Aberrant Selection and Function of Invariant NKT Cells in the Absence of AP-1 Transcription Factor Fra-2

Victoria J. Lawson, Diane Maurice, Jonathan D. Silk, Vincenzo Cerundolo and Kathleen Weston

*J Immunol* 2009; 183:2575-2584; Prepublished online 20 July 2009; doi: 10.4049/jimmunol.0803577

http://www.jimmunol.org/content/183/4/2575

**Supplementary Material** [http://www.jimmunol.org/content/suppl/2009/07/21/jimmunol.0803577.DC1](http://www.jimmunol.org/content/suppl/2009/07/21/jimmunol.0803577.DC1)

**References** This article cites 54 articles, 26 of which you can access for free at: [http://www.jimmunol.org/content/183/4/2575.full#ref-list-1](http://www.jimmunol.org/content/183/4/2575.full#ref-list-1)

**Subscription** Information about subscribing to *The Journal of Immunology* is online at: [http://jimmunol.org/subscription](http://jimmunol.org/subscription)

**Permissions** Submit copyright permission requests at: [http://www.aai.org/About/Publications/JI/copyright.html](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](http://jimmunol.org/alerts)
Aberrant Selection and Function of Invariant NKT Cells in the Absence of AP-1 Transcription Factor Fra-2

Victoria J. Lawson, Diane Maurice, Jonathan D. Silk, Vincenzo Cerundolo, and Kathleen Weston

The journal article discusses the role of the AP-1 family, specifically the AP-1 transcription factor Fra-2, in the regulation of normal iNKT cell development and function. It highlights that in the absence of Fra-2, there is a marked increase in the number of both thymic and peripheral iNKT cells, indicating altered selection of iNKT cells. The study shows that iNKT cells expressing the lower affinity TCRV8.2 chain are selected into the iNKT lineage, as these cells undergo positive selection. The signaling requirements for the divergence of iNKT cells from other T cell lineages are also considered, with a focus on the role of Fra-2 in this process.

D Bermingham, a diverse array of thymocytes with differing Ag specificities is generated through rearrangement of TCR genes and random pairing of TCRα- and TCRβ-chains. These cells undergo selection on the basis of their affinity for peptide-MHC, and those surviving selection commit to, and acquire the effector functions associated with, CD4+ “helper”, CD8+ “cytotoxic”, or CD4+CD8+ regulatory lineages. In parallel, thymocytes bearing the Vα14-Jα18 TCRα-chain rearrangement are selected into the iNKT lineage (1–3).

In contrast to conventional T cells, which are selected on peptide-MHC complexes displayed by thymic epithelial cells, iNKT cells are selected from the CD4+CD8+ double-positive (DP) thymocyte population by other DP thymocytes (4, 5), and respond to glycolipid Ags presented by the nonpolymorphic, MHC class I-like molecule CD1d (6). While the identity of the selecting ligand for iNKT cells remains contentious, mature iNKT cells very rapidly produce a range of cytokines, and this, along with their ability to indirectly modulate the activity of other immune cells, endows them with potent immunoregulatory properties (1).

The signaling requirements for the divergence of iNKT cells from other T cell lineages are not fully understood. In common with other T cells, iNKT cells undergo positive and negative selection. The canonical murine iNKT TCRα-chain, Vα14-Jα18, is found paired with a restricted set of TCRβ chains, usually Vβ7, Vβ8.2, and Vβ2 (8, 9). Positive selection was demonstrated in CD1d-deficient mice engineered to overexpress the Vα14-Jα18 TCRα-chain. In these mice, iNKT cells possess a broad repertoire of Vβ chains paired with Vα14-Jα18, and in some cases, respond to α-GalCer (10); the absence of these variant TCRs in normal animals suggests that cells expressing them fail positive selection. iNKT cells are also subject to negative selection, as evidenced by the dose-dependent disappearance of iNKT cells from thymic organ culture upon the addition of agonist α-GalCer, and by the loss of iNKT cells in mice overexpressing CD1d, with the lowest affinity Vβ2 chain being overrepresented among remaining iNKT cells (11). In addition to TCR signaling, the SLAM-SAP-Fyn signaling cascade is required by iNKT cells, with Slamf1-, Slamf6-, Sh2d1a (SAP)-, and Fyn-deficient mice all exhibiting severe defects in the iNKT cell lineage (1, 2, 12). SLAM or TCR signaling activates the NF-κB pathway, which is essential for iNKT cell maturation, predominantly regulating survival (1, 2). Downstream of the TCR, the Tec kinases Itk and Rtk are required by iNKT cells (13), at least partly because they regulate the transcription factor Tbx21, which controls terminal maturation (14).

Selection or postselection maturation of iNKT cells is also affected by transcription factors that influence T cell or NK cell selection or development. Rorc (4), Runx1 (4), Tox (15), Egr2 (16), and Gata3 (17), affect both T cell and iNKT development; Ets-1 (18), Irf1 (19), and Mef2 (20) have roles in NK and iNKT cell development. Importantly, recent work has now identified the transcription factor PLZF as a “signature” factor essential from an early stage in iNKT cell development for lineage
establishment, postselection maturation, and effector differentiation (21, 22).

The AP-1 family consists of dimerized bZIP proteins, either dimers of Jun family members (c-Jun, JunD, and JunB) or heterodimers between Jun and Fos family members (c-Fos, FosB, Fra-1, and Fra-2). The wider AP-1 superfamily includes the MAF and ATF protein families. AP-1 activity is integral to many cellular processes, including proliferation and apoptosis, and consequently it is central to development of many tissues (23). Within the immune system, AP-1 lies downstream of several signaling cascades (24) and contributes to regulation of the IL-2 and IL-4 promoters (25, 26). A role for AP-1 in iNKT development is suggested by experiments showing that overexpression of the AP-1 superfamily member BATF (B cell-activating transcription factor), a repressor (27), causes a severe reduction in the number of iNKT cells and affects the ability of those remaining to switch on key cytokines, including IL-2 and IL-4, in response to stimulation (28, 29).

AP-1 family member Fra-2 came to our attention in a screen for transcription factors differentially expressed during thymocyte selection. Here we show that when Fra-2 is deleted just before thymocyte selection, conventional T cell development is unaffected, but there is a pronounced increase in the number of thymic iNKT cells from a very early point in their development. Furthermore, these iNKT cells show alterations in TCRβ use and widespread dysregulation of AP-1-responsive genes, suggesting that AP-1 activity during or immediately after selection is required for normal development of the iNKT cell lineage. In the periphery, we find that Fra-2-deficient iNKT cells produce increased amounts of IL-4 and, most unusually, IL-2, in response to Ag. Taken together, our results demonstrate a role for Fra-2 in both the development and function of iNKT cells.

