The Journal of Immunology

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J Immunol 2001; 166:3757-3762; ;
doi: 10.4049/jimmunol.166.6.3757
http://www.jimmunol.org/content/166/6/3757

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Lymphocytes from Autoimmune MRL *lpr/lpr* Mice Are Hyperresponsive to IL-18 and Overexpress the IL-18 Receptor Accessory Chain[1]

Detlef Neumann,[‡] Elda Del Giudice,[*] Antonio Ciaramella,[*] Diana Boraschi,[†] and Paola Bossù[2*]

MRL *lpr/lpr* mice spontaneously develop a severe autoimmune lupus syndrome characterized by strong autoantibody production and massive lymphoproliferation, in which IFN-γ plays a major pathogenic effect. The role of the IFN-γ-inducing cytokine IL-18 in the autoimmune syndrome of *lpr/lpr* mice has been investigated. In response to IL-18, lymph node cells of *lpr/lpr* mice produce significant amounts of IFN-γ and proliferate more potently as compared with cells from +/+ mice. Cells likely responsible for such hyperresponsiveness to IL-18 include NK cells and the CD4$^+$/CD8$^+$ self-reactive T lymphocytes characteristically present in lymph nodes of *lpr/lpr* mice. Analysis of the expression of IL-18R complex revealed that mRNA for the IL-18Rα-chain is constitutively expressed at similar level both in +/+ and *lpr/lpr* lymphocytes. In contrast, the expression of the accessory receptor chain IL-18Rβ is low in unstimulated +/+ cells but significantly high in *lpr/lpr* cells. Thus, the abnormally high expression of the IL-18R chain IL-18Rβ could be one of the causes of the hyperresponsiveness of *lpr/lpr* cells to IL-18 at the basis of consequent enhancement of IFN-γ production and development of IFN-γ-dependent autoimmune pathology. *The Journal of Immunology,* **2001, 166: 3757–3762.**

The MRL *lpr/lpr* mouse spontaneously develops a severe autoimmune syndrome closely resembling human systemic lupus erythematosus, characterized by progressive lymphadenopathy, hypergammaglobulinemia, autoantibody production, and immunocomplex formation eventually leading to endorgan disease such as vasculitis, arthritis, and fatal renal failure (1, 2). The *lpr* mutation of the *fas* gene impairs Fas molecule functions (3), thus mutant mice show a defect in lymphocyte apoptosis responsible for impaired deletion of autoreactive T lymphocytes (4–6). Defective Fas is also at the basis of lymphadenopathy, which is mainly attributable to the accumulation of a peculiar subset of T cells, defined as CD3$^+$B220$^+$CD4$^-$CD8$^-$ double-negative (DN)[3] cells, likely deriving from self-reactive T lymphocytes (7, 8). Because of the deficiency of Fas-dependent apoptosis, autoreactive B cells also persist in *lpr/lpr* mice and are responsible for autoantibody-dependent pathological features (9) as well as for the activation of autoreactive T cells (10, 11). In consideration of the key role played by autoreactive T lymphocyte activation in this lupus model, several studies have been addressed to clarify the involvement of T cell-related cytokines in the pathology. IL-12, IL-4, and IFN-γ have been found to be involved in the pathogenesis of the *lpr* syndrome (12–17). Although deletion of either gene for IFN-γ or IL-4 in *lpr/lpr* mice results in the reduction of lymphadenopathy, endorgan disease, and early mortality (14), the important role of Th1-type cytokines in this pathology is indicated by the observation that the ratio of IFN-γ- to IL-4-secreting cells increases with disease progression (15), that DN T cells and autoantibodies are absent only in IFN-γ-deficient mice (14), and that in *lpr/lpr* mice lacking the IFN-γR the kidneys are significantly protected from glomerulonephritis damage (16).

