMingen), and 5 × 10⁶/ml T-depleted irradiated spleen cells from BALB/c (IL-4−/−×IL-4Ra−/−) mice as APC. After 6 h, cells were washed, fixed in 4% paraformaldehyde, and permeabilized with 0.1% saponin in PBS containing 1% FCS. Cells were first blocked with 2.4G2 (10 µg/ml) then stained simultaneously with CyChrome-anti-CD4, FITC-anti-IFN-γ, and PE-anti-IL-4 (all obtained from PharMingen) and analyzed by FACS. For the 5C7×RAR2 culture, cells were stimulated with 3 µM PCC peptide, 3 µM monensin, and T-depleted irradiated spleen cells (5 × 10⁶/ml) from B10.A IL-4−/− mice.

**Results**

Conventional T cells from IL-4Ra−/− mice produce an early burst of IL-4

To address whether naive conventional CD4⁺ T cells are capable of producing an initial burst of IL-4 upon TCR, we crossed DO11.10 TCR transgenic mice on a homozygous scid background to the BALB/c IL-4Ra−/− mutants. Such mice would lack NK T cells, and the scid mutation would guarantee exclusive expression of the appropriate transgenic TCR. To further exclude IL-4 production from the splenic APC population and consumption of the IL-4 produced in the cultures, we used irradiated T-depleted spleen cells from (IL-4×IL-4Ra−/−) double-deficient mice as APC. LN cells from DO11.10/scid IL-4Ra−/− and IL-4Ra−/− mice were cultured with 3 µM OVA peptide, and supernatants were obtained at 48 h (Fig. 1). Such supernatants contained 50 pg/10⁶ cells/ml of IL-4. We failed to detect any IL-4 when IL-4Ra+/− cells were cultured with peptide alone. However, the addition of anti-IL-4Ra mAb resulted in the appearance of a comparable amount of IL-4 in supernatants from such cells, arguing that the “early” IL-4 was rapidly consumed by the CD4⁺ T cells. Thus, both IL-4Ra+/− and IL-4Ra−/− DO11.10 cells respond to OVA, resulting in the production of 50 pg of IL-4 per million cells within 48 h.

IL-4 made by conventional CD4⁺ T cells can result in Th2 polarization

To assess whether the small amount of IL-4 produced within 48 h after stimulation was capable of generating a Th2 response, LN cells from DO11.10/scid/IL-4Ra−/− and IL-4Ra−/− mice were stimulated for a 5-day priming period with OVA peptide in the presence of irradiated T-depleted spleen cells from IL-4−/−IL-4Ra−/− donors. Amounts of IL-4 in the supernatants and the frequency of IL-4-producing cells were measured after restimulation with peptide and APCs (Fig. 2 and Table I).

When no other additions were made to the initial priming culture, the frequency of IL-4-producing cells was at the borderline of detectability (Fig. 2, “peptide alone”) and the amounts of supernatant IL-4 were low (Table I). The addition of IL-4 to the IL-4Ra−/− cells dramatically increased the frequency of IL-4-producing cells and the amount of supernatant IL-4. As expected, no change was seen in the IL-4Ra−/− cells cultured with IL-4.
If IL-4Rα⁺/⁻ cells were cultured without added IL-4 but in the presence of anti-IFN-γ and anti-IL-12, 4% of the cells produced IL-4, and supernatant IL-4 content was 4823 pg/10⁶ cells. When anti-IL-4 Ab was added to such cultures, the number of IL-4-secreting cells fell to 1.7%, and the amount of supernatant IL-4 was reduced to 1121 pg/10⁶ cells. This argues that the endogenous production of IL-4 by IL-4Rα⁺/⁻ cells was sufficient to cause the induction of a modest number (~3%) of IL-4-producing cells but only if IFN-γ and IL-12 were neutralized.

The data also show that there is a small number IL-4-producers even in the absence of stimulation through the IL-4 receptor. The latter conclusion is supported by results with the IL-4Rα⁺/⁻ cells primed in the presence of anti-IFN-γ and anti-IL-12, with or without IL-4 or anti-IL-4. In these cultures, supernatant IL-4 levels ranged from 386–646 pg/10⁶ cells, slightly less than that obtained in the IL-4Rα⁺/⁻ cells cultured with anti-IFN-γ, anti-IL-12, and anti-IL-4 (1121 pg/10⁶ cells) (Table I).

These results lead us to conclude that the modest amount of IL-4 produced by DO11.10/scid/IL-4Rα⁺/⁻ and IL-4Rα⁻/⁻ CD4⁺ T cells in response to Ag stimulation is sufficient to polarize a small proportion of the responding cells (i.e., the IL-4Rα⁺/⁻ cells) when IFN-γ and IL-12 are neutralized.