Materials and Methods

Mice

Mice were kept in-house in accordance with U.K. Home Office regulations and the project was approved by the local ethics committee. Strains used were: CD4cre (30), Fra-2f/f (31), MHC-deficient mice (b2m−/− and H2-Ab1−/−) (32, 33), Rag2−/− (34), and B6.SJL-Ptprca/H9262 (CD45.1) (35). Fra-2f/f and Fra-2−/−CD4cre animals were obtained on a mixed C57BL/6/129 background, but were backcrossed onto C57BL/6 for at least four generations; all other strains were inbred. Mice were analyzed at 5–8 wk of age, and in all experiments on a mixed background, littermates were used as controls.

Lymphocyte isolation

Lymphocytes were prepared by gentle disaggregation of tissue through a 40-μm nylon filter. Single-cell suspensions from spleen were red-cell lysed. Hepatic lymphocytes were isolated by centrifugation of disaggregated liver at 500 × g, resuspension of the cell pellet in 33% Percoll, centrifugation at 680 × g, and red-cell lysis.

Flow cytometry

Fluorescently labeled or biotin-conjugated anti-mouse Abs from eBioscience or BD Biosciences were used. Biotin-conjugated Abs were visualized using streptavidin-conjugated fluorophores. Allophycocyanin-conjugated CD1d tetramer loaded with PBS57 was obtained from the National Institutes of Health Tetramer Facility. Following extracellular staining, intracellular cytokine staining was performed using Cytofix/CytoPerm buffers (BD Biosciences) with brefeldin A (eBioscience). Data were acquired on a LSRII cytometer (BD Biosciences) and analyzed using FlowJo (Tree Star). Cells were sorted on a FACSAria (BD Biosciences) or a MoFlo (Dako).

Bone marrow chimeras

Bone marrow cells (105) from Fra-2−/− or Fra-2−/−CD4cre (CD45.2−) donors were mixed with 106 cells from C57BL/6 CD45.1 donors, and recipient sublethally irradiated Rag2−/− mice received 2 × 105 total cells in 200 μl of PBS via tail vein injection. Reconstituted mice were analyzed 6–8 wk later.

Quantitative RT-PCR

RNA was isolated using TRizol reagent (Invitrogen). cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen). For real-time PCR, gene-specific primers were used with the SYBR Green Master mix (Qiagen), or gene-specific assays were purchased from Applied Biosystems. Sample values were normalized to expression of hypoxanthine phosphoribosyltransferase or actin.

iNKT cell activation

For in vivo activation, 0, 1, 2, 4, or 5 μg of α-GaICer in PBS-0.05% Tween 20 vehicle was injected i.p. For in vitro activation, total spleen and liver lymphocytes were cultured for 90 min with 10 ng/ml PMA and 1 μg/ml ionomycin.

Cell cycle and apoptosis

For proliferation assays, mice were injected i.p. with 1.8 μg of BrdU (Fluka). After 1 h cells were harvested and analyzed using the BD FITC BrdU flow kit. For apoptosis, thyocytes were cultured overnight without stimulation, and apoptotic and dead cells were visualized with annexin V (NeXins Research) and 4′,6-diamino-2-phenylindole.

Microarray analysis

Total thymic NKT cells were sorted from three Fra-2−/− mice and three Fra-2−/−CD4cre littermates. RNA extraction and chip hybridization are described, and the annotated data sets are available, with accession number P Trey_KGW_0509, at http://bioinformatics.pricier.mann. ac.uk/vce.

Data were analyzed with Bioconductor, the “affy” package’s robust multichip average (RMA) method, and an empirical Bayes t test.

OVA-specific T and B cell responses

Mice were injected i.v. with 400 μg of OVA (Sigma-Aldrich) with or without 1 μg of α-GaICer (Axoxera). Seven days postinjection, PBLs were stained with H-2Kβ/SIINFEKL tetramer. Thirteen days postinjection, OVA-specific helper T cell and B cell responses were analyzed by ELISpot and ELISA, respectively. ELISpot was performed using the IFN-γ and IL-4 ELISpot ALP kits (Mabtech), with the following peptides: SIINFEKL (OVA237-246), TETWSNVMEERKIKV (OVA256-265) (“TETW”), and ISQAYHAHAEINAGR (OVA323-339) (“ESQ”). Spots were detected with biotinylated rat anti-mouse IFN-γ and IL-4, streptavidin alkaline phosphatase polymer, and the Bio-Rad alkaline phosphatase substrate kit. For ELISA, serial dilutions of serum were added overnight to OVA-coated plates at 4°C. OVA-specific IgG were detected with HRP-conjugated goat anti-mouse IgG (Dako).

Results

An increased number of iNKT cells in Fra-2−/−CD4cre mice

Up-regulation of CD69 on DP thymocytes correlates with the onset of signaling through the TCR, and it indicates the commencement of thymic selection and of the CD4 vs CD8 lineage decision process. We observed that Fra-2 levels increased as naive DP thymocytes became CD69+. This was the case for wild-type (WT) as well as CD8 lineage-restricted MHC class II+ and CD4 lineage-restricted β2-microglobulin−/− mice (Fig. 1A), suggesting that Fra-2 might play a role in thymocyte selection.

To investigate the consequences of loss of Fra-2 during thymocyte development, we bred F2−/− inducible knockout mice (31) to CD4cre transgenic mice to generate mice lacking Fra-2 from the late double-negative (DN4) stage of thymocyte development onward. Deletion at the Fosl2 locus, encoding Fra-2, was almost complete in DP cells (Fig. 1B), and there was a corresponding loss of Fra-2 mRNA (Fig. 1C). However, this loss of Fra-2 had no effect on the development of either CD4 or CD8 lineage T cells, or of regulatory T cells. Numbers and percentages of all these populations showed no difference between Fra-2−/−CD4cre mice and Fra-2−/− littermates (Tables I and Fig. 1D) in the thymus and in the periphery.

Having failed to find any effect of Fra-2 loss on the development of conventional αβ T lineages, we looked at iNKT cells, which can be identified using glycosphingolipid-loaded CD1d tetramer (36,
The increased number of iNKT cells developing in Fra-2f/fCD4cre mice could be due to a defect in the selecting DP thymocyte population, either in CD1d Ag presentation or in expression of the SLAM family of proteins. Alternatively, the defect could be intrinsic to the very small number of thymocytes that are iNKT cell precursors. We found no gross difference in the levels of either CD1d or Slamf1 between Fra-2f/fCD4cre mice and littermate controls (Fig. 2, A and B), making it unlikely that the selecting DP population was abnormal. Therefore, to confirm that the increase in iNKT cell numbers was due to a cell-intrinsic defect, we set up a competitive reconstitution experiment. Equal amounts of CD45.1 (WT) and CD45.2 (Fra-2f/fCD4cre or Fra-2f/f) bone marrow were used to reconstitute sublethally irradiated Rag2−/− mice.