IL-18, originally named IFN-γ-inducing factor (IGIF), is a cytokine capable of inducing IFN-γ production in primed T cells (18) and augmenting the NK activity and proliferation of spleen cells (18, 19). IL-18 does not induce development of Th1 cells or expression of IFN-γ by itself, but it synergizes with IL-12 (20–22), which induces IL-18R expression (23–25). Structurally, IL-18 is closely related to the IL-1 family (26) and shares with IL-1β the maturation mechanism through caspase-1 (IL-1β-converting enzyme) (27). IL-18 binding to its target cells is mediated by specific plasma membrane receptors, which strictly resemble the IL-1R complex. The previously orphan receptor IL-1R-related protein was identified as a low-affinity receptor for IL-18 (28) and renamed IL-18Rα (29). Recently, a second receptor subunit, the accessory protein-like molecule (AcPL, or IL-18Rβ), has been cloned (30). Like the IL-1R accessory protein, IL-18Rβ does not bind IL-18 directly but forms the active signaling receptor complex with IL-18Rα bound to IL-18.

The investigation on the role of IL-18 in autoimmune pathologies has just begun. In the development of autoimmune Th1-dependent insulitis in nonobese diabetic mice an association between the active stage of the disease and the expression of IL-18 was found (31, 32). Neutralizing Abs to IL-18 prevent the development of experimental autoimmune encephalomyelitis (33). More recently, enhanced expression of IL-18 has been observed in the gut mucosal tissues of Crohn's disease patients (34, 35) and in synovial tissues of rheumatoid arthritis patients (36).

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Received for publication March 6, 2000. Accepted for publication January 2, 2001.

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[1] This work has been conducted under a research contract with Consorzio Autoimmunità Tardiva, Pomezia, Italy, within the Programma Nazionale Farmaci seconda fase of the Ministero dell'Università e della Ricerca Scientifica e Tecnologica. D.N. was supported in part by a grant of the Hannover Medical School (HILF).

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[3] Abbreviations used in this paper: DN, CD4$^-$ CD8$^-$ double negative; IGIF, IFN-γ-inducing factor; AcPL, accessory protein-like; HPRT, hypoxanthine phosphoribosyltransferase; LN, lymph node.

0022-1767/01/$02.00

In this study, the involvement of IL-18 in the development of autoimmune murine lupus has been investigated. Lymph node (LN) cells from MRL *lpr/lpr* mice have been found to hyperreact to stimulation with IL-18, as compared with control +/+ mice, both in terms of IFN-γ production and cell proliferation. This hyperreactivity could be ascribed to the constitutively up-regulated expression of the accessory receptor protein IL-18Rβ in *lpr*/*lpr* cells. It is proposed that both NK/NKT cells and the autoreactive T lymphocytes characteristic of the *lpr*/*lpr* syndrome are the cells responsible for hyperresponsiveness to IL-18. Thus, these data suggest that constitutive hyperexpression of the signaling chain IL-18Rβ in LN cells from *lpr*/*lpr* mice could be one of the events at the basis of the IFN-γ-dependent autoimmune pathology in murine lupus.