When these cells were recultured for five additional days under the same conditions, they proved to be excellent IL-4-producers when challenged; 29.3% produced IL-4 (Fig. 2B), and supernatant IL-4 content was 157,000 pg/10⁶ cells (Table I). Only 4.2% of the cells primed in the presence of anti-IL-4, anti-IL-12, and anti-IFN-γ produced IL-4 after the second round of priming. This strongly implies that the enhanced IL-4 production observed after the second round of stimulation is in response to endogenously produced IL-4, and not simply due to repetitive stimulation.

In contrast to the wild-type (WT) cells, the twice-primed IL-4Rα⁺/⁻ cells had a frequency of IL-4-secreting cells ranging from 2.2–3.0%, and the amount of supernatant IL-4 ranged from 1600–2140 pg/10⁶ cells in the cultures containing anti-IFN-γ and anti-IL-12. The latter provides a more rigorous reflection of the IL-4-independent component of the polarization process than the result obtained with heterozygous cells.

Because these cells are derived from BALB/c mice, the possibility remained that the IL-4 response we observed was peculiar to

Table I. IL-4 levels (picograms per milliliter) in cultures from in vitro primed DO11.10/scid/IL-4Rα WT and KO LN, and 5CC7 LN cells

<table>
<thead>
<tr>
<th>LN Donor</th>
<th>Priming</th>
<th>Peptide Alone</th>
<th>IL-4</th>
<th>IL-4, anti-IFN-γ, anti-IL-12</th>
<th>Anti-IL-4, anti-IFN-γ, anti-IL-12</th>
<th>Anti-IL-4, anti-IFN-γ, anti-IL-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4R⁺/⁻</td>
<td>One round</td>
<td>271</td>
<td>94,493</td>
<td>88,639</td>
<td>1121</td>
<td>4,823</td>
</tr>
<tr>
<td>IL-4R⁻/⁻</td>
<td>121</td>
<td>181</td>
<td>646</td>
<td>386</td>
<td>540</td>
<td></td>
</tr>
<tr>
<td>5CC7</td>
<td>64</td>
<td>23,368</td>
<td>19,403</td>
<td>456</td>
<td>379</td>
<td></td>
</tr>
<tr>
<td>IL-4R⁺/⁻</td>
<td>Two rounds</td>
<td>630</td>
<td>329,000</td>
<td>95,348</td>
<td>3,305</td>
<td>91,281</td>
</tr>
<tr>
<td>IL-4R⁻/⁻</td>
<td>146</td>
<td>293</td>
<td>1,600</td>
<td>2,140</td>
<td>1,703</td>
<td></td>
</tr>
<tr>
<td>5CC7</td>
<td>Not done</td>
<td>125,000</td>
<td>131,000</td>
<td>1,119</td>
<td>17,094</td>
<td></td>
</tr>
</tbody>
</table>

*LN cells (1 × 10⁷/ml) from DO11.10/scid/IL-4Rα⁺/⁻ and IL-4Rα⁻/⁻ mice were stimulated in 24-well plates with 3 μM OVA peptide for 5–6 days with T-depleted spleens from BALB/c as APC under the conditions indicated and described in Fig. 2. For the analysis, cells were washed, and 1 × 10⁶ cells were restimulated with 3 μM OVA or pigeon cytochrome c peptide and 5 × 10⁶ (IL-4 × IL-4Rα⁺/⁻) as APC. IL-4 was measured by ELISA in supernatants taken at 24 h. For the 5CC7 cultures, supernatant IL-4 levels were obtained with heterozygous cells.
this mouse strain, which has been reported to “default” to Th2 cells upon priming in the absence of added IL-4 (28). To address this, we performed a similar experiment with cells from 5CC7×RAG2 TCR transgenic mice, which are on a B10.A background. These cells were primed with 3 μM PCC peptide. As shown in Fig. 2, the 5CC7 cells showed trends in IL-4 production comparable to the BALB/c IL-4Ra−/− mice. More importantly, a small percentage of IL-4 producers were detected after the first round of priming with pigeon cytochrome c peptide in the presence of anti-IFN-γ and anti-IL-12. This was strikingly augmented after the second priming. This further confirms that vigorous Th2 responses develop similarly in CD4+ T cells from BALB/c and B10.A backgrounds, particularly when IFN-γ and IL-12 are neutralized.