The iNKT cell lineage

We found that equal proportions of TCRγhigh cells in the reconstituted recipients were derived from the CD45.1 and CD45.2 donors (Fig. 2, C, upper panels, and D, left panel), whether the CD45.2 donor was Fra-2f/f or Fra-2f/fCD4cre. Having established that the selecting DP populations in the recipient animals were essentially the same irrespective of CD45.2 donor, we next looked at iNKT development in recipient mice. When a Fra-2f/fCD45.2 donor was used, equal proportions of the iNKT cells were derived from each of the CD45.1 and CD45.2 donors. However, when the CD45.2 donor was Fra-2f/fCD4cre, on average twice as many of the iNKT cells in the reconstituted recipients were derived from the CD45.2 donor (Fig. 2, C, lower panels, and D, right panel). Therefore, Fra-2f/fCD4cre iNKT cells are better able than Fra-2f/f iNKT cells to develop using an equivalent selecting population. This indicates that the cause of increased numbers of iNKT cells in a Fra-2f/fCD4cre mouse is independent of the selecting DP population, and therefore is likely to be intrinsic to the iNKT lineage.

Fra-2 deletion acts at or immediately after iNKT cell selection

To determine where in iNKT cell development loss of Fra-2 had caused the increase in cell number, we analyzed thymic iNKT cells according to their developmental stage using expression of CD44 and NK1.1 (38, 39) (Fig. 3A, populations A–D). The earliest CD1d tetramer+ iNKT cells have no expression of NK1.1 or of CD44. As the cells mature, they up-regulate CD44 to become first CD44int+, then CD44high+. They then either exit the thymus or up-regulate NK1.1 to become CD44highNK1.1+. By percentage, no significant difference was observed between iNKT subsets (Fig. 3, A and B).

Given that there was a 2.5-fold increase in the absolute number of iNKT cells in a Fra-2f/fCD4cre thymus, these data show that all CD1d tetramer+ populations were overrepresented in the absence of Fra-2. The loss of Fra-2 therefore affects the development of iNKT cells at or before the earliest CD44−NK1.1−CD1d+ tetramer+ population we analyzed, suggesting that it acts either during the selection process itself or shortly afterwards, perhaps in the CD24hightetramer+CD69high population, which normally comprises a few hundred cells in the thymus, and constitutes the earliest iNKT developmental intermediate yet to be detected (40).

We next analyzed the Fra-2f/fCD4cre thymic iNKT population for changes in cell cycling or apoptosis. First, we used annexin V staining to identify apoptotic cells. As Fig. 3C shows, we observed no difference in the amount of cell death following 24 h in culture, between Fra-2f/fCD4cre and Fra-2f/f iNKT cells. Additionally, we saw no differences in cell death in DP cells (Fig. 3C) or in other populations in the thymus (data not shown). This excludes the possibility that the gain in iNKT cell numbers is due to enhanced survival of DP thymocytes, which would allow them to make more

\[ \text{expression of Fra-2} \]

\[ \text{expression of Fra-2} \]

\[ \text{expression of Fra-2} \]

\[ \text{expression of Fra-2} \]

\[ \text{expression of Fra-2} \]
Fra-2 regulates iNKT development and function

Fra-2f/f or Fra-2f/fCD4cre mice. Black line, CD150; gray area, isotype control.

Table I. T cell numbers in Fra2f/f and Fra2f/fCD4cre mice

<table>
<thead>
<tr>
<th></th>
<th>Fra2f/f</th>
<th>n</th>
<th>Fra2f/fCD4cre</th>
<th>n</th>
<th>p Value (Student’s t test, 2-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus size ($\times 10^6$ cells)</td>
<td>130 (31.0)</td>
<td>9</td>
<td>123 (29.1)</td>
<td>6</td>
<td>0.6576</td>
</tr>
<tr>
<td>% CD4 SP thymus</td>
<td>8.8 (1.4)</td>
<td>11</td>
<td>9.4 (2.2)</td>
<td>9</td>
<td>0.5309</td>
</tr>
<tr>
<td>% CD8 SP thymus</td>
<td>3.3 (1.5)</td>
<td>11</td>
<td>3.9 (0.9)</td>
<td>9</td>
<td>0.2943</td>
</tr>
<tr>
<td>Regulatory T thymus (CD25+GITR+ % CD4 SP)</td>
<td>3.4 (0.4)</td>
<td>3</td>
<td>4.0 (1.0)</td>
<td>3</td>
<td>0.4009</td>
</tr>
<tr>
<td>Spleen size ($\times 10^6$ lymphocytes)</td>
<td>38.3 (11.9)</td>
<td>10</td>
<td>36.0 (11.1)</td>
<td>7</td>
<td>0.6900</td>
</tr>
<tr>
<td>T cells spleen ( % Thy1.2+ )</td>
<td>31.9 (4.1)</td>
<td>6</td>
<td>30.4 (3.6)</td>
<td>5</td>
<td>0.5758</td>
</tr>
<tr>
<td>CD4 SP spleen (% lymphocytes)</td>
<td>18.9 (2.9)</td>
<td>15</td>
<td>19.7 (3.5)</td>
<td>12</td>
<td>0.4975</td>
</tr>
<tr>
<td>CD8 SP spleen (% lymphocytes)</td>
<td>12.5 (2.6)</td>
<td>15</td>
<td>12.8 (2.4)</td>
<td>12</td>
<td>0.7213</td>
</tr>
<tr>
<td>Regulatory T spleen (CD25+GITR+ % CD4 SP)</td>
<td>6.5 (0.3)</td>
<td>3</td>
<td>6.6 (1.2)</td>
<td>3</td>
<td>0.8906</td>
</tr>
<tr>
<td>NKT thymus (% TCRβ6, CD1d-tetramer+ )</td>
<td>0.18 (0.07)</td>
<td>18</td>
<td>0.43 (0.18)</td>
<td>14</td>
<td>0.0008</td>
</tr>
<tr>
<td>NKT spleen (% TCRβ6, CD1d-tetramer+ lymphocytes)</td>
<td>0.68 (0.31)</td>
<td>13</td>
<td>0.99 (0.41)</td>
<td>15</td>
<td>0.0181</td>
</tr>
<tr>
<td>NKT liver (% TCRβ6, CD1d-tetramer+ lymphocytes)</td>
<td>15.26 (5.39)</td>
<td>9</td>
<td>19.06 (4.40)</td>
<td>8</td>
<td>0.1187</td>
</tr>
<tr>
<td>Memory CD8 spleen (% CD8+, CD44+CD122hi/hi lymphocytes)</td>
<td>9.6 (1.3)</td>
<td>3</td>
<td>9.31 (2.18)</td>
<td>3</td>
<td>0.8550</td>
</tr>
</tbody>
</table>

*Numbers are mean and SD (in parentheses) of number of mice per genotype. SP, single positive.

