Pro-Th1 Cytokines Promote Fas-Dependent Apoptosis of Immature Peripheral Basophils

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Pro-Th1 Cytokines Promote Fas-Dependent Apoptosis of Immature Peripheral Basophils

Elke Schneider, Marie-Béatrice Tonanny, Mariette Lisbonne, Maria Leite-de-Moraes, and Michel Dy

We have previously characterized immature hemopoietic cells of the basophil lineage as a lin−c-kit+ population, which responds to IL-3 by enhancing its histamine synthesis through histidine decarboxylase activation. Herein, we show both in vitro and in vivo that exposure to the pro-Th1 cytokines IL-12 and IL-18 promotes Fas-dependent apoptosis of these cells in the spleen. This conclusion was supported by the following findings: 1) A 24-h treatment with IL-12 plus IL-18 enhanced Fas expression and annexin staining among basophil precursor-enriched lin−c-kit+ splenocytes. 2) Fas or Fas ligand deficiency in mutant mice abolished the inhibitory effect of IL-12 plus IL-18 on IL-3-induced histamine production. 3) The large spectrum inhibitor of the caspase cascade, benzoyloxycarbonyl-Val-Ala-Asp fluoromethylketone, significantly reduced the effect of IL-12 plus IL-18. The inhibition of histamine production was mediated through NK cells, since it failed to occur upon stimulation of spleen cells from NK cell-deficient mice or after NK cell depletion. IL-12 plus IL-18 rendered NK cells cytotoxic against Fas-transfected target cells and promoted their production of IFN-γ and TNF-α, which are both essential for sensitizing histamine-producing cells to the Fas death pathway. This is the first evidence that pro-Th1 cytokines can promote apoptosis of immature peripheral histamine-producing cells, thus limiting Th2 immune responses. Comparable in vivo data as well as increased histamine production in the spleen of aged Fas-deficient lpr mice support its physiological relevance. The Journal of Immunology, 2004, 172: 5262-5268.

Histamine is a well-known mediator in a variety of physiological and pathological situations. It can be released from basophil or mast cell granules or newly synthesized from histidine upon activation of histidine decarboxylase (EC 4.1.1.22). The latter feature is typical for a subset of hemopoietic precursors we have identified as immature basophils using phenotypic and morphological criteria (1). These cells constitute an important source of histamine, which they newly synthesize, along with IL-4 and IL-6 (2), in response to cytokines (3), aggregated IgE (4), or calcium ionophore (5). In this respect, they resemble the non T-non B cells described by Seder et al. (6) but are quite distinct from mast cells or precursors of this lineage (7).

Several recent studies support the notion that histamine influences the outcome of the immune response by modulating the production of cytokines controlling the Th1/Th2 balance (8, 9). The most clear-cut evidence comes from studies demonstrating its anti-Th1 effect, which is due to its capacity to inhibit the production of the pro-Th1 cytokine IL-12 by APCs (10), while stimulating that of IL-10 (11). Consequently, it endows these cells with a DC2 phenotype, enabling them, in turn, to promote a Th2 response (12-14).

Along with mast cells, basophils are associated with allergic reactions. They are not easily detected in mice by simple morphological criteria based on light microscopy (15). Furthermore, their precursors express too low levels of FcεR in normal conditions for positive sorting. Yet, using their unique capacity to synthesize histamine in response to IL-3 as a means of identification, we have been able to show that this population is present at appreciable concentrations in murine bone marrow and spleen (2, 7, 16). In accordance with its biological activities, it is potentially capable of amplifying the Th2 orientation of the immune response in two ways, either by reducing the Th1 response through the production of histamine or by generating the pro-Th2 cytokines IL-4 and IL-6.

Taking into account the current debate regarding the “hygiene hypothesis” (17), we addressed the question whether basophil precursors were modified by a Th1 polarization of the immune response. To this end, we examined the effect of the pro-Th1 cytokines IL-12 plus IL-18 on basophil precursors. We found that this treatment decreased histamine synthesis in the spleen, but not in the bone marrow, through Fas-dependent apoptosis. This finding prompted us to investigate the cellular and molecular mechanisms involved, in particular the role of NK cells.