Thus, in the absence of exogenous IL-4, a limited amount of IL-4 is produced by naive cell populations; such production is sufficient so that a substantial degree of polarization can be achieved through repetitive culture. Endogenously produced IFN-γ and IL-12 will block this effect, presumably by limiting the responsiveness of the T cells to the very modest amounts of IL-4 produced early in the first culture.

IL-4 has been shown to enhance survival of T and B cells (as reviewed in Ref. 29). To exclude the possibility that the increase in IL-4 production in the IL-4Ra+/− cell population compares to IL-4Ra−/− cells reflects preferential survival of the IL-4Ra-expressing cells, we measured the total number of live cells recovered after each round of priming. There was no appreciable difference in either IL-4Ra+/− or −/− groups cultured with or without IL-4 (Table II). This makes it quite unlikely that the enhanced differentiation of IL-4Ra+/− cells into IL-4 producers can be accounted for by a proliferation mechanism.

**Anti-IgD injection results in IL-4 production by CD4+ T cells from IL-4Ra−/− mice**

Our results indicate that naive CD4+ T cells secrete an early burst of IL-4 that is capable of driving Th2 responses in vitro. We next asked whether such IL-4R-independent IL-4 production is functionally active during an in vivo response. A potent stimulus of IL-4 production and of IgE responses is treatment of mice with goat Ab to mouse IgD (30). Although the stimulus is specific for B-cells, Ag-specific CD4+ T cells (30) are required to induce the response. It has also been shown that NK T cells do not contribute to IL-4 production because CD11c− mice that are deficient for NK T cells retain IL-4-dependent IgE responses after anti-IgD treatment (31).

To determine whether the capacity of T cells to produce IL-4 in response to anti-IgD required prior priming through the IL-4 receptor, WT and IL-4Ra−/− mice were treated with anti-IgD. As shown in Fig. 3A, the frequency of cells with cytosolic IL-4 in response to anti-IgD was comparable between the heterozygous controls and IL-4Ra−/− mice. Nearly all IL-4 producers were in

---

**Table II. Cell recoveries (×10⁶) in cultures from in vitro primed DO11.10/scid/IL-4Ra WT and KO LN, and 5CC7 LN cells**

<table>
<thead>
<tr>
<th>Priming</th>
<th>Peptide Alone</th>
<th>IL-4</th>
<th>IL-4, anti-IFN-γ, anti-IL-12</th>
<th>Anti-IL-4, anti-IFN-γ, anti-IL-12</th>
<th>Anti-IFN-γ, anti-IL-12</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-4Ra+/−</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>One round</td>
<td>9.5</td>
<td>10.8</td>
<td>19.0</td>
<td>16.3</td>
<td>25.6</td>
</tr>
<tr>
<td>IL-4Ra−/−</td>
<td>14.6</td>
<td>12.7</td>
<td>24.6</td>
<td>27.6</td>
<td>24.4</td>
</tr>
<tr>
<td>5CC7</td>
<td>13.3</td>
<td>8.6</td>
<td>16.8</td>
<td>15.8</td>
<td>18.6</td>
</tr>
<tr>
<td><strong>IL-4Ra+/−</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Two rounds</td>
<td>7.5</td>
<td>9.5</td>
<td>12.5</td>
<td>9.2</td>
<td>13.0</td>
</tr>
<tr>
<td>IL-4Ra−/−</td>
<td>8.3</td>
<td>6.9</td>
<td>12.9</td>
<td>10.3</td>
<td>9.8</td>
</tr>
<tr>
<td>5CC7</td>
<td>not done</td>
<td>5.0</td>
<td>13.1</td>
<td>7.4</td>
<td>9.5</td>
</tr>
</tbody>
</table>

*LN cells (1 × 10⁶/ml) from DO11.10/scid IL-4Ra+/− and IL-4Ra−/− mice and 5CC7 × RAG2 mice were stimulated in 24-well plates as described in Fig. 2 and Table I. Cells were recovered after one and two rounds of in vitro priming, and viable cells were counted by trypan blue exclusion.

---

**FIGURE 3.** Conventional CD4+ T cells produce IL-4 after anti-IgD treatment. IL-4Ra+/− and IL-4Ra−/− mice were injected s.c. with 200 µl of goat anti-mouse IgD plasma. Spleens were removed at 5 days, stimulated with 10 ng/ml of PMA, 500 ng/ml of ionomycin, and 3 µM monensin (PharMingen) for 6 h, and analyzed for intracellular IL-4 production by cytoplasmic staining and flow cytometry. Top, IL-4 production by total CD4+ T cells. Bottom, The frequency of Vβ8+ IL-4-producing cells after gating on CD4+ cells. Untreated controls shown were from IL-4Ra+/− mice.