~2% of total thymocytes incorporated BrdU in this period. Approximately 1% of iNKT cells had incorporated BrdU, and when iNKT cells were separated by CD44 expression (Fig. 3D, two left plots), it was apparent that more CD44$^+$ than CD44$^-$ iNKT cells were in cycle. However, no difference was observable between Fra-2$^{CD4cre}$ and Fra-2$^{CD4cre}$ iNKT cells, either at 1 h after labeling, or if cells were cultured in the presence of BrDU for 24 h before analysis (data not shown). The lack of any appreciable difference between normal and Fra-2-deficient iNKT cells in terms of their ability to cycle or die lends further weight to the idea that loss of Fra-2 causes a very early increase in the iNKT population, and that this amplification simply persists throughout iNKT cell ontogeny.

Fra-2$^{CD4cre}$ iNKT cells exhibit changes in TRBV repertoire and coreceptor usage

We next investigated whether the repertoire of T cell receptors used by Fra-2$^{CD4cre}$ iNKT cells was normal. Whereas the percentage of DP cells expressing the canonical iNKT invariant distal TRCγ rearrangements, including the invariant iNKT Vα14-18 (41).

To analyze cell cycle in Fra-2$^{CD4cre}$ iNKT cells in vivo, we injected mice with BrdU 1 h before analysis. As shown in Fig. 3D, approximately 1% of iNKT cells had incorporated BrdU in this period. Approximately 1% of iNKT cells had incorporated BrdU, and when iNKT cells were separated by CD44 expression (Fig. 3D, two left plots), it was apparent that more CD44$^+$ than CD44$^-$ iNKT cells were in cycle. However, no difference was observable between Fra-2$^{CD4cre}$ and Fra-2$^{CD4cre}$ iNKT cells, either at 1 h after labeling, or if cells were cultured in the presence of BrDU for 24 h before analysis (data not shown). The lack of any appreciable difference between normal and Fra-2-deficient iNKT cells in terms of their ability to cycle or die lends further weight to the idea that loss of Fra-2 causes a very early increase in the iNKT population, and that this amplification simply persists throughout iNKT cell ontogeny.

Fra-2$^{CD4cre}$ iNKT cells exhibit changes in TRBV repertoire and coreceptor usage

We next investigated whether the repertoire of T cell receptors used by Fra-2$^{CD4cre}$ iNKT cells was normal. Whereas the percentage of DP cells expressing the canonical iNKT invariant TRCγ repertoire

FIGURE 2. The Fra-2$^{CD4cre}$ iNKT phenotype is cell intrinsic. A. Representative CD1d histograms of DP thymocytes from Fra-2$^{CD4cre}$ mice. Black line, CD150; gray area, isotype control. B. CD150 vs CD45.1 status of TCRβ6, T cells (top panels), cells in dot plots correspond to those gated as TCRβ6, in histogram) and iNKT cells (lower panels, cells in lower dot plots correspond to those gated as TCRβ6, as shown) in populations from a representative reconstituted thymus. D. Ratio of CD45.2 (Fra-2$^{CD4cre}$) to CD45.1 (WT competitor) cells in populations from reconstituted thymus. Each point represents data from one mouse; means are indicated by a horizontal line. CD45.2 bone marrow donors are as labeled on the x-axis. The difference between the means is significant only for iNKT cells; p = 0.04, 2-tailed student’s t test.

FIGURE 3. Fra-2 acts at or near the iNKT lineage commitment step. A. CD44 vs NK1.1 FACS profiles of gated PBS57-CD1d-tet$^{+}$TRCγ6, iNKT cells. Developmental progression of cells follows gates labeled A–D. B. Percentage of total PBS57-CD1d-tet$^{+}$TRCγ6, iNKT cells from Fra-2$^{CD4cre}$ (black bars) and Fra-2$^{CD4cre}$ (gray bars) mice falling into each of gates A–D; n = 8, error bars show SD. C. Live (annexin V$^-$, DAPI$^-$) cells from Fra-2$^{CD4cre}$ (black bars) and Fra-2$^{CD4cre}$ (gray bars) thymus directly ex vivo or cultured for 24 h; n = 3, error bars show SD. D. BrDU incorporation by total thymocytes and iNKT cells from Fra-2$^{CD4cre}$ (black bars) and Fra-2$^{CD4cre}$ (gray bars); n = 4, labels show averages ± SD.

To analyze cell cycle in Fra-2$^{CD4cre}$ iNKT cells in vivo, we injected mice with BrdU 1 h before analysis. As shown in Fig. 3D, approximately 1% of iNKT cells had incorporated BrdU in this period. Approximately 1% of iNKT cells had incorporated BrdU, and when iNKT cells were separated by CD44 expression (Fig. 3D, two left plots), it was apparent that more CD44$^+$ than CD44$^-$ iNKT cells were in cycle. However, no difference was observable between Fra-2$^{CD4cre}$ and Fra-2$^{CD4cre}$ iNKT cells, either at 1 h after labeling, or if cells were cultured in the presence of BrDU for 24 h before analysis (data not shown). The lack of any appreciable difference between normal and Fra-2-deficient iNKT cells in terms of their ability to cycle or die lends further weight to the idea that loss of Fra-2 causes a very early increase in the iNKT population, and that this amplification simply persists throughout iNKT cell ontogeny.
Fra-2f/fCD4cre iNKT cells. In the thymus, most Fra-2f/f iNKT cells
ligand is limiting, there is a skew in TCR V8.2 usage (8). We
therefore indicate a preference for iNKT cells bearing a TCR
Fra-2f/fCD4cre mice, but the skew toward TCRV8.2 (12), was on average 3.4-fold up-regulated (two probe sets). Un-
Known about the importance of this is unclear. As iNKT cells forced to express V8.2 might
be expected, given that all these stages were amplified upon loss of Fra-2. However, further
supporting our contention that Fra-2 loss affects a very early event in iNKT ontogeny, when we searched for specific genes associated
with either iNKT or DP development in the data set, we saw that there were changes in expression of several key genes involved in
the earlier stages of iNKT development (Table II), as well as some, such as Rag1, Rag2, and Dntt, associated with preselection
DP. Of note, Cd24a (HSA), the diagnostic surface marker of the earliest detectable CD24+ tetramer+ CD69+ iNKT thymic preci-
sor (40), was on average 21-fold up-regulated (three separate probe
sites in their upstream regions (43), we found 72 genes, of which
42 were up-regulated and 30 down-regulated (supplemental Table
D). Therefore, loss of Fra-2 is suf-
cient to cause a significant perturbation of AP-1 target genes in
iNKT cells, likely by affecting the balance between repressive and
activating AP-1 family complexes.