Materials and Methods

Animals

Six- to 10-wk-old pathogen-free, male or female wild-type C57BL/6 and mutant lpr/lpr, gld/gld, c−/− mice were bred in our animal facility. IFN-γ−/− and RAG2−/− strains, on the same genetic background, were kindly provided by Dr. J. Di Santo (Pasteur Institute, Paris, France).

Cytokines and Abs

Murine IL-18, IL-12, IFN-α, and IL-3 were purchased in recombinant form from R&D Systems (Abingdon, U.K.). The following mAbs to mouse leukocytes were provided by PharMingen (San Diego, CA): unlabeled and PE-conjugated hamster CD95 (Fas; J02), FITC-conjugated CD45R/B220 (RA3-6B2), PE-conjugated pan-NK cells (DX5), biotinylated mouse NK1.1 (PK136), CD3 (145-2C11), CD19 (MB19-1), Gr-1 (RB6-8C5), TER 119, c-kit (ACK45), PE-conjugated anti-TNF-α (MP6-XT22), and isotype controls. CyChrome-streptavidin was used to reveal NK1.1+ cells, PE-conjugated anti-IFN-γ (XMH1.2) and rat IgG1 (isotype control) were purchased from Caltag Laboratories (Le Perray en Yvelines, France).
Preparation of splenocytes enriched for histamine-producing cells

Total spleen cells were incubated for 20 min at 4°C with a mixture of the following biotinylated mAbs: Thy1.2 (30-H12), CD19, Gr-1, c-kit, and TER 119. After incubation with streptavidin-coated beads, unlabeled cells were recovered as described above and assessed for their line-c-kit<sup>+</sup> cell enrichment. In some experiments, the depletion of splenic line<sup>+</sup> cells was performed with the SpinSep kit developed by StemCell Technologies (Vancouver, Canada) according to the manufacturer’s instructions.

Treatment with cytokines and/or anti-Fas mAb

Total spleen cells (10<sup>7</sup>/ml), line<sup>+</sup> (10<sup>6</sup>/ml), and NK1.1<sup>+</sup> fractions (10<sup>7</sup>/ml) were plated into Falcon 3047 multiwell plates (2 ml/well) and incubated for 24 h in the presence of IL-18 (100 ng/ml), IL-12 (10 ng/ml), IFN-γ (20 ng/ml), TNF-α (10 ng/ml), and anti-CD95 (Fas) mAb (5 μg/ml), alone or in combination, in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. Supernatants were then collected and stored at −20°C until IFN-γ assay. In some experiments, the caspase inhibitor benzylxycarbonyl-Val-Ala-Asp-fluoromethylketone (Bachem, Bubendorf, Switzerland) was added at a concentration of 50 μM 2 h before other factors. After the 24-h pretreatment, cells were counted and incubated for another 48 h in 96-well microtiter plates (5 × 10<sup>3</sup> cells/well). After 24-h incubation, the wells were analyzed for Fas expression relative to the isotype control (dotted line). Annexin V staining was performed after a 24-h incubation with (thick line) or without (thin line) IL-12 plus IL-18, c-kit<sup>+</sup> cells were analyzed for Fas expression relative to the isotype control (dotted line). Annexin V staining was performed after a 24-h incubation with (thick line) or without (thin line) IL-12 plus IL-18, as compared with freshly isolated line-c-kit<sup>+</sup> cells (dotted line).

FIGURE 1. The effect of IL-12 plus IL-18 on histamine production is Fas-dependent and accompanied by increased Fas expression and annexin V staining among basophil precursor-enriched spleen cells. A, Splenocytes from wild-type, lpr, and gld C57BL/6 mice were preincubated for 24 h with or without IL-12 (10 ng/ml) plus IL-18 (100 ng/ml). Anti-Fas mAb (5 μg/ml) was added in some experiments performed with the FasL-deficient strain. B, Wild-type splenocytes were depleted of mature cells using the SpinSep procedure from StemCell Technologies according to the manufacturer’s instructions. After a 24-h incubation with (thick line) or without (thin line) IL-12 plus IL-18, c-kit<sup>+</sup> cells were analyzed for Fas expression relative to the isotype control (dotted line). C, Annexin V staining was performed after a 24-h incubation with (thick line) or without (thin line) IL-12 plus IL-18, as compared with freshly isolated line-c-kit<sup>+</sup> cells (dotted line).

