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Lymphoadenopathy in IL-2-Deficient Mice: Further Characterization and Overexpression of the Antiapoptotic Molecule Cellular FLIP

Patricia Chastagner, Jay Reddy, and Jacques Thèze

IL-2 was originally identified as a potent T cell growth factor. It was subsequently demonstrated that IL-2 also exerts proapoptotic effects under certain conditions. Inactivation of IL-2 by gene targeting in mice showed that whereas IL-2 is not essential for the generation, clonal expansion, or differentiation of lymphocytes to effector cells, it has a unique role in preventing the accumulation of activated lymphocytes. IL-2−/− mice show lymphoadenopathy and autoimmune reactions, suggesting that the proapoptotic effects of IL-2 may predominate in vivo. In this study, we confirm that lymph nodes (LNs) are enlarged in IL-2−/− animals, but surprisingly, we found that their spleens are almost normal in size. Subsequent to this observation, we compare lymphocytes from LNs and spleens of IL-2−/− and IL-2+/− animals to analyze molecular and cellular correlates of the immunopathological disorders found in IL-2-deficient mice. LN lymphocytes from IL-2−/− are selectively activated and show an enhanced survival capacity and an increased ability to proliferate in vitro when compared with LN cells from IL-2+/− mice and splenocytes from IL-2−/− and IL-2+/− mice. Because the apoptosis inhibitor FLIP has been shown in vitro to participate in the IL-2 control of activation-induced cell death, we analyze its expression in IL-2−/− mice. FLIP was found to be selectively overexpressed in the LNs of IL-2−/− mice, but no overexpression was found in spleen cells or thymocytes. These results suggest that FLIP, in conjunction with other IL-2-regulated genes previously characterized in our laboratory, is involved in controlling lymphoadenopathy in IL-2−/− mice.

Flow cytometric analysis

Splenocytes and LN cells were used as single-cell suspensions. Lymphocyte subsets were characterized by staining with FITC-labeled mAb. When indicated, the activation of the different cell subsets was measured by adding PE-conjugated anti-CD69 mAb during the incubation period. Flow cytometry was performed using a FACScan flow cytometer and Lysis software (BD Biosciences, Mountain View, CA).

The following mAbs were prepared in the Pasteur Institute Department of Immunology: FITC-conjugated anti-CD3 mAb (clone C113.4), FITC-conjugated anti-CD4 mAb (clone GK1.5), FITC-conjugated anti-CD8 mAb (clone H35β), FITC-conjugated anti-β2M mAb (clone R6β2), FITC-conjugated anti-NK1.1 (clone PK136), and FITC-conjugated anti-Mac-1 mAb (clone M1/70). FITC-labeled anti-IL-2Rα mAb (clone 5A2) was characterized in the laboratory and used as previously described (12). PE-labeled anti-CD69 mAb (clone H12.2F3) was obtained from Pharmingen-Clinisciences (Montrouge, France).

Cell proliferation and cell survival assays

LN and spleen cells were cultured (10⁶ cells/well) in 96-well flat-bottom microplates in a final volume of 200 μl. Anti-CD3 mAb (clone 145-2C11; BD PharMingen, Paris, France) was used at 0.2 μg/ml. IL-2, IL-4, and IL-9 were used at the indicated concentrations. After a 48-h incubation, cultures were pulsed with [3H]Tdr and harvested 16 h later.

For cell survival assays, single-cell suspensions (10⁶/ml) were prepared from a pool of mesenteric, inguinal, and popliteal LNs, or from spleens taken from IL-2−/− and IL-2+/+ mice. Propidium iodide (150 μg/ml) was added to the cell suspension and the lymphocytes were analyzed for size and granularity (forward light scatter/side light scatter) using a FACScan flow cytometer. This easily distinguished dead from live cells (13).

Statistical analysis

The statistical analysis was performed using two-tailed Student’s t test. A p value of <0.05 was considered as significant.