Comparing our data to a microarray analysis of maturing thymic
iNKT subsets A–D (see Fig. 3A for definition, and Ref. 46), we
found that there was no strong trend toward any developmental
stage (supplemental Fig. 3A), as might be expected, given that all
these stages were amplified upon loss of Fra-2. However, further
supporting our contention that Fra-2 loss affects a very early event in
iNKT ontogeny, when we searched for specific genes associated
with either iNKT or DP development in the data set, we saw that there were changes in expression of several key genes involved in
the earlier stages of iNKT development (Table II), as well as some, such as Rag1, Rag2, and Dntt, associated with preselection
DP. Of note, Cd24a (HSA), the diagnostic surface marker of the earliest detectable CD24+ tetramer+ CD69+ iNKT thymic preci-
sor (40), was on average 21-fold up-regulated (three separate probe
sets), and Slamf1, also found in this subset of thymic iNKT cells
(12), was on average 3.4-fold up-regulated (two probe sets). Un-
expectedly, Plzf, a key gene for iNKT development (21), was on
average 4.3-fold down-regulated (three probe sets), although it has
been reported that the Plzf promoter is AP-1 regulated (47). Il4
transcripts were also 4.5-fold reduced, consistent with a skew to-
ward a less mature subset (29). We also found that, as shown by
our surface marker analysis, TCRV8.2 (4.8-fold; one probe set)
and both the CD8α- (13-fold average; three probe sets) and CD8β-
chain (21.5-fold; one probe set) genes were overexpressed in Fra-
2f/fCD4cre iNKT cells. Array data were not of use in analyzing expression of multiple TCR Vβ genes, as only one other TCR-
specific probe set, for TCRVβ13, was on the chip used for hy-
bridization. TCRVβ13 was 3.9-fold overexpressed, but the signif-
icance of this is unclear, in iNKT cells forced to express Vβ13 are
unresponsive to lipid Ag stimulation (10).

AP-1 activity is dysregulated in NKT cells
To investigate the mechanism of action of Fra-2 in NKT cell de-
velopment, we performed a microarray experiment, comparing
FACS-sorted total thymic NKT cells from Fra-2f/f and Fra-
2f/f CD4cre mice. From those genes significantly differentially ex-
pressed (p < 0.019), and more than 2-fold changed, more were
up-regulated (1316) than down-regulated (658), indicating that
Fra-2 exerts a net repressive influence on transcription in NKT
cells (see supplemental Table II for a complete list of genes).
Searching for genes with cross-species conserved AP-1 binding
sites in their upstream regions (43), we found 72 genes, of which
42 were up-regulated and 30 down-regulated (supplemental Table
D), in line with Fra-2’s ability to act as both an activator and
repressor of transcription (44, 45). A further search for genes
potentially regulated by the AP-1 superfamily member ATF-2 yielded
15 up-regulated and 4 down-regulated genes (supplemental Table I),
suggesting that Fra-2 acts as a repressor of ATF-2, with which it is
known to dimerize (17). Expression of two members of the AP-1
family itself was also significantly changed; these were c-Jun (9-fold
up-regulated; confirmed by quantitative RT-PCR (supplemental Fig.
3B)) and BATF (1.9-fold down-regulated). Therefore, loss of Fra-2 is
sufficient to cause a significant perturbation of AP-1 target genes in
iNKT cells, likely by affecting the balance between repressive and
activating AP-1 family complexes.

Vα14-Jα18 α-chain rearrangement was close to zero in both con-
trol and Fra-2-deficient animals (Fig. 4A, left side), both Fra-2f/f
and Fra-2f/fCD4cre iNKT cells expressed the Vα14-Jα18 α-chain
rearrangement (Fig. 4A, right side) paired with the typical reperto-
ire of TCRβ chains, including Vβ7, Vβ8.2, Vβ2 (1) (Fig. 4B),
demonstrating that they are true “invariant” NKT cells. However,
there was a skew in the percentage of iNKT cells using each
TCRβ variant in a Fra-2f/fCD4cre mouse, with an increase in the
use of TCRV8.2 (Fig. 4B). TCRβ use is informative about
selection of iNKt cells: in a CD1d+/− mouse, where selecting
ligand is limiting, there is a skew in TCR Vβ repertoire toward
the use of Vβ7, which has led others to suggest that this TCR
has the highest ligand affinity (10, 42). By absolute count the
number of cells carrying all three TCRβ chains is increased in
Fra-2f/fCD4cre mice, but the skew toward TCRV8.2 might
therefore indicate a preference for iNKt cells bearing a TCR
with lower ligand affinity.

CD8 expression levels can affect iNKt cell selection, as either
gain or loss of CD8αβ causes perturbations in Vβ usage (8). We
therefore assessed levels of CD8 and CD4 on thymic and liver
Fra-2f/fCD4cre iNKt cells. In the thymus, most Fra-2f/f iNKt cells
were CD4−, with CD8 expression ranging from CD8− through
CD8dim to CD8bright, such that most cells were either CD4− CD8−
or CD4+ CD8dim (Fig. 4C, top left panel). However, the CD4+ CD8−
population was gone in Fra-2f/fCD4cre iNKt cells, so that they
were mostly CD4+ CD8dim (Fig. 4C, top right panel). There was
a similar shift in CD8 expression in liver iNKt cells (Fig. 4C, lower
panels). The staining was due to CD8β heterodimers rather than
CD8α homodimers, as shown by an increase in staining for both
CD8α and CD8β (Fig. 4D, compare solid black line (Fra-2f/f
CD4cre) with grey shaded area (Fra-2f/f); isotype control is black-
shaded). The alteration in CD8 expression on Fra-2f/fCD4cre
iNKt cells may lend support to the idea that the iNKt population
in Fra-2f/fCD4cre thymus contains cells that would not normally
pass selection. Fra-2f/fCD4cre iNKt cells also showed an unusual
pattern of NK receptor expression (supplemental Fig. 2), although
the significance of this is unclear.
Fra-2f/fCD4cre iNKT cells are able to drive an immune response

Numbers of peripheral Fra-2f/fCD4cre iNKT cells were slightly elevated in the spleen and were not significantly changed in the liver, but might display abnormal functional properties due to loss of Fra-2. First, to investigate whether Fra-2f/fCD4cre mice were able to effectively mount an iNKT-driven immune response, we looked at several parameters, beginning with the immediate trans-activation of other immune cells in response to α-GalCer activation of iNKT cells. Six hours after injection of α-GalCer, we analyzed iNKT cell-mediated activation of B, T, and dendritic cells via the up-regulation of activation markers CD69 on B and T cells, and CD86 on dendritic cells. We found no difference in the degree of activation when we compared Fra-2f/f and Fra-2f/fCD4cre mice and CD86 on dendritic cells. We found no difference in the degree of activation when we compared Fra-2f/f and Fra-2f/fCD4cre mice and CD86 on dendritic cells. We found no difference in the degree of activation when we compared Fra-2f/f and Fra-2f/fCD4cre mice and CD86 on dendritic cells. We found no difference in the degree of activation when we compared Fra-2f/f and Fra-2f/fCD4cre mice and CD86 on dendritic cells.