Table 1. Effect of IL-12 and/or IL-18 on IL-3-induced histamine release by spleen and bone marrow cells<sup>a</sup>

<table>
<thead>
<tr>
<th>Spleen cells</th>
<th>Bone marrow cells total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>line-c-kit&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>16.6 ± 1.5</td>
</tr>
<tr>
<td>IL-12</td>
<td>13.1 ± 1.7*</td>
</tr>
<tr>
<td>IL-18</td>
<td>17.1 ± 1.9*</td>
</tr>
<tr>
<td>IL-12 + IL-18</td>
<td>4.6 ± 0.6**</td>
</tr>
</tbody>
</table>

<sup>a</sup>Splenocytes and bone marrow cells were preincubated for 24 h in culture medium alone or along with IL-12 (10 ng/ml) and/or IL-18 (100 ng/ml). They were then incubated for 48 h with IL-3 and assayed for histamine synthesis. Data represent mean ± SEM from 10 separate experiments for total spleen cells and 3 separate experiments for line-c-kit<sup>+</sup> spleen cells and total bone marrow cells.

<sup>b</sup>ND, Not done.

<sup>c</sup>NS.

<sup>d</sup>p < 0.05; <sup>e</sup>p < 0.01 (ANOVA plus Dunnett’s test); <sup>f</sup>p < 0.001 (Student’s t test) NS: not significant (ANOVA plus Dunnett’s test), relative to respective controls preincubated in medium alone.

In vivo treatment

Wild-type and C56BL/6-lpr/lpr mice received a single i.v. injection of IL-12 (0.2 μg/mouse) plus IL-18 (0.25 μg/mouse) or saline and were sacrificed 2 h later when splenocytes were assayed for spontaneous and IL-3-induced histamine production and Fas-dependent cytotoxicity.

Cell preparations

Spleen and bone marrow cells were prepared as previously described (18) and adjusted to a final concentration of 10<sup>7</sup> and 2.5 × 10<sup>6</sup>/ml, respectively. They were incubated in MEM, supplemented with 1% sodium pyruvate (100 mM), 1% g-aminobutyric acid, 1% penicillin, 100 μg/ml streptomycin (all from Life Technologies, Grand Island, NY), and 10% horse serum (Biowest, Nuaille, France), referred to as culture medium.

Mononuclear spleen cells from C57BL/6-<sup>c</sup>-<sup>v</sup> mice, chosen for their lack of NKT and CD8 lymphocytes, were labeled with biotinylated anti-NK1.1 (clone PK136) in PBS containing 2% FCS. After washing, they were preincubated in microtiter plates, and optimal concentrations of IL-3 were added directly to the wells after 24 h without washing.

Apoptosis assay

Apoptosis was evaluated among gated basophil precursor-enriched line-c-kit<sup>+</sup> cells of the spleen, using annexin V staining after a 24-h incubation of line cells with or without IL-12 (10 ng/ml) plus IL-18 (100 ng/ml).

CTL assay

Target cell lysis by total spleen cells and sorted NK cells was measured by the Just Another Method (19) assay using the L1210 cell line transfected with Fas (L1210 Fas) and the nontransfected control (L1210) as target cells (20). Briefly, total spleen cells, pretreated for 24 h with IL-12 plus IL-18 or culture medium alone, were incubated at various target:effector ratios with L1210 or L1210 Fas cells (2.5 × 10<sup>5</sup>) labeled with [3H]thymidine (Amersham, Les Ulis, France) for 18 h. Assays were performed in 96-well U-bottom plates in a total volume of 200 μl/well. After incubation, cells were harvested and radioactivity was determined using a beta counter (LKB Wallac, Gaithersburg, MD). Percent lysis was calculated as follows: 100 − [(experimental release − maximum release/experimental release) × 100]. Maximum release was estimated by adding 20 μl of a DNase I solution (10 mg/ml; Roche Diagnostics, Indianapolis, IN).

IFN-γ and histamine assays

IFN-γ production was measured by ELISA (sensitivity: 40 pg/ml) as previously described (21). Histamine was determined by an automated continuous flow fluorometric technique (22), whose limit of sensitivity is 0.5 μm.
ng/ml. The specificity of this assay has been verified by a RIA (Immuno
tech, Marseille, France).