Materials and Methods

Mice and cell preparation

MRL +/+ and MRL *lpr/lpr* mice obtained from The Jackson Laboratory (Bar Harbor, ME) were housed and bred under specific pathogen-free conditions in the animal facility at Dompé Research Center. Control C57BL/6 mice were obtained from Harlan-Nossan (Correzzana, Italy). Age- and sex-matched animals were euthanized and LN or spleen dissected. Single-cell suspensions were prepared by teasing the organs in complete medium (RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 50 μM 2-ME, and 50 μg/ml gentamicin sulfate; Life Technologies, Paisley, U.K.) and routinely analyzed by cytofluorometry (FACScan; Becton Dickinson, Mountain View, CA) with mAbs specific for CD4, CD8, CD3, CD19, CD11c, or B220 (BD PharMingen, San Diego, CA). For experimental procedures, pooled cells derived from axillary and inguinal LN of three or more mice were cultured for the indicated times in complete medium alone or containing murine IL-18 and/or murine IL-12 (PeproTech, Rocky Hill, NJ). In some experiments, spleen or LN $CD4^+/CD8^+$ and $CD4^-/CD8^-$ cell subpopulations were isolated by immunomagnetic separation with anti-CD4 (L3T4; clone GK-1.5) and anti-CD8 (Ly2; clone 53-6.7) (both kind gifts of Matthias Hoffman, Medical School Hannover, Hannover, Germany) with a MiniMACS system (Miltenyi Biotec, Auburn, CA). In some instances, a depletion of NK/NKT cells was performed with anti-NK cell microbeads (Miltenyi Biotec), with the rat IgM anti-mouse pan-NK cells mAb DX5. Distribution of reactivity to DX5 is overlapping with that of NK-1.1, as determined with the PK136 Ab (BD PharMingen). After separation, cells were checked cytofluorometrically for CD3, CD4, CD8, B220, and pan-NK (determined with DX5). The unfractionated population in the LN of +/+ mice was: 83% $CD3^+$, 57% $CD4^+$, 30% $CD8^+$, 38% $B220^+$; in the LN of *lpr/lpr* mice was: 71% $CD3^+$, 37% $CD4^+$, 25% $CD8^+$, 20% $B220^+$, 5% NK^+; in the spleen of +/+ mice was: 31% $CD3^+$, 19% $CD4^+$, 14% $CD8^+$, 37% $B220^+$; and in the spleen of *lpr/lpr* mice was: 26% $CD3^+$, 13% $CD4^+$, 12% $CD8^+$, 38% $B220^+$, 7% NK^+.

In the $CD4^+/CD8^+$-depleted population, the contaminant $CD3^+$ cells were ~2% in +/+ and 1% in *lpr*/*lpr* LN and 0% in the spleen of both strains.

In the $CD4^+/CD8^+$-enriched population, total $CD3^+$ were 92% in +/+ and 86% in *lpr/lpr* LN and 74% in +/+ and 78% in *lpr/lpr* spleen.

In the NK^- population, the depletion was complete (0% NK^+ cells) in both strain organs.

NK-enriched populations (>45% DX5-positive cells) were also prepared, but it was not possible to obtain cell numbers sufficient for functional studies.

Generation of autoreactive and alloreactive T cell lines

Autoreactive and alloreactive T cell lines were generated from LN cells from MRL +/+ and *lpr/lpr* mice on repeated stimulation with syngeneic or allogeneic cells after a modification of the original procedure (4, 37). Briefly, autoreactive cells were obtained by culturing *lpr/lpr* LN cells at high density (6×10^6 cells/well of Cluster[24] plates; Costar, Cambridge, MA). After 7–10 days cells were washed and stimulated in culture for 5–7 days with mitomycin C-treated syngeneic (from MRL *lpr/lpr* mice) spleen cells in the presence of murine IL-2 (10 U/ml; PeproTech). Stimulated cells were recovered on separation from dead cells on a Lympholyte-M gradient (Cedarlane, Hornby, Ontario, Canada) and restimulated in culture following the same procedure. Similarly, alloreactive cells were obtained by culturing +/+ or *lpr/lpr* LN cells with mitomycin C-treated allogeneic (from BALB/c mice) spleen cells in the presence of IL-2 for 5–7 days and restimulated with allogeneic spleen cells following the same procedure described for autoreactive cells. After at least five cycles of stimulation, proliferating cells were characterized cytofluorometrically for marker expression and, on stimulation with PMA/ionomycin, for cytokine expression. Autoreactive and alloreactive Th1 cell lines with stable and comparable phenotype ($CD3^+$, $CD4^+$, $CD8^-$, $B220^-$ and IFN-γ^+, IL-4^-) were selected and analyzed for proliferation and production of IFN-γ in response to stimulation with IL-12 and IL-18.

IFN-γ production

Unfractionated, NK-depleted, and CD4/CD8-enriched or -depleted LN or spleen cells (2.5×10^5 cells/well) or cells from autoreactive and alloreactive lines (1×10^5 cells/well) were incubated in 0.2 ml of culture medium containing the appropriate stimuli in 96-well microculture plates (Cluster[96]; Costar). After 48 h of incubation, cell-free supernatants were harvested and the IFN-γ concentration was determined using a specific ELISA (Endogen, Woburn, MA).