We next analyzed the immediate effects of α-GalCer activation of iNKT cells, other lymphocytes, 3 days after the i.p. injection of 1, 2, or 4 μg of α-GalCer; gray bars, TEWT; white bars, ISQ; n = 3, data shown as averages ± SD. E, ELISPOT of spleen, 13 days after OVA/α-GalCer immunization; for IFN-γ production in response to: black bars, SIINFEKL; gray bars, TEWT; white bars, ISQ; n = 3, data shown as averages ± SD. F, ELISA for IgG in serum 13 days after OVA/α-GalCer immunization, where a 12-fold change was observed. Fra-2f/f, black bars; Fra-2f/fCD4cre, gray bars.

Peripheral Fra-2f/fCD4cre iNKT cells have an enhanced response to α-GalCer

We next analyzed the immediate effects of α-GalCer activation upon iNKT cells themselves, looking first at proliferation in response to Ag. We assayed iNKT cell proliferation by measuring the size of the iNKT cell population as a percentage of total spleen lymphocytes, 3 days after the i.p. injection of 1, 2, or 4 μg of α-GalCer (48). At all doses, the relative size of the iNKT cell compartment increased more in Fra-2f/fCD4cre animals than in littermate controls, with the most marked difference being with injection of 4 μg of α-GalCer, where a 12-fold change was observed in Fra-2f/fCD4cre iNKT cells relative to an 8.4-fold change in control cells (Fig. 6A).

By administering BrdU 1 h pre-mortem, we were able to ask if a greater percentage of Fra-2f/fCD4cre iNKT cells were cycling 3 days after α-GalCer administration. We found that the same percentages of iNKT cells were in cycle at this time point,

### Table II. Changes in expression of selected genes in Fra2-deficient thymic iNKT cells

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Il2rb (CD122)</td>
<td>-5.67</td>
</tr>
<tr>
<td>Il4</td>
<td>-4.49</td>
</tr>
<tr>
<td>Znhi16 (PLZF)</td>
<td>-4.32</td>
</tr>
<tr>
<td>CD38</td>
<td>-3.70</td>
</tr>
<tr>
<td>Tcet1 (TCF-1)</td>
<td>2.27</td>
</tr>
<tr>
<td>Rorc</td>
<td>2.70</td>
</tr>
<tr>
<td>Ccr9</td>
<td>3.24</td>
</tr>
<tr>
<td>Slamf1 (CD150)</td>
<td>3.39</td>
</tr>
<tr>
<td>Terh-V13</td>
<td>3.91</td>
</tr>
<tr>
<td>Terh-V8.2</td>
<td>4.79</td>
</tr>
<tr>
<td>CD8a</td>
<td>12.52</td>
</tr>
<tr>
<td>CD8b1</td>
<td>20.48</td>
</tr>
<tr>
<td>CD24a (HSA)</td>
<td>21.02</td>
</tr>
</tbody>
</table>

A fold change indicates Fra-2f/fCD4cre/Fra-2f/f. Values were derived from averages of triplicate microarrays of sorted thymic iNKT cells of each genotype.

Fra-2f/fCD4cre iNKT cells are able to drive an immune response.

By administering BrdU 1 h pre-mortem, we were able to ask if a greater percentage of Fra-2f/fCD4cre iNKT cells were cycling 3 days after α-GalCer administration. We found that the same percentages of iNKT cells were in cycle at this time point.

---

*Fra-2f/fCD4cre iNKT cells are able to drive an immune response.*

Numbers of peripheral Fra-2f/fCD4cre iNKT cells were slightly elevated in the spleen and were not significantly changed in the liver, but might display abnormal functional properties due to loss of Fra-2. First, to investigate whether Fra-2f/fCD4cre mice were able to effectively mount an iNKT-driven immune response, we looked at several parameters, beginning with the immediate trans-activation of other immune cells in response to α-GalCer activation of iNKT cells. Six hours after injection of α-GalCer, we analyzed iNKT cell-mediated activation of B, T, and dendritic cells via the up-regulation of activation markers CD69 on B and T cells, and CD86 on dendritic cells. We found no difference in the degree of activation when we compared Fra-2f/f and Fra-2f/fCD4cre mice and CD86 on dendritic cells. We found no difference in the degree of activation when we compared Fra-2f/f and Fra-2f/fCD4cre mice and CD86 on dendritic cells. We found no difference in the degree of activation when we compared Fra-2f/f and Fra-2f/fCD4cre mice and CD86 on dendritic cells. We found no difference in the degree of activation when we compared Fra-2f/f and Fra-2f/fCD4cre mice and CD86 on dendritic cells. We found no difference in the degree of activation when we compared Fra-2f/f and Fra-2f/fCD4cre mice and CD86 on dendritic cells. We found no difference in the degree of activation when we compared Fra-2f/f and Fra-2f/fCD4cre mice and CD86 on dendritic cells. We found no difference in the degree of activation when we compared Fra-2f/f and Fra-2f/fCD4cre mice and CD86 on dendritic cells. We found no difference in the degree of activation when we compared Fra-2f/f and Fra-2f/fCD4cre mice and CD86 on dendritic cells. We found no difference in the degree of activation when we compared Fra-2f/f and Fra-2f/fCD4cre mice and CD86 on dendritic cells. We found no difference in the degree of activation when we compared Fra-2f/f and Fra-2f/fCD4cre mice and CD86 on dendritic cells.