Table I. Cell counts and weight of the LNs and spleens of IL-2−/− and IL-2+/+ mice

<table>
<thead>
<tr>
<th>LNs</th>
<th>No. of Cells (×10⁶)</th>
<th>Weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cervical</td>
<td>Inguinal</td>
</tr>
<tr>
<td>1st mo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−/−</td>
<td>48.2 ± 20</td>
<td>18.7 ± 8.1</td>
</tr>
<tr>
<td>+/+</td>
<td>10.3 ± 2.43</td>
<td>8.38 ± 0.75</td>
</tr>
<tr>
<td>+/−</td>
<td>3.03 ± 0.04</td>
<td>0.002</td>
</tr>
<tr>
<td>Ratio†</td>
<td>4.66</td>
<td>2.23</td>
</tr>
<tr>
<td>3rd mo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−/−</td>
<td>20.9 ± 7.66</td>
<td>26.3 ± 11.9</td>
</tr>
<tr>
<td>+/+</td>
<td>8.30 ± 1.40</td>
<td>8.65 ± 0.58</td>
</tr>
<tr>
<td>+/−</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>Ratio†</td>
<td>2.52</td>
<td>3.04</td>
</tr>
</tbody>
</table>

* Ratio of results obtained with IL-2−/− animals vs results obtained with IL-2+/+ animals.
FIGURE 1. Lymphocyte subsets and CD69 expression in the LNs and spleens of IL-2−/− and IL-2+/+ mice. A, LN cells were prepared as single-cell suspensions, stained with FITC-labeled mAb specific for each subset, and analyzed by flow cytometry. The percentage of each subset was calculated and the weight of the LNs and spleens from IL-2−/− and IL-2+/+ animals (Table I).

Comparison of the reactivity and survival capacity of lymphocytes from IL-2−/− and IL-2+/+ mice.

The capacity of the lymphocytes to proliferate in response to suboptimal anti-CD3 concentrations (0.2 μg/ml) was evaluated in the presence of a limited concentration of IL-2 (2 nM) unable to induce alone a detectable T cell proliferation. The results presented in Fig. 2A demonstrate that LN cells from IL-2−/− mice showed a greater capacity to proliferate than LN cells from IL-2+/+ animals (p < 0.002). In contrast, under the same experimental conditions, spleen cells from IL-2−/− and IL-2+/+ animals proliferated equally well.

In a subsequent series of experiments, the capacity of LN and spleen lymphocytes to proliferate in response to IL-2 alone was tested using cells from both strains (Fig. 2B). Surprisingly, LN T cells from IL-2−/− animals showed a substantial proliferative capacity in response to IL-2 in the absence of any costimulatory signal. This IL-2 proliferation was specific because IL-4 and IL-9 did not induce any [3H]TdR incorporation under the same experimental conditions (Fig. 2, C and D). As expected, IL-2Rα was detected at the cell surface of IL-2−/− lymphocytes after they had been induced to proliferate by IL-2 (Fig. 2E).

It has been demonstrated that lymphocytes from IL-2−/− mice are more resistant to Fas-mediated apoptosis than the corresponding cells from IL-2+/+ mice (6). In this study, we examined the ability of IL-2−/− lymphocytes to survive in vitro (Fig. 3). Whole LN cells from IL-2−/− or IL-2+/+ animals were followed in culture in the absence of any stimulation. The LN cells from IL-2−/− mice survived longer than those from IL-2+/+ animals (Fig. 3A). This is not the consequence of spontaneous, measurable proliferation in the LN cultures from IL-2−/− mice (Fig. 3B). In contrast, compared between IL-2−/− and IL-2+/+ mice. B, upper panels. LN lymphocytes were stained with PE-labeled anti-CD69 mAb and analyzed by flow cytometry. Lower panels, LN lymphocytes were stained with FITC-labeled anti-CD3 mAb followed by PE-labeled anti-CD69 mAb. PE-labeled isotype control was used as background. The percentage of CD69+ cells above background is indicated. C, LN cells and splenocytes were stained with FITC-labeled mAb specific for CD4, CD8, or B220, and by PE-labeled anti-CD69 mAb. The percentage of double positive cells is given.
spleen cells from IL-2−/− and IL-2+/− mice survived equally well. It should be noted that the spleen cells survived for a shorter period than the LN lymphocytes.