Cell proliferation

Unfractionated LN cells (2.5×10^5 cells/well) or cells from autoreactive and alloreactive lines (1×10^5 cells/well) were incubated in 0.2 ml of culture medium containing the appropriate stimuli in 96-well microculture plates (Cluster[96]; Costar). Microcultures were incubated in moist air at 37°C for 48 h and exposed for an additional 6 h to 0.5 μCi/well tritiated thymidine (sp. act. 185 GBq/mmol; Amersham, Buckinghamshire, U.K.). Incorporated radioactivity was then assessed and results were expressed either as mean cpm ± SEM of triplicate cultures or as fold-increase vs control cells.

RT-PCR

Total RNA was prepared from up to 1.0×10^7 cells by using the RNeasy Total RNA Isolation Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed into cDNA in a total volume of 50 μl by using pdN6 primers (Boehringer Mannheim, Mannheim, Germany) and Moloney murine leukemia virus reverse transcriptase (Stratagene, La Jolla, CA). PCR amplification was conducted in a total volume of 25 μl 1× PCR buffer (Stratagene) containing 2.5 μl of the first-strand cDNA, 50 μM of each dNTP (Stratagene), 1 μM of each primer (Endogen, Milano, Italy), and 1 U *Taq* DNA polymerase (Stratagene). The oligonucleotides used were: hypoxanthine phosphoribosyltransferase (HPRT) 5′ (5′-GTTGGATACAGGCCAGACTTTGTT-3′), HPRT 3′ (5′-GATTCAACTTGCGCTCATCTTAGGC-3′), IL-18Rα 5′ (5′-GTGCACAGGAATGAAACAGC-3′), IL-18Rα 3′ (5′-ATTTAAGGTCCAATTGCGACGA-3′), IL-18Rβ 5′ (5′-GGAGTGGGAAATGTCAGTAT-3′), and IL-18Rβ 3′ (5′-CCGTGCCGAGAAGGATGTAT-3′). Cycle parameters were annealing 0.5 min at 55°C, elongation 1 min at 72°C, and denaturation 0.5 min at 95°C. Resulting PCR products were separated in a 2% agarose gel and visualized by ethidium bromide staining. For semiquantitative PCR, sequences of the housekeeping gene HPRT, IL-18Rα, and IL-18Rβ were amplified out of each cDNA batch with 27, 28, 29, and 30 amplification cycles. After gel analysis, bands were scanned (Personal Densitometer with ImageQuant software; Molecular Dynamics, Sunnyvale, CA) and their densities were assessed. For each number of cycles, the ratio between densities of receptor bands and of the corresponding HPRT band was calculated and expressed as relative units.

Results

LN cells from MRL lpr/lpr *mice overreact to IL-18*

The ability of IL-18 to induce IFN-γ production and proliferation in LN cells from young MRL *lpr/lpr* mice was assessed in comparison to MRL +/+ mice and to control unrelated C57BL/6 mice. As shown in Fig. 1 (*left*), IL-18 could directly induce significant IFN-γ production only in *lpr/lpr* LN cells, whereas it had no effect on LN cells from C57BL/6 or +/+ mice, which could only be activated in the presence of a costimulus like IL-12 (Fig. 1, *right*). Also, in the case of synergistic stimulation of LN cells with IL-18 and IL-12, cells from *lpr/lpr* mice were more sensitive than control cells to activation for IFN-γ production (Fig. 1, *right*). Similarly, in the absence of costimulation, IL-18 could induce significant proliferation of *lpr/lpr* LN cells (Fig. 2, *left*; data not shown). In synergism with IL-12 (which had no significant effect by itself), LN cells from *lpr/lpr* mice underwent potent IL-18-induced proliferation, much higher than that of the other strains (Fig. 2).