Flow cytometric analysis and intracytoplasmic cytokine staining

After blocking Fc receptor functions with rat anti-mouse CD16/CD32 mAb (2.4G2), cells were labeled with appropriate Abs using three-color immuno-fluorescence. Cells were analyzed on a FACScan cytofluorometer (BD Biosciences, Mountain View, CA) using CellQuest software. RBC and debris were excluded on the basis of forward and side scatter parameters. At least 10,000 cells were acquired within the live gate.

For intracytoplasmic staining of IFN-γ or TNF-α, spleen cells were incubated for 6 h in culture medium alone or along with IL-12 (100 ng/ml) plus IL-18 (10 ng/ml). Cells were washed twice and stained with biotinylated NK1.1 mAb following CyChrome-streptavidin as a second step reagent and FITC-conjugated B220 mAb. After fixation with 4% paraformaldehyde for 5 min, cells were washed in staining buffer supplemented with 0.5% saponin for cell permeabilization. They were then treated for 30 min with PE-conjugated anti-IFN-γ mAb, PE-conjugated anti-TNF-α mAb, or their isotype controls in the same buffer. After washing, cells were resuspended in staining buffer and analyzed on a FACScan cytometer using CellQuest software. At least 10,000 events were acquired in the NK1.1<sup>bright</sup> region in each run.

Statistical analyses

The standard Student’s t test was used to establish statistical significance between two series of data. One-way ANOVA with Dunnett’s test was used for multiple comparison with one control.

Results

IL-12 plus IL-18 inhibit histamine release by spleen cells

In the present study, we used the proinflammatory cytokines IL-12 and IL-18 to evaluate the effect of a Th1 microenvironment on histamine-producing cells in spleen and bone marrow. As shown in Table I, a 24-h exposure of splenocytes to both molecules resulted in a substantial decrease of IL-3-induced histamine release, while either factor alone had a slight or no effect, respectively. Similar results were obtained with basophil precursor-enriched lin-c-kit<sup>+</sup> spleen cells, thus excluding the involvement of mast cells and other mature subsets, such as T and B lymphocytes, in this biological activity. Histamine secretion was also reduced by IL-12 plus IL-18 in the absence of IL-3 (5.1 ± 0.5 ng of histamine per10<sup>6</sup> unstimulated cells vs 1.2 ± 0.1 ng/10<sup>6</sup> cells exposed to IL-12 plus IL-18; means ± SEM from three separate experiments). In contrast, histamine levels were enhanced rather than diminished in supernatants of bone marrow cells stimulated in the same conditions.

IL-12 plus IL-18-induced decrease of IL-3-induced histamine is mediated through the Fas death pathway

Knowing that IL-12 and IL-18 are potent inducers of Fas ligand (Fasl),<sup>3</sup> we investigated the involvement of the Fas-dependent apoptosis in the decrease of histamine in spleen cell supernatants. As shown in Fig. 1A, histamine release was only slightly inhibited when spleen cells were recovered from Fas- or FasL-deficient mice of the lpr or gld genotype. The requirement of Fas-Fasl interactions was further corroborated by the observation that an efficient response to IL-12 plus IL-18 could be restored to gld splenocytes

<sup>3</sup> Abbreviation used in this paper: Fasl, Fas ligand.
when the missing FasL was replaced by cross-linking with anti-Fas mAb (Fig. 1A). It is also clear from Fig. 1 that a 24-h treatment with IL-12 plus IL-18 increased not only the level of Fas expression among basophil precursor-enriched Lin<sup>-</sup> c-kit<sup>+</sup> splenocytes (Fig. 1B), but also enhanced annexin V staining within this population (Fig. 1C). Finally, in the same line of evidence, the broad spectrum caspase inhibitor benzoylcarbonyl-Val-Ala-Asp-fluoromethylketone reduced the effect of IL-12 plus IL-18 on IL-3-induced histamine secretion from 75.2 ± 2.5% to 35.7 ± 3.4% inhibition (means ± SEM from four separate experiments).

