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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.

Interleukin-2 is an inflammatory and immunoregulatory pleiotropic cytokine predominantly produced by CD4+ T lymphocytes. It exerts its effects on various cell types such as T, B, NK cells, and monocytes by binding to its receptors (1). IL-2 has numerous functions including survival, activation, growth, and differentiation of immune cells (2). In addition, IL-2 negatively controls the immune response as suggested by the phenotype of IL-2-deficient mice because they show accumulation of activated lymphocytes. This may explain the lymphoadenopathy and colitis characterizing the phenotype of these animals. Thus, immune cell activation and differentiation are induced in the absence of IL-2, but the negative control of the immune response cannot be engaged in the absence of this cytokine (3). The molecular mechanisms implicated in these IL-2-dependent positive and negative regulatory circuits are still under investigation (4).

To understand the pleiotropic functions of IL-2 at the molecular level, we previously characterized IL-2-inducible genes using a cDNA subtraction approach (5). Of these genes, those coding for cytoskeleton proteins (α tubulin and β catenin), oncogene-regulating proteins (CTCF, JIF-1), and transcriptional factors (E2F4, CREB, Zix-1) were further studied in vivo using IL-2−/− animals. These genes are underexpressed in the spleens and lymph nodes (LNs) of IL-2−/− mice, suggesting that they may play a role in determining the phenotype of IL-2−/− animals by influencing gene expression, oncogene regulation, and cellular adhesion that may lead to abnormal lymphocyte activation. Alternatively, the phenotype of IL-2−/− mice may be explained by impaired Fas- or TNF-mediated cell death (6, 7).

IL-2 has been shown to play a dual role during specific T cell activation. Its presence is initially mandatory for clonal expansion and later for sensitizing T cells to activation-induced cell death (AICD; Ref. 8). A number of recent reports have indicated that FLIP (also known as FLICE/caspase-8 inhibitory protein) plays an important role in the control of AICD (9). In vitro, the down-regulation of FLIP after T cell activation is mediated by IL-2, and no such effect is observed in cells from IL-2 knockout mice (10). By competitively inhibiting the binding of procaspase-8 to the adaptative molecule Fas-associated death domain protein (FADD), cellular FLIP (cFLIP) inhibits the Fas-mediated apoptosis pathway. The two forms of cFLIP (the long form (FLIPL) and the short form (FLIPS)) are inhibitory (11).

In this study, we compared the activation status and the proliferative and survival capabilities of lymphocytes from the LNs and spleens of IL-2−/− and IL-2+/+ mice and tested the hypothesis that FLIP overexpression in vivo may contribute to the selectively altered phenotype of LN lymphocytes in IL-2−/− mice. This work further confirms the pleiotropic effects of IL-2 at the molecular level.

Materials and Methods

Mice

IL-2−/− and IL-2+/+ mice on the 129/01A × C57BL/6 background were bred under routine conditions in the animal facilities of the Pasteur Institute.
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 2C11.4, BD PharMingen, Paris, France); FITC-conjugated anti-CD4 mAb (clone GK1.5); FITC-conjugated anti-CD8 mAb (clone H35β); FITC-conjugated anti-B220 mAb (clone RA3β2B); FITC-conjugated anti-NK1.1 (clone PK136), and FITC-conjugated anti-Mac-1 mAb (clone M1/70). FITC-labeled anti-IL-2Rα mAb (clone 2C113.4), FITC-conjugated anti-NK1.1 (clone PK136), and granulometry (forward light scatter/side light scatter) using a FACScan flow cytometer. This easily distinguishes dead from live cells (13).

Mac-1 mAb (clone M1/70). FITC-labeled anti-IL-2Rα mAb (clone 2C113.4), FITC-conjugated anti-NK1.1 (clone PK136), and FITC-conjugated anti-Mac-1 mAb (clone M1/70). FITC-labeled anti-IL-2Rα mAb (clone 2C113.4), FITC-conjugated anti-NK1.1 (clone PK136), and FITC-conjugated anti-Mac-1 mAb (clone M1/70). FITC-labeled anti-IL-2Rα mAb (clone 2C113.4), FITC-conjugated anti-NK1.1 (clone PK136), and FITC-conjugated anti-Mac-1 mAb (clone M1/70). FITC-labeled anti-IL-2Rα mAb (clone 2C113.4), FITC-conjugated anti-NK1.1 (clone PK136), and FITC-conjugated anti-Mac-1 mAb (clone M1/70).

Cell proliferation and cell survival assays

LN and spleen cells were cultured (10^5 cells/well) in 96-well flat-bottom microtiter plates 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 assay, single-cell suspensions (10^7/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 distinguishes dead from live cells (13).

Western blot analysis

Lysates were prepared from the spleens and LNs of IL-2−/− and IL-2+/+ mice and analyzed as already described (7, 14). After electrophoresis on a 12% polyacrylamide gel, the proteins (100 μg/sample) were transferred to nitrocellulose membranes (Millipore Corporative, Paris, France). The immunoblots were incubated with rabbit anti-cFLIP polyclonal Abs (Alexis, Santa Cruz Biotechnology, Santa Cruz, CA) and peroxidase-conjugated anti-mouse Abs (1/10,000; Amersham, Les Ulis, France). Bands corresponding to the amount of cFLIP or β-actin were measured using NIH Image software (National Institutes of Health, Bethesda, MD). The cFLIP signal was normalized to that of β-actin and the cFLIP/β-actin ratio calculated.