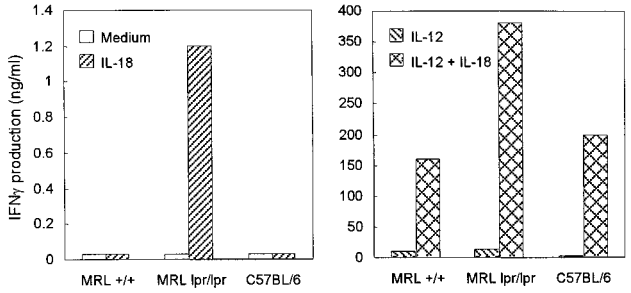

FIGURE 1. Enhanced production of IFN-γ by *lpr/lpr* LN cells in response to IL-18. *Left*, LN cells from MRL +/+, *lpr/lpr*, and C57BL/6 mice were cultured for 48 h in the absence or presence of 100 ng/ml murine IL-18. IFN-γ production in the culture supernatant was measured with a specific ELISA able to detect cytokine concentrations >0.03 ng/ml. *Right*, IFN-γ production by LN cells was analyzed after 48 h of stimulation with murine IL-12 (10 ng/ml) in the absence or presence of 100 ng/ml murine IL-18. Results are the mean from duplicate determinations within single representative experiments with errors <10% (not shown). Similar results were obtained in three different experiments.

In the attempt to define the cell population responsible for IL-18 hyperresponsiveness within *lpr/lpr* LN, LN subpopulations enriched or depleted in $CD4^+$ and $CD8^+$ cells were assayed for their ability to produce IFN-γ in response to cytokine stimulation. After the experimental procedure for enrichment/depletion, IFN-γ production in unstimulated cells or in cells exposed to either IL-12 or IL-18 alone was usually very low and often below detection limits (data not shown). Significant and reproducible levels of IFN-γ could be detected in response to IL-18 in synergism with IL-12. As shown in Fig. 3 (*top*), in LN from control +/+ mice IFN-γ production in response to the combination of IL-18 and IL-12 is to be attributed both to the subpopulation depleted of $CD4^+/CD8^+$ cells (NK-, APC-, and B-enriched cells) and to the population enriched in $CD4^+/CD8^+$ (85–90% T cells) and is also significant in the NK-depleted population (NK-negative T and non-T cells). This would suggest a major role for NK/NKT cells and possibly for B cells, but also the contribution of T cells in the responsiveness to IL-18. As already observed, IFN-γ production in response to IL-18 (in combination with IL-12) was much higher in *lpr/lpr* LN as compared with +/+ cells. Also in this case, most of the activity could be attributed to CD4/CD8-depleted cells (enriched in NK, APC and B cells, and also containing DN lymphocytes), but highly

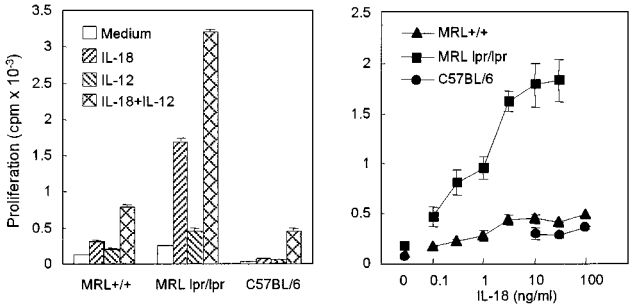

FIGURE 2. Enhanced proliferation of *lpr/lpr* LN cells in response to IL-18. *Left*, Proliferative response of LN cells MRL +/+, *lpr/lpr*, and C57BL/6 mice after 48 h of stimulation with medium alone or containing IL-18 (100 ng/ml), IL-12 (10 ng/ml), or the combination of the two cytokines. *Right*, Dose-dependent effect of IL-18 in inducing LN cell proliferation in synergy with IL-12 (10 ng/ml). Results are the mean ± SEM of triplicate determinations within single representative experiments. Similar results were obtained in four different experiments.