We next analyzed the immediate effects of α-GalCer activation of iNKT cells, other lymphocytes, 3 days after the i.p. injection of 1, 2, or 4 μg of α-GalCer; gray bars, TEWT; white bars, ISQ; n = 3, data shown as averages ± SD. E, ELISPOT of spleen, 13 days after OVA/α-GalCer immunization; for IFN-γ production in response to: black bars, SIINFEKL; gray bars, TEWT; white bars, ISQ; n = 3, data shown as averages ± SD. F, ELISA for IgG in serum 13 days after OVA/α-GalCer immunization, where a 12-fold change was observed. Fra-2f/f, black bars; Fra-2f/fCD4cre, gray bars.

Peripheral Fra-2f/fCD4cre iNKT cells have an enhanced response to α-GalCer

We next analyzed the immediate effects of α-GalCer activation upon iNKT cells themselves, looking first at proliferation in response to Ag. We assayed iNKT cell proliferation by measuring the size of the iNKT cell population as a percentage of total spleen lymphocytes, 3 days after the i.p. injection of 1, 2, or 4 μg of α-GalCer (48). At all doses, the relative size of the iNKT cell compartment increased more in Fra-2f/fCD4cre animals than in littermate controls, with the most marked difference being with injection of 4 μg of α-GalCer, where a 12-fold change was observed in Fra-2f/fCD4cre iNKT cells relative to an 8.4-fold change in control cells (Fig. 6A).

By administering BrdU 1 h pre-mortem, we were able to ask if a greater percentage of Fra-2f/fCD4cre iNKT cells were cycling 3 days after α-GalCer administration. We found that the same percentages of iNKT cells were in cycle at this time point.
regardless of genotype (Fig. 6B), so the enhanced amplification of iNKT cells in response to α-GalCer must be established earlier in the Ag response. Staining for activated caspase 3 three days after Ag challenge also showed that there was little apoptosis in the iNKT cell population (Fig. 6C), suggesting that cell death was not a factor in determining iNKT cell population size at this time point.

**Fra-2-deficient iNKT cells have an enhanced cytokine response**

Transgenic expression of the AP-1 repressor BATF results in a size at this time point.

Enhanced expansion of Fra-2f/fCD4cre iNKT cells. A. Relative size of the iNKT cell population as a percentage of spleen lymphocytes 3 days after i.p. injection of α-GalCer at the concentrations shown; “t0” data (left-hand panels) as Fig. 1, shown for comparison. Fra-2f/f, n = 3; Fra-2f/fCD4cre, n = 3. B. BrdU incorporation by spleen iNKT cells, 3 days after i.p. α-GalCer injection and 1h after BrdU injection; black bars, Fra-2f/f; gray bars, Fra-2f/fCD4cre; n = 3, error bars show SD. C. Anti-active caspase 3 staining of spleen iNKT cells, 3 days after i.p. α-GalCer injection; black bars, Fra-2f/f; gray bars, Fra-2f/fCD4cre; n = 3, error bars show SD.

Given that selection of iNKT cells in a Fra-2f/fCD4cre mouse gives rise to mature iNKT cells bearing an unusual repertoire of TCRs and ancillary receptors, IL-2 and IL-4 production might be increased in Fra-2f/fCD4cre iNKT cells because of alterations in TCR signaling, rather than as a direct consequence of loss of Fra-2. To distinguish between these two possibilities, we bypassed TCR signaling by using PMA and ionomycin to activate iNKT cells ex vivo. Intracellular staining showed that 1.5 h after PMA/ionomycin treatment, there was an even larger difference between the percentage of Fra-2f/fCD4cre and iNKT cells staining positive for IFN-γ, IL-4, and IL-2 following 90 min of in vitro stimulation with PMA/ionomycin of iNKT cells from liver and spleen. D. Intracellular staining for IFN-γ, IL-4, and IL-2 in TCRβ1−CD1d tetramer T cells from liver and spleen; black bars, Fra-2f/f; gray bars, Fra-2f/fCD4cre; n = 3, error bars show SD.

To determine whether T cells from Fra-2f/fCD4cre animals were also abnormally responsive to Ag, total spleen and liver lymphocytes were stimulated with PMA and ionomycin. After excluding iNKT cells by gating out tetramer+ lymphocytes, we found that a significant percentage of T cells were making IL-2 and IL-4, but not IFN-γ, after this short period of stimulation; T cells from littersmate controls, as expected, were not producing these cytokines (Fig. 7D; compare gray bars (Fra-2f/fCD4cre) with black (Fra-2f/f) bars). Therefore, Fra-2 deficiency causes the rapid up-regulation of IL-2 and IL-4 production upon Ag stimulation of both conventional T and iNKT cells.
Discussion

In this study, we have shown that loss of the AP-1 transcription factor Fra-2 causes a 2.5-fold increase in thymic iNKT cells, suggesting that normally, Fra-2 can limit the number of DP cells able to enter the iNKT lineage. Furthermore, it does this selectively, as T cell development is unaffected. These data confirm and extend previous studies on the important role of AP-1-regulated transcription in iNKT cell ontology, and they show that the presence of Fra-2 is necessary to maintain correct regulation of AP-1 targets.

In terms of iNKT cell effector function, we have also demonstrated that Fra-2-f/fCD4cre NKT cells differ from WT iNKT cells in their immediate response to Ag, producing unusually high amounts of IL-2 and IL-4, and proliferating abnormally. Fra-2-f/fCD4cre T cells are also able rapidly to produce IL-2 and IL-4.

Our data on iNKT development are consistent with an important role for Fra-2 during or immediately after selection into the iNKT lineage. We argue this for the following reasons: first, loss of Fra-2 causes a gain in iNKT cell numbers from very early on in iNKT ontogeny, and second, the proportional increase in use of the lower avidity Vβ8.2 receptor indicates that in a Fra-2-f/fCD4cre thymus, some of the extra iNKT cells may be “rescued” lower avidity cells. Support for the notion that selection is affected also comes from the phenotype of BATF transgenic animals: in addition to its later effects on cytokine secretion, overexpression of the AP-1 inhibitor BATF partially blocks iNKT development before the CD44^low to CD44^+ transition (29). Interestingly, in the remaining iNKT cells escaping the block, there is a reduction in surface expression of the invariant TCR, coupled with a skew away from usage of Vβ8.1/8.2, suggesting that the opposite effect to that seen with Fra-2 deficiency may be occurring (28).

How might loss of Fra-2 lead to an increase in iNKT cell numbers? The increase could be due to perturbation of the selection process itself, or to increased cell cycling or decreased apoptosis immediately following selection. Alterations in cycling or death in pre-selection thymocytes can be excluded, since development of most T cells are unaffected by the loss of Fra-2. We were unable to detect changes in either apoptosis or cell cycle in the thymic iNKT subset, even gating on CD44^low cells. This excludes gross alterations in either process, although data from our microarray analysis, showing that Cd24a and Slamf1 are both overrepresented in the earliest pre-selection thymocytes can be excluded, since development of most T cells are unaffected by the loss of Fra-2. We were unable to detect changes in either apoptosis or cell cycle in the thymic iNKT subset, even gating on CD44^low cells. This excludes gross alterations in either process, although data from our microarray analysis, showing that Cd24a and Slamf1 are both overrepresented and I4 is underrepresented in total thymic iNKT cells when Fra-2 is missing, strongly suggest that there is an increase in the earliest iNKT precursor subset (12, 29, 40). However, due to the very low numbers of these cells in the thymus, attempts to verify this experimentally have not yielded statistically significant data.