The physiological relevance of the control of histamine-producing cells by the Fas pathway is corroborated by the increased histamine synthesis in spleens from 3- to 6-mo-old Fas-deficient C57BL/6-lpr/lpr mice. Splenocytes of this genotype produced 12.8 ± 4.0 ng of histamine per 10<sup>6</sup> unstimulated and 68.6 ± 6.3 ng per 10<sup>6</sup> IL-3-induced cells, as compared with the wild type, the production of which was 4.4 ± 0.7 and 21.2 ± 2.2 ng/10<sup>6</sup> cells, respectively (means ± SEM from three separate experiments; p < 0.001). These data suggest that histamine-producing cells accumulate in the spleen when they can no longer be eliminated by Fas-FasL interactions.

**Effect of IL-12 plus IL-18 on histamine-producing cells depends on the NK1.1<sup>+</sup> population**

Spleen cells exposed for 24 h to IL-12 plus IL-18 acquire the capacity to kill target cells in a Fas-dependent manner, as demonstrated by a more efficient lysis of Fas-transfected than nontransfected L1210 cells (Fig. 2A). Stimulation with IL-12 or IL-18 alone was sufficient to render spleen cells cytotoxic, albeit less than both factors together (Fig. 2B). Depletion of NK1.1<sup>+</sup> cells nearly abrogated the capacity of spleen cells exposed to IL-12 plus IL-18 to kill L1210 cells through the Fas pathway (Fig. 2C), indicating that they were the main effector cells.

Indeed, even though both NK and NKT cells can express FasL in response to IL-12 plus IL-18 (23, 24), NK cells are clearly preponderant in our experimental setup. The inhibitory effect of IL-12 plus IL-18 was effectively maintained in NKT and CD8 cell-deficient β<sub>m<sup>-/-</sup></sub> mice (Fig. 3), but drastically diminished after depletion of the remaining NK cells. Furthermore, the inhibition of histamine production reappeared when positively selected NK cells were added back to the depleted β<sub>m<sup>-/-</sup></sub> splenocytes (Fig. 3).

**NK cell-derived IFN-γ and TNF-α contribute to the inhibitory effect of IL-12 plus IL-18**

We have previously reported that histamine-producing cells are not susceptible to Fas cross-linking, unless IFN-γ is present (25). As shown in Table II, spleen cells did effectively generate this cytokine during the 24-h preincubation with IL-12 plus IL-18 and, to a much lesser extent, in response to IL-12 alone. IFN-γ production was similar in spleen cells from NKT cell-deficient C57BL/6 β<sub>m<sup>-/-</sup></sub>−/− mice were depleted for NK1.1<sup>+</sup> cells as described in Materials and Methods. Data represent means ± SEM from six individual experiments for wild-type splenocytes and two individual experiments for mutant mice.

<table>
<thead>
<tr>
<th>Spleen Cell Populations</th>
<th>Culture medium</th>
<th>IL-12</th>
<th>IL-18</th>
<th>IL-12 + IL-18</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>&lt;0.04</td>
<td>3.30 ± 1.40</td>
<td>0.14 ± 0.05</td>
<td>95.25 ± 14.30</td>
</tr>
<tr>
<td>C57BL/6-β&lt;sub&gt;m&lt;sup&gt;-/-&lt;/sup&gt;&lt;/sub&gt; NK1.1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>&lt;0.04</td>
<td>ND*</td>
<td>ND</td>
<td>114.8/94.4</td>
</tr>
<tr>
<td>C57BL/6-β&lt;sub&gt;m&lt;sup&gt;-/-&lt;/sub&gt;&lt;/sub&gt; NK1.1&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>&lt;0.04</td>
<td>ND</td>
<td>ND</td>
<td>40.5/37.9</td>
</tr>
</tbody>
</table>

* IFN-γ production was measured by ELISA in supernatants from spleen cells (10<sup>7</sup>/ml) incubated for 24 h with IL-12 and/or IL-18 or in culture medium alone. Splenocytes from NKT cell-deficient C57BL/6 β<sub>m<sup>-/-</sub></sub>−/− were depleted for NK1.1<sup>+</sup> cells as described in Materials and Methods. Data represent means ± SEM from six individual experiments for wild-type splenocytes and two individual experiments for mutant mice.