**FIGURE 4.** IL-4 produced by anti-IgD treated IL-4Ra−/− mice can functionally drive IgE class switching. BALB/c and IL-4Ra−/− mice were injected s.c. with 200 µl of goat anti-mouse IgD plasma. Spleen cells (40 × 10⁶) from BALB/c nu/nu mice were injected i.v. into IL-4Ra−/− mice 2 days after anti-IgD injection according to the method described (45). Mice were bled 8 days after anti-IgD treatment, and total serum IgE levels were measured by ELISA. The results are from three mice per group ± SD and represent two individual experiments.
the CD4+ T cell population. In addition, using quantitative, competitive RT-PCR, the number of IL-4 transcripts in total spleen cells from anti-IgD treated IL-4Ra−/− mice was actually 2-fold greater than that of WT mice (data not shown).

The IL-4-producing CD4+ T cells do not appear to be from the subset of NK T cells; the proportion of IL-4-producing cells from the IL-4Ra−/− mice that are Vβ8+ is 21% (1.5 of 7.2% total IL-4 producers), which is only slightly greater than the frequency of Vβ8+ cells that did not produce IL-4 (13.5%) (Fig. 3B). NK T cells have been shown to have a frequency of Vβ8+ cells of ~55%. Thus, it seems likely that conventional CD4+ T cells can be stimulated by anti-IgD to produce IL-4 even if these cells are incapable of responding to IL-4.

Because the capacity of conventional CD4+ T cells from IL-4Ra−/− mice to produce IL-4 in response to anti-IgD injection, we next asked whether this IL-4 was functionally capable of stimulating B cell Ig class switching to IgE, which is generally an IL-4-dependent event. We transferred WT spleen cells from BALB/c nu/nu mice to IL-4Ra−/− recipients and asked whether anti-IgD could induce IgE expression in the reconstituted animals. As shown in Fig. 4, anti-IgD-treated IL-4Ra−/− mice had very low levels of IgE, but when reconstituted with nu/nu spleen cells, they developed IgE levels of 60.4 μg/ml, equivalent to the response of intact BALB/c mice. It is interesting to note that even without the addition of WT spleen cells, IgE was modestly increased in the IL-4Ra−/− mice after anti-IgD treatment (undetectable to 308 ng/ml). IL-4-independent IgE production has been observed in other systems; in IL-4 and IL-4R knockout mice, IgE production of ~0.1–1% of normal is often observed (32). Thus, anti-IgD injection induced IL-4Ra−/−-independent IL-4 production by a cell population that can mediate Ig class switching, further supporting the notion that these are “conventional” CD4+ T cells.

Discussion

It is clear that in vivo and in vitro, IL-4 plays a major role in the induction of Th2 responses (1–5, 33). The cellular source of this “inducing” IL-4 has been a matter of great interest that has been investigated by several groups. In this report, we contribute to these studies by quantitating the early burst of IL-4 occurring after initial Ag stimulation of CD4+ IL-4Ra−/− T cells. Cells from IL-4Ra mutant mice provide a unique opportunity to measure such early IL-4 production because IL-4 is not consumed by these cells, nor can IL-4-dependent, IL-4-producing cells appear in the culture. Furthermore, the absence of IL-4Ra assures that there is no possibility that these cells would be previously exposed to IL-4 in vivo and therefore “primed”, either by IL-4 produced by endogenously activated CD4+ cells or by non-T cell sources of IL-4 such as mast cells.

We examined Ag-specific IL-4 production by LN cells obtained from DO11.10/scid mice that were either mutant or wild type at the IL-4Ra locus. Under these conditions, ~50 pg/ml were produced by 106 cells. This is an amount that is less than that required to optimally polarize naive CD4+ T cells to become Th2 cells (generally 500-1000 pg/ml) but may still be sufficient to cause a degree of polarization.

Therefore, we asked whether T cells from WT DO11.10/scid mice could use their endogenously secreted IL-4 to differentiate into Th2 cells. Our data demonstrate that LN cells from DO11.10/ scid mice stimulated with 3 μM OVA peptide prime for IL-4 production when IFN-γ and IL-12 were neutralized. Under these conditions, ~4% of the T cells became IL-4 producers. When stimulated through a second round, this rose to 30%, presumably due to the substantial amounts of IL-4 produced in the first round. Thus the early IL-4 secreted by naive T cells appears to be sufficient to prime for a delayed, but eventually quite striking, Th2 response, if IL-12 and IFN-γ are neutralized.