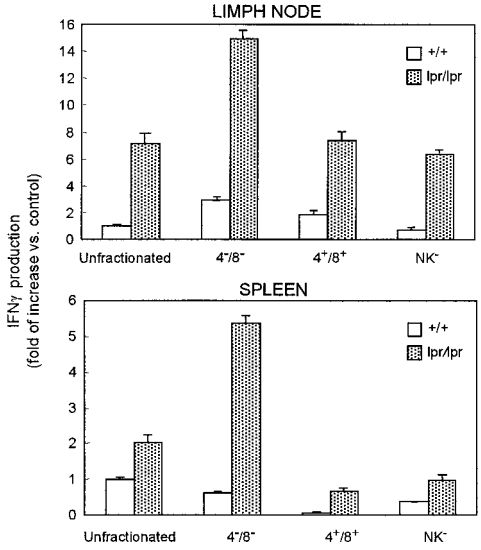

FIGURE 3. IFN-γ production by LN and spleen subpopulations. LN (*top*) and spleen cells (*bottom*) from MRL +/+ (□) and *lpr/lpr* (▨) were used unfractionated or were fractionated into three different subpopulations: $CD4^-/CD8^-$ (T-depleted cells); $CD4^+/CD8^+$(T-enriched cells); and NK^- (NK-depleted cells). IFN-γ production was evaluated in response to 48 h of stimulation with IL-18 (100 ng/ml) and IL-12 (10 ng/ml) and expressed as fold-increase vs control (IFN-γ production of unfractionated +/+ cells = 1). IFN-γ production by unfractionated +/+ spleen cells in response to IL-18 plus IL-12 was 23.3 ± 0.8 times higher than that of LN cells (mean ± SD of two separate experiments). Data are representative of five experiments performed and are reported as mean ± SD of triplicate cultures in a single experiment or of duplicate/triplicate experiments.

significant responsiveness to IL-18 could also be found in CD4/CD8-enriched cells and in NK-depleted cells. This observation was confirmed by data obtained by stimulating *lpr/lpr* LN subpopulations with IL-18 alone (without IL-12). Although levels of IFN-γ produced were very low, it was possible to observe that the majority of IL-18-responsive cells were $CD4^-/CD8^-$ (777.7 ± 31.3 pg IFN-γ/10^7 cells) but that a significant response could be attributed to $CD4^+/CD8^+$ cells (86.4 ± 4.5 pg IFN-γ/10^7 cells) and in particular to NK-depleted $CD4^+/CD8^+$ cells (64.3 ± 8.6 pg IFN-γ/10^7 cells). In contrast, it was not possible to detect IFN-γ production in +/+ LN subpopulation stimulated with IL-18 alone (data not shown).

Control experiments were performed by isolating lymphocyte subpopulations in the spleen of either +/+ or *lpr/lpr* mice (Fig. 3, *bottom*). Although the responsiveness to IL-18 (in combination with IL-12) was more abundant than in LN cells, the pattern of responsiveness was similar to that observed in LN cells, i.e., the majority of IL-18-responsive cells were in the CD4/CD8-depleted population, but a significant responsiveness could be observed also in CD4/CD8-enriched and/or NK-depleted cells, in particular in *lpr/lpr* mice. Thus, both NK and T cells are apparently responsible for enhanced responsiveness to IL-18 in *lpr/lpr* mice. To establish whether autoreactive T cells in the LN may take part into this hyperresponsiveness to IL-18, LN cells from *lpr/lpr* and +/+ mice were cultured to generate autoreactive (from *lpr/lpr*) and alloreactive (from both strains) T cell lines. As shown in Fig. 4, autoreactive $CD4^+$ T cell lines generated from *lpr/lpr* LN were hyperresponsive to IL-18 both in terms of IFN-γ production (Fig. 4, *left*) and proliferation (Fig. 4, *right*), as compared with alloreactive lines generated from either +/+ or *lpr/lpr* LN.

Thus, it can be suggested that, whereas $CD4^-/CD8^-$ cells (possibly NK cells) are the main cell population responsible of IFN-γ