* *Not determined.*

**FIGURE 4.** Intracellular staining of IFN-γ in NK cells. Splenocytes (10<sup>7</sup>/ml) from C57BL/6 six mice were incubated for 6 h with (thick line) or without (thin line) IL-12 plus IL-18. They were then stained with NK1.1-CyChrome and B220-FITC mAbs followed by intracellular labeling with PE-conjugated anti-IFN-γ mAb, as compared with the isotype control (dotted line). The cells analyzed were gated from the NK1.1<sup>+</sup>B220<sup>-</sub> population.
In vivo treatment with IL-12 plus IL-18 induces decreased histamine production by spleen cells from wild-type but not from \#lpr\#lpr mice

As shown in Fig. 7A, decreased histamine release in response to IL-3 occurred also in vivo 2 h after a single i.v. injection of IL-12 plus IL-18. It was increased rather than inhibited in mice of the \#lpr\#lpr genotype, confirming the involvement of the Fas pathway. This conclusion was further corroborated by the marked Fas-dependent cytotoxicity exerted by spleen cells from wild-type mice injected 2 h before with IL-12 plus IL-18 (Fig. 7B) that was abolished after depletion of NK1.1\(^+\) cells, similarly to what we observed in vitro.

Discussion

There is increasing evidence that in addition to its role in allergic responses histamine exerts more subtle regulatory functions during the immune response by modulating the synthesis of cytokines directing the Th1/Th2 balance of the immune response (8, 9). In contrast, a number of studies have established that in addition to mature mast cells and basophils, which constitute its main cellular stores, histamine can be provided by other cell populations, in which case it is newly synthesized in response to various stimuli rather than released by degranulation (26, 27). Basophil precursors are an important source of this “nascent” histamine, which they generate together with IL-4 and IL-6 in response to growth factors like IL-3 and GM-CSF or aggregated IgE (3, 4). These distinctive features can be used as a means of identification, even though they are not easily purified to homogeneity because of the lack of specific markers.

In the present study, we provide evidence for a negative regulation of basophil precursors by IL-12 plus IL-18, which results in a substantial reduction of IL-3-induced histamine synthesis in the spleen. This effect depends on Fas-FasL interactions since it did not occur in mice deficient for either molecule and was partially abolished in the presence of the large spectrum inhibitor of the caspase cascade benzylxycarbonyl-Val-Ala-Asp-fluoromethylketone. Exposure to IL-12 plus IL-18 increased Fas expression and annexin V staining among basophil precursor-enriched \#c-kit\#c-kit spleen cells, lending further support to the notion that histamine-producing cells do effectively undergo Fas-dependent apoptosis.

NK cells are essential in mediating this biological activity. They become effectively cytotoxic against Fas-transfected L1210 target cells upon stimulation with IL-12 plus IL-18, while their depletion from the spleen abolishes the effect, both in terms of histamine production and cytotoxicity. The requirement of NK cells for the inhibitory effect of IL-12 plus IL-18 is probably the main reason why histamine-producing cells are insensitive to Fas-induced cell death in the bone marrow, where NK and NKT cells are rare and/or functionally different (28). In accordance with this conclusion, medullary cells did not acquire the capacity to kill Fas-transfected targets in response to IL-12 plus IL-18 and produced only minute amounts of IFN-\(\gamma\) and no detectable TNF-\(\alpha\) (data not shown).

We have previously reported that Fas cross-linking alone induces no significant decrease of histamine production by spleen cells (26). This observation is confirmed herein, since treatment with IL-12 and IL-18 alone enabled spleen cells to kill Fas-transfected targets, while reduced histamine synthesis occurred only when sufficient amounts of IFN-\(\gamma\) were generated during the pre-treatment with both cytokines. Yet, even the concomitant induction of IFN-\(\gamma\) and FasL does not entirely account for the inhibitory effect of IL-12 plus IL-18, which persists, to a large extent, in IFN-\(\gamma\)-/- mice. It is possible that IFN-\(\alpha\) and/or \(\beta\), which can both sensitize histamine-producing cells to the Fas death pathway (26), replace IFN-\(\gamma\) in this context. Another likely candidate for this effect is TNF-\(\alpha\), whose production is strongly increased in response to IL-12 and IL-18 (Ref. 13; data not shown).