The results presented in Figs. 2 and 3 demonstrate that LN lymphocytes from IL-2−/− mice have a greater ability to proliferate and survive in vitro than LN lymphocytes from IL-2+/− animals. No such difference was found between spleen lymphocytes from the two mouse strains under study.

LN cells from IL-2−/− mice express more FLIP than those from IL-2+/− mice

Cell extracts prepared from IL-2−/− and IL-2+/− LN cells were subjected to Western blot analysis using anti-FLIP Ab. Fig. 4A presents the results of a typical analysis of cell extracts prepared from five IL-2−/− and seven IL-2+/− animals. The Ab recognized a 55-kDa band readily detectable in the LN cell extracts from the IL-2−/− mice. The same band was expressed at a far lower intensity in the LN cell extracts from the IL-2+/− animals. As a control, we analyzed the expression of β-actin by Western blotting. Data concerning β-actin expression confirmed the different intensities of FLIP expression in IL-2−/− and IL-2+/− animals.

As previously observed when analyzing gene expression by IL-2−/− mice, cFLIP expression was found to be heterogeneous (5). This might be due to the genetic heterogeneity of the animals used. The data were further analyzed by calculating the FLIP/β-actin ratio as indicated in Materials and Methods (Fig. 4B). The ratio in IL-2−/− was 2.5-fold that in IL-2+/− mice, and the difference was statistically significant (p < 0.01). When the same analysis was performed with cell extracts from spleens of IL-2−/− and of IL-2+/− animals, the ratios were found to be comparable (Fig. 4C).

FLIP L and FLIP S mRNA expression in the LNs and spleens of IL-2−/− and IL-2+/− mice

FLIP expression was further quantified at the mRNA level by analyzing FLIP mRNA expression using semiquantitative RT-PCR. Fig. 5A presents the data obtained with mRNA preparations from the LNs, spleens, and thymuses of IL-2−/− and IL-2+/− mice.
IL-2 REGULATION OF FLIP EXPRESSION IN VIVO

FLIP<sub>L</sub> mRNA was clearly overexpressed in the LNs of IL-2<sup>−/−</sup> when compared with the LNs of IL-2<sup>+/+</sup> mice. In contrast, the spleens and thymuses from IL-2<sup>−/−</sup> and IL-2<sup>+/+</sup> mice expressed comparable levels of FLIP<sub>L</sub> mRNA. Some variability in the FLIP<sub>L</sub> mRNA levels was observed in the different experimental groups. The results for FLIP<sub>L</sub> mRNA expression were quantified (Fig. 5B), and the ratio was calculated using β-actin mRNA as an internal marker. The results show that the LN cells from IL-2<sup>−/−</sup> animals expressed five times more FLIP<sub>L</sub> mRNA than those from IL-2<sup>+/+</sup> animals, whereas spleens from both strains expressed comparable levels. In a similar manner to spleen, the thymuses from IL-2<sup>−/−</sup> and IL-2<sup>+/+</sup> animals expressed comparable levels of FLIP<sub>L</sub> mRNA.

Additional experiments were performed to verify that the LN cells from IL-2<sup>−/−</sup> animals selectively overexpressed FLIP mRNA. The results obtained by semiquantitative analysis of RT-PCR data were verified using IL-2Rγ mRNA as internal marker because this gene, like β-actin, is constitutively expressed. Fig. 6A confirms that FLIP<sub>L</sub> mRNA is overexpressed in the LN cells from IL-2<sup>−/−</sup> animals. The results obtained with IL-2<sup>−/−</sup> and IL-2<sup>+/+</sup> mice were statistically different (p < 0.003). This analysis also confirmed that spleen cells from IL-2<sup>−/−</sup> and IL-2<sup>+/+</sup> mice express similar amounts of FLIP<sub>L</sub> mRNA. We also verified that FLIP<sub>R</sub> mRNA follows the same pattern of expression as FLIP<sub>L</sub> mRNA (Fig. 6B). Because LN cells from IL-2<sup>−/−</sup> mice are overactivated (Fig. 1), it was important to verify that FLIP mRNA overexpression was not dependent on lymphocyte activation. To do this, we compared FLIP<sub>L</sub> mRNA expression in LN cells from IL-2<sup>−/−</sup> and MRL mice which express an intact IL-2 gene but show abnormal T cell activation (17). The data shown in Fig. 6C clearly demonstrate that FLIP mRNA overexpression correlates with a lack of IL-2, not with lymphocyte activation. The results reported in Figs. 5 and 6 establish that in the absence of IL-2, FLIP mRNA is overexpressed in the LNs of IL-2<sup>−/−</sup> mice.