Semiquantitative RT-PCR

This technique has already been used in the laboratory in the past (15, 16). Total RNA was extracted from the spleens and LNs of IL-2−/−, IL-2+/+, and MRL/lpr mice using RNA PLUS reagent (Quantum Bioprobe, Montreuil, France). The first-strand CDNA was synthesized from 1 μg of mRNA using oligo(dT)12-18 (25 ng/μl) by incubating the mixture at 70°C for 10 min followed by quick chilling on ice. The following were then added according to the manufacturer’s instructions: 5 × first-strand buffer, dNTP mix, avian myeloblastosis virus reverse transcriptase (Promega, Lyon, France), and RNasin (2 U/μl; Promega) to a final volume of 20 μl. The mixture was sequentially incubated at 42°C for 60 min, 95°C for 5 min, and cooled to 4°C. cDNA mixture (2 μl) was used for PCR amplifications using gene-specific primers. The reactions were performed using a Thermal Cycler (PerkinElmer, Wellesley, MA).

The PCR products were size fractionated on 1.5% agarose gel, transferred onto Hybond-N membranes (Amersham), and hybridized with specific probes. For semiquantitative analyses, gels were exposed on Kodak storage phosphor screens (Kodak, Rochester, NY), and the radioactive signal was measured using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). β-Actin and IL-2Ry, which are constitutively expressed in all lymphomononuclear cells, were used as internal controls. A semiquantitative analysis of cFLIP mRNA expression was obtained by dividing the radioactive signals for cFLIP mRNA (band intensity after exposure of the x-ray film) by the radioactive signals for β-actin mRNA or IL-2Ry mRNA and expressing the result as a ratio, i.e., FLIP/β-actin or FLIP/IL-2Ry.

The sequence of the primers and probes used are as follows. FLIPp, sense, 5'-TATGCAAGATGCGCCAAAA-3'; antisense, 5'-CAGGGTTG AGGCCTATTCC-3'; and probe, 5'-GACTCTAAGGCCCTGCACAC-3'. FLIPs, sense, 5'-TGGATCAGCTACTGAGCAGA-3' and antisense, 5'-TGTTGAATTTTATTACCATCTGAGACTGT3'-; and probe, 5'-TGGAGAGAGGCCAGCTCTCTTTTGCT-3'. IL-Ry, sense, 5'- TCCAGCTTGCATCTGGTCCG-3'; antisense, 5'-CAAGGTCTCATGTCGAGGCACGCAGAAG-3'; and probe, specific cDNA fragment, β-Actin; sense, 5'-TGGAAATCTGTGACAGCAAGAAGC-3'; and antisense, 5'-TAAACCGAGCTCAGTAAAGTCCG-3'; and probe, specific cDNA fragment.

Statistics

Mean ± SD are reported. Student’s t test was used to evaluate the significance of the results.

Results

Characterization of the lymphoproliferation and activation status of lymphocytes from IL-2−/− mice

The enlargement of the LNs in IL-2−/− animals was analyzed over time (Table I). By measuring the total number of cells, it was found that cervical LNs were more enlarged at 1 mo than 3 mo old (Table I). By measuring the total number of cells, it was found that cervical LNs were more enlarged at 1 mo than 3 mo old (Table I). By measuring the total number of cells, it was found that cervical LNs were more enlarged at 1 mo than 3 mo old (Table I). By measuring the total number of cells, it was found that cervical LNs were more enlarged at 1 mo than 3 mo old (Table I).

The ratio was always <2 and the difference was not statistically significant (p > 0.05). These results were verified by measuring
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 have been induced to proliferate by IL-2 (Fig. 2E).

Il-2 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,
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+/− mice. 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 < 0.014). 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).

**FLIPL and FLIPS 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.
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>S</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 over-activated (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 over-expressed 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>S</sub> has only the two DEDs. Furthermore, an unanticipated role played by FLIP<sub>L</sub> has recently been described in the regulation of the transcription NF-kB 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>S</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
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, different 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, 3555...
CREB, ZbX-1) may under some circumstances increase the susceptibility of lymphocytes to activation and proliferation in IL-2−/− mice. More recently, we found that TNF-β, TNF-α, 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 multiple IL-2-regulated genes may be involved directly or indirectly in the lymphoproliferation seen in IL-2−/− mice.

The respective roles played by FLIP and other genes regulated by IL-2 deserve further discussion. Because all of the IL-2-induced genes described previously are equally underexpressed in the LNs and spleens of IL-2−/− animals, this suggests that FLIP overexpression is very critical in the in vivo selective LN lymphoproliferation described in this study. Underexpression of the genes previously described by our laboratory would be necessary but not sufficient to observe the lymphoproliferation. By contrast, FLIP overexpression would be required to reduce susceptibility to apoptosis and induce the activation and lymphoproliferation observed in the LNs of IL-2−/− mice. In this context, it is also interesting to compare the characteristics of IL-2-deficient mice and recently generated FLIP transgenic animals. In these mice, FLIP has been placed under the CD2 promoter. Peripheral T cells in these FLIP transgenic animals are like IL-2−/− T lymphocytes protected against Fas-induced apoptosis in vitro. However, unlike IL-2-deficient mice, FLIP transgenic animals do not show an accumulation of activated T cells in vivo. This strongly suggests that constitutive expression of FLIP in T cells is not always sufficient to elicit alone the lymphoproliferation observed in IL-2−/− mice. Our observation that lymphocyte expansion in IL-2−/− mice involves all the lymphocyte subsets suggests that FLIP must be overexpressed in more than one cell type to explain the IL-2−/− phenotype. However, a direct role of FLIP overexpression in vivo has been suggested by demonstrating autoimmunity as a consequence of retrovirus-mediated expression of FLIP in T lymphocytes.

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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