Given the skew in TCRVβ usage toward Vβ8.2, suggested by others to be of lower avidity (10, 42), it seems possible that selection into the iNKT lineage is affected in Fra-2-deficient thymuses, either by increased positive selection or by a net decrease in negative selection. We consider the latter to be unlikely, as were negative selection to be affected, the higher avidity Vβ3-containing TCR, and possibly other potentially autoreactive TCRs, would be overrepresented, rather than the lower avidity Vβ8.2 TCR. A change in positive selection could occur via increased ancillary signaling, perhaps via Slamf1, which was up-regulated in our microarray, or by a change in the TCR signal itself: Fra-2-f/fCD4cre iNKT cells do not fully down-regulate CD8α and CD8βf, and as a result the whole population is CD4^+CD8^+. While a functional interaction between the invariant NKT TCR and CD8 coreceptor has yet to be demonstrated, modeling suggests that it is possible (Y. Jones and J. Brown, unpublished observations), raising the idea that CD8 has a role as a coreceptor for iNKT cells, potentially enhancing the selection signal.

By analogy with T cell development (49), enhanced positive selection of lower affinity iNKT cells might also be caused by increased intracellular signaling. Intracellular signaling during iNKT cell selection and development could potentially be perturbed in the absence of Fra-2, likely via the dysregulation of AP-1 target genes evident from our microarray data; this could then affect the selection process. AP-1 activity is induced upon TCR signaling to protein kinase Cθ (50) during T cell development, and furthermore, the Tec kinases Itk and Rlk are required for optimal AP-1 activity (51). Since both protein kinase Cθ and the Tec kinases are essential for iNKT cell development and function (13, 52), we speculate that AP-1 may be a target of these pathways.

Comparison with the BATF transgenic mice shows that at all points, Fra-2-f/fCD4cre mice have the opposite phenotype. As BATF is a repressor of AP-1 activity (27), this suggests that a net activation of AP-1-regulated genes may be the driving factor in the increase in iNKT cell number when Fra-2 is deleted. Our microarray data show that indeed, there are significant changes in the gene set regulated by the AP-1 superfamily when Fra-2 is absent. Therefore, despite JunB, JunD, Jun, and Fos all being expressed by iNKT cells (29), Fra-2 must be necessary for the full complement of normal AP-1-regulated transcription to occur. Whether this is via an effect on complexes containing Fra-2, or due to a more generalized alteration in the balance of AP-1 activation vs repression, is unclear. Interestingly, loss of Fra-2 led to a 10-fold up-regulation of Jun, an activating member of the AP-1 family, as well as a small down-regulation of BATF, and it would be interesting to determine whether Jun also has a specific role in iNKT development. The role of Atf2, whose target genes underwent a net activation in Fra-2-f/fCD4cre iNKT cells, is also unexplored and may be pertinent, as expression of a dominant interfering Atf2 mutant severely inhibits NK cell development (17). The pleiotropic effects on AP-1-regulated transcription caused by tampering with Fra-2 are well illustrated by our observations regarding the Il4 gene, which is coordinately regulated by AP-1 and NFAT (26): although our microarray results suggest that Il4 mRNA is down-regulated in Fra-2-deficient total thymic iNKT cells, IL-4 is superinduced in peripheral iNKT cells in the absence of Fra-2 (see below). Although we do not know the reason for this anomaly, it seems very likely that loss of Fra-2 may lead to complex context-specific effects dependent on which other AP-1 family members are present.

Once committed, thymic iNKT cells developed ostensibly as normal in the absence of Fra-2, exhibiting no overt change in their capacity to proliferate and survive. Mature peripheral Fra-2-f/fCD4cre iNKT cells remained able to stimulate T and dendritic cells to a similar degree to controls, although it is not clear why their ability to stimulate B cells was slightly attenuated. However, there were some marked abnormalities; Fra-2-deficient iNKT cells proliferated more in response to α-GalCer than did their normal counterparts, and when treated with either α-GalCer or PMA/ionomycin, they produced normal amounts of IFN-γ, but made very large quantities of both IL-2 and IL-4. IL-2 is normally produced at very low levels by iNKT cells, but it is known to stimulate their proliferation, and this effect is enhanced by IL-4 (53). Interestingly, Fra-2-deficient T cells were also able to make large amounts of IL-2 and IL-4 immediately upon activation, rather than as a delayed response, making them more similar to iNKt cells than to normal T cells in this respect. However, detailed analysis of their surface phenotype indicated they were not expressing iNKT markers (Table I and data not shown) and hence had not undergone a lineage switch like that caused by overexpression of the transcription factor PLZF, which endows conventional T cells with an iNKT-cell like phenotype and effector function (22). The increased
and (in the case of T cells) abnormally early IL-2 and IL-4 production we observed in Fra-2f/fCD4cre peripheral iNKT and T cells shows that the damping of expression of these genes, which are known to be direct targets of AP-1 (25, 26) and were repressed in BATF transgenics (28), is lost in the absence of Fra-2 in the periphery: whether this is part of Fra-2’s role as a repressor, or is a consequence of Fra-2’s regulation of, for example, BATF or c-Jun, is unclear.

In conclusion, as we have shown herein that Fra-2 is an important regulator of the development of iNKT cells, an exploration of Fra-2’s effects on other innate and T cell populations may be timely. For example, it would be of interest to determine whether Fra-2 is involved in the αβγδ fate decision influenced by Jun (54), or in development of the NK lineage, which depends on many of the same transcription factors as iNKT cell development (14, 18). We anticipate that fuller analysis of the roles of other AP-1 proteins in iNKT cells, and the role of Fra-2 in other lineages, will be fruitful areas for future study.

Acknowledgments
We are indebted to Erwin Wagner for the gift of Fra-2f/f mice, Richard Mitter and the Cancer Research UK microarray facility, the FACS facilities of Cancer Research UK London Research Institute, National Institute of Medical Research (London), and Institute of Cancer Research, Demelza Bird and Mark Allen for technical assistance, and Rose Zamoyksa and Gitta Stockinger for helpful advice and comments.

Disclosures
The authors have no financial conflicts of interest.

References