Discussion

Our results establish that IL-2 participates in vivo in the negative regulation of FLIP, the negative regulator of FAS- and TNF-mediated death pathways (9). The absence of IL-2 results in increased levels of FLIP, and lymphocytes from IL-2<sup>−/−</sup> mice show increased resistance to spontaneous cell death as well as increased susceptibility to anti-CD3 stimulation. However, this regulation is complex and is not observed in all lymphoid organs; FLIP is overexpressed in the LNs but not in the spleen and thymuses of IL-2<sup>−/−</sup> mice. The known cellular and biochemical properties of FLIP may explain, at least in part, some of the immunological disorders observed in IL-2-deficient animals.

The role of FLIP in the control of T cell death has already been documented. FLIP expression levels have been found to be initially up-regulated then down-regulated in primary T cells after antigenic stimulation. TCR ligation-induced down-regulation of FLIP has been correlated with sensitization of the T cells to AICD (18–22). FLIP inhibits the Fas-mediated pathway. Fas signaling is initiated by oligomerization of the receptor by Fas ligand. The cytoplasmic domain of cross-linked Fas then binds the adaptative molecule FADD. This is followed by the binding of procaspase-8 to FADD throughout the interaction of death effector domains (DEDs). Caspase is then activated leading to a cascade of catalytic caspase activation culminating in apoptosis. Owing to its structural homology with caspase-8, FLIP competitively inhibits the binding of procaspase-8. In procaspase-8, the FLIP<sub>L</sub> has two DEDs followed by a caspase-like sequence, whereas the FLIP<sub>R</sub> has only the two DEDs. Furthermore, an unanticipated role played by FLIP<sub>R</sub> has recently been described in the regulation of the transcription NF-κB and in extracellular signal-regulated kinase-mediated gene expression, and thus, in the regulation of the proliferation and/or differentiation of Fas-stimulated cells (23). Overexpression of FLIP in the LNs of IL-2<sup>−/−</sup> animals has been described in this study at both the mRNA and protein levels. Using semiquantitative RT-PCR and either β-actin or IL-2Rγ as internal controls, we demonstrated that FLIP mRNA levels in the LNs of IL-2<sup>−/−</sup> animals are 2- to 5-fold greater than those in the LNs from IL-2<sup>+/+</sup> mice. In contrast, FLIP levels are comparable in both strains for the spleen and for the thymus. This latter result is in agreement with our previous data showing that IL-2 does not affect gene expression in this primary organ (5, 16). Furthermore, we demonstrated that FLIP<sub>L</sub> and FLIP<sub>R</sub> mRNA follow the same pattern of expression. It is worth noting that FLIP overexpression is not linked to lymphocyte activation because lymphocytes from MRL mice do not overexpress the corresponding mRNA. This is a critical result because MRL and IL-2-deficient mice have a comparable phenotype, including an apoptosis defect (due to Fas ligand mutation in MRL mice) and lymphoproliferation, but the IL-2 gene is intact in MRL mice (24, 25). Therefore, these results