These experiments also show that a small percentage of transgenic CD4+ T cells from IL-4Ra−/− mice can acquire IL-4-producing capacity when primed in the absence of IFN-γ and IL-12. Whether this represents IL-4-independent in vitro priming or the response of cells that were actually primed in vivo is not certain. The latter is rendered unlikely by the fact that the cells used were derived from mice on a scid background, which should be much less likely to be primed by environmental Ags.

Our results demonstrate that there is an IL-4-independent in vivo pathway of IL-4 secretion by “conventional” T cells. Injection of anti-IgD into IL-4Ra mutant mice resulted in the same frequency of IL-4-producing CD4 T cells as in WT mice (Fig. 3). Although Kaplan et al. have reported that that IL-4-secreting cells in STAT6−/− mice express the NK Ag DX5, it is most unlikely that the cells making IL-4 in response to anti-IgD are NK T cells. IL-4 production in response to anti-IgD occurs normally in WT T cell-deficient mice (CD1−/− and B2 m−/−) (31, 34), and our analysis of the frequency of Vβ8+ CD4 T cells in the IL-4Ra−/− mice makes it unlikely that the IL-4 producers are NK T cells (Fig. 3).

Further evidence for the IL-4-independent appearance of IL-4-producing Th2 cells is from a study in IL-4Ra−/− and in Stat6−/− mice infected with Schistosoma mansoni. CD4+ T cells from these mice produce IL-4, although at a lower frequency than do CD4+ T cells from infected WT mice (35). Careful analysis of the CD4 cells from the mutant mice and of schistosome egg Ag-specific T cell clones derived from them showed that the cells had the capacity to produce IL-5 as well as IL-4, strengthening the contention that they were authentic Th2 cells.

Another in vivo system in which IL-4-independent induction of Th2 responses occurs is in BCL6−/− mice. These mice develop a severe Th2-type myocarditis and pulmonary vasculitis, marked by eosinophil infiltration and by the production of IL-4, IL-5, IL-10, and IL-13 (36). BCL6 has been shown to be capable of opposing Stat6-induced transcription of some genes (36); nonetheless, a similar syndrome occurs in (BCL6×STAT6)−/− mice, indicating that in the absence of BCL6, a STAT6-independent pathway of priming for IL-4 production is revealed (37).

Ouyang and colleagues have recently reported that a proportion of DO11.10 TCR transgenic STAT6−/− cells develop into IL-4 producers when primed in vitro (38). They demonstrated that in such cells GATA3 mRNA levels were elevated, and that the DNase I hypersensitivity had been induced in the second intron of the IL-4 gene, consistent with the concept that the pathway of Th2 priming, although IL-4-independent, was still GATA3 dependent. This result and the results from the (BCL6×STAT6)−/− mice are consistent with the existence of a single common pathway for Th2 differentiation involving GATA3. This pathway is most efficiently stimulated through IL-4Ra/STAT6 activation, but it may also be induced through some other mechanism(s), possibly through TCR and accessory molecule engagement with appropriate APCs. Such IL-4-independent priming has been reported when human DC2 dendritic cells, but not DC1 cells, have been used to stimulate naive (CD45RO+) CD4 T cells to become IL-4 producers (39).

Furthermore, it has been reported that APCs that fail to express ICAM-1 efficiently induce Th2 responses (40, 41).

Thus, these results indicate that some conventional CD4+ T cells can produce IL-4 in response to Ag-mediated T cell activation, and that such IL-4 can then drive the bulk of the responding CD4 T cells to differentiate into polarized Th2 cells. The factors that govern the initial production of IL-4 are a complex interplay...
of peptide dose, costimulatory molecules, the strength and repeti-
tion of the stimulus, and the presence of down-regulatory cyto-
kines, such as IFN-γ (19, 21, 42–44). The DO11.10scid IL-
4Rα−/− mice may be useful in dissecting these parameters.

Acknowledgments

We thank Dragana Jankovic for helpful discussions and critical reading of the manuscript, Susan Barbieri and Calvin Eigsti for assistance with FACS sorting, and the staff of the National Institute of Allergy and Infectious Diseases Animal Care Unit for outstanding technical assistance.

References

linkage of FcεRI or to calcium ionophores. Nature 339:64.
cation of messenger RNA for IL-4 in human eosinophils with granule localiza-
induced early IL-4 production is dependent upon IL-5 and eosinophils. J. Exp. Med. 184:1871.
amounts of IL-4 and IFN-γ upon activation by anti-CD3 or CD1. J. Immunol. 158:2259.
ablation of IL-4-producing cells. Cell 75:985.