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**FIGURE 5.** The inhibition of IL-3-induced histamine production in response to IL-12 plus IL-18 is only slightly reduced in IFN-\(\gamma\)-deficient mice. Spleen cells from IFN-\(\gamma\)-/- and wild-type mice were exposed for 24 h to IL-12 plus IL-18, washed, and incubated for another 48 h in the presence of IL-3 with or without 20 ng/ml IFN-\(\gamma\). Data are means \(\pm\) SEM from three experiments. *p < 0.05

**FIGURE 6.** TNF-\(\alpha\) participates in the sensitization of histamine-producing cells to Fas-induced apoptosis. Spleen cells from C57BL/6 (A) \#RAG2\#RAG2-/- and \#RAG2\#RAG2-/- mice (B) were incubated for 24 h with different stimuli, washed, and tested for their histamine release in response to IL-3 during an additional 48-h incubation. Data represent means \(\pm\) SEM from three separate experiments performed with wild-type mice and a typical example of two with \#RAG2\#RAG2-/- and \#RAG2\#RAG2-/- strains (the results of the second experiment are indicated in parentheses). For intracellular staining of TNF-\(\alpha\) in NK cells (D), splenocytes \((10^7/ml)\) from C57BL/6 mice were incubated for 6 h with (continuous line) or without (broken line) IL-12 plus IL-18. After staining with NK1.1-CyChrome and B220-FITC mAbs, intracellular labeling was performed with PE-conjugated anti-TNF-\(\alpha\) mAb, as compared with the isotype control (dotted line). The cells analyzed were gated from the NK1.1\(^{hi}\) or B220\(^{hi}\) population (C).

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in its original terms (31), it remains likely that the pattern of adult
fections of the Th1 type may provide protection against future
olours which sensitize its target cells to apoptosis.
role belongs to NK cells, which provide both FasL and the mol-
the spleen. There may be other factors involved, yet the pivotal
purpose is TNF-α/H9251
lpr/lpr
mice were sacri-
FIGURE 7. In vivo treatment with IL-12 plus IL-18 decreases IL-3-
induced histamine production and confers FasL-dependent cytotoxicity to
NK cells. C57BL/6 and C57BL/6-lpr/lpr mice were sacrificed 2 h after i.v.
injection of IL-12 plus IL18 or saline. A, Spleen cells from wild-type and
mutant mice were tested for their capacity to secrete histamine during a
48-h incubation with IL-3. Data are means ± SEM from four experiments.
*p < 0.05. B, Cytotoxic effect of spleen cell from mice having received
IL-12 plus IL-18 ([square]), as compared with depleted NK1.1 splenocytes
(▲) and saline-injected controls (▲).
purpose is TNF-α, which is effectively generated by NK cells in
the spleen. There may be other factors involved, yet the pivotal
role belongs to NK cells, which provide both FasL and the mole-
cules which sensitize its target cells to apoptosis.
Considering the increasing prevalence of allergic diseases in
western countries, several theories have been proposed as a possible
explanation. The most widely discussed is the so-called hy-
giene hypothesis, which states that the confrontation with infec-
tions of the Th1 type may provide protection against future
development of allergies. Even though this theory has been refuted
in its original terms (31), it remains likely that the pattern of adult
immune responses is determined by “danger” signals encountered in
the past, even though they will not necessarily give rise to in-
fec tions. Given the implication of basophils in allergic reactions, it
is interesting to note that the Th1 microenvironment can effectively
diminish their immediate precursors and eventually attenuate the
severity of this type of disease.
Both in vitro and in vivo treatment with IL-12 and IL-18 di-
ninished the peripheral histamine production, thus limiting its po-
tential pro-Th2 effect through inhibition of IL-12 and stimulation of
IL-10 production (11, 12). Since histamine-producing cells un-
dergo apoptosis in these conditions, the concomitant production of
the pro-Th2 cytokines IL-4 and IL-6 is likewise abolished (2). Fur-
thermore, we have recently demonstrated that IL-12 plus IL-18 pro-
mote apoptosis of NKT cells, which are another important
source of IL-4 (21, 24).
In conclusion, the proinflammatory cytokines IL-12 and IL-18
not only amplifies the Th1 orientation of the immune response by
inducing FasL, IFN-γ, and TNF-α, but the cytokines generated in
these conditions cooperate to diminish the pool of basophil pre-
cursors, thus reducing the availability of mature effectors of aller-
gic reactions.

Acknowledgments
We thank A. Arnould and F. Machavov for their technical assistance.

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