**FIGURE 4.** cFLIP protein expression by the LNs and spleens from IL-2<sup>−/−</sup> and IL-2<sup>+/+</sup> animals. A, Cellular extracts prepared from the LN of IL-2<sup>−/−</sup> (6) and IL-2<sup>+/+</sup> (7) mice were subjected to Western blot analysis. Anti-cFLIP Abs and anti-β-actin mAb (clone C-2) were used to reveal the corresponding proteins. Peroxidase-conjugated Abs and the ECL system were used to visualize the protein bands. cFLIP corresponds to the 55-kDa protein band. B, cFLIP expression was quantified as described in Materials and Methods. The mean of cFLIP/β-actin ratio (normalized signal) is reported for LN cell extracts from IL-2<sup>−/−</sup> (7) and IL-2<sup>+/+</sup> (7) mice. C, The ratio of the normalized signals (~Actin: +Actin) are reported for the LNs and the spleens.
clearly establish the role played by IL-2 in the in vivo regulation of FLIP.

Preferential overexpression of FLIP in the LNs of IL-2−/− mice seems to correlate with many immunological characteristics defined in the IL-2-deficient phenotype. LN size is preferentially increased in IL-2−/− mice, whereas the enlargement of the spleen is small and not statistically significant (Table I). The cellular expansion found in the LNs of IL-2−/− mice does not alter the proportion of the different subsets and all the lymphocytes are concerned by the activation process (CD69 expression). In contrast, expression of the CD69 marker by spleen lymphocytes is comparable in IL-2−/− and IL-2+/− mice. This corresponds to background lymphocyte activation we observe systematically in the mice from our animal facilities. We also noted spontaneous NF-κB activation in the LNs from IL-2−/− mice (data not shown). The Western blots we conducted were unable to detect any processed FLIP responsible for the induction of the proliferative signals (23). However, spontaneous NF-κB activation may, at least in part, explain the increased susceptibility of the T lymphocytes from IL-2−/− mice to proliferate in response to limited amounts of anti-CD3 and IL-2. In the course of this investigation, we noted that lymphocytes from IL-2−/− mice proliferated intensely in response to IL-2 alone. In the absence of IL-2, some signaling circuits may be induced and are ready to function on exposure to this cytokine.

At the cellular level, overexpression of FLIP may explain the increased ability of lymphocytes from IL-2−/− mice to survive in vitro (Fig. 3) and their resistance to Fas-induced apoptosis, as previously described (26). In vivo, this may induce resistance to AICD and lead to autoimmune reactions, which are a hallmark of the IL-2-deficient phenotype (26–28). However, differences results suggest that LN T cells from IL-2−/− mice may be controlled by normal regulatory circuits. In transfer experiments, wild-type cells of hemopoietic origin present in the same animal are able to prevent hyperactivation of LN cells from IL-2−/− mice (29).

Different mechanisms have been put forward to explain the phenotype of IL-2−/− mice. A lack of CD4+CD25+ “professional suppressor” T cells has been suggested as a possible mechanism responsible for autoimmune reactions in IL-2−/− mice (29–32). Their absence in IL-2−/− mice might also explain the uncontrolled lymphocyte activation. In contrast, our data suggest that the IL-2−/− phenotype is under multigenic control (5, 7). We have previously identified IL-2-inducible genes coding for cytoskeleton proteins (α tubulin and β catenin), oncogene-regulating proteins (CTCF, JIF-1), and transcriptional factors (E2F4, CREB, ZhX-1). Under some conditions, underexpression of β catenin may lead to faulty adhesion and release of lymphocytes from normal regulatory control by cell-cell contact. Similarly, underexpression of CTCF and JIF-1 and low levels of transcriptional factors (E2F4,
CREB, ZhX-1) may under some circumstances increase the susceptibility of lymphocytes to activation and proliferation in IL-2−/− mice. More recently, we found that TNF-α, TNF-β, and lymphotoxin-β are underexpressed in vivo in IL-2−/− mice, and under some conditions, this may also contribute to the uncontrolled proliferation of their lymphocytes. Hence, we propose that regulatory dysfunction of IL-2-inducible genes is very critical in the in vivo selective LN lymphoproliferation observed in IL-2−/− mice. This study has shed additional light on the complex network of regulatory functions directly or indirectly controlled by IL-2, and further supports the notion that the pleiotropic molecular effects of IL-2 are under multigenic control.

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