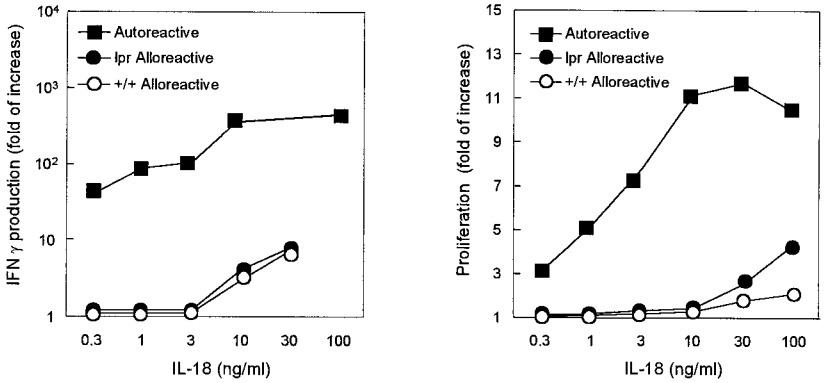

FIGURE 4. Autoreactive T cells from *lpr/lpr* LN are hyperresponsive to IL-18. *Left*, IFN-γ production by autoreactive and alloreactive T cells derived from MRL +/+ and *lpr/lpr* LN in response to IL-18. *Right*, Proliferation of autoreactive and alloreactive T cells derived from MRL *lpr/lpr* and +/+ LN in response to IL-18. Results, expressed as fold-increase vs control (unstimulated cells = 1), are the mean of duplicate or triplicate determinations within single representative experiments with errors <10% (not shown). Similar results were obtained in four different experiments.

production in response to IL-18, a significant fraction of responsive cells is $CD4^+/CD8^+$ (mostly T cells). In *lpr/lpr* LN, this $CD4^+/CD8^+$ population is at least in part represented by autoreactive T cells and could be responsible for the subsequent lymphoproliferative syndrome characteristic of *lpr/lpr* LN.

LN cells from MRL lpr/lpr *mice constitutively express mRNA for IL-18Rβ*

To assess the role of the IL-18R complex in the hyperresponsiveness to IL-18 of *lpr/lpr* LN cells, we analyzed the expression of mRNA for the two chains forming the active IL-18R complex, i.e., the IL-18 binding chain IL-18Rα and the IL-18R accessory protein IL-18Rβ.

IL-18Rα mRNA expression was clearly detectable in unstimulated +/+ and *lpr/lpr* LN cells, as well as in LN from the unrelated strain C57BL/6 (Fig. 5, *left*). The IL-18Rα expression was not further increased by treatment with IL-12.

Analysis of the IL-18R accessory chain revealed that in +/+ LN cells mRNA expression for IL-18Rβ was rather low in the absence of stimulation, but that it could be significantly up-regulated by IL-12 (Fig. 5, *right*). At variance with +/+ cells, *lpr/lpr* LN cells constitutively expressed high levels of IL-18Rβ mRNA, comparable to those observed in +/+ cells triggered by IL-12, which could not be further increased by treatment with IL-12 (Fig. 5, *right*).

Expression of IL-18R chains was evaluated by semiquantitative RT-PCR on Th1 autoreactive and alloreactive cell lines. Because of the state of activation of the cultured cells, all cell lines were found positive for expression of both receptor chains. However, it was possible to observe a general and consistent tendency of autoreactive *lpr/lpr* cells to express higher levels of IL-18Rβ, in particular when compared with +/+ alloreactive cells (data not shown).

Discussion

In the development of the progressive lupus-like pathology in the mouse strain MRL *lpr/lpr*, Th1 cytokines, and in particular IFN-γ, apparently play an important role. In fact, deletion of the IFN-γ gene in *lpr/lpr* mice leads to disappearance of DN T cells, autoantibodies, and early mortality (17). Also, the predominance of IgG2a and IgG3 vs IgG1 autoantibody isotypes in sera, accompanied by IFN-γ hyperproduction in LN cells and splenocytes, indicates an active role for Th1 cells (13–15). The cytokine IL-18 (IGIF, IL-1γ) is one of the main stimulators of IFN-γ production in Th1 cells and could also induce Th1 cell proliferation in synergism with IL-12 (18, 20–25). Thus, it could be hypothesized that alterations in IL-18 production or responsiveness could be at the basis of the pathologically high levels of IFN-γ in *lpr/lpr* mice.

To investigate this issue, the ability of IL-18 to activate LN cells from MRL *lpr/lpr* mice was assessed. Young *lpr/lpr* mice (4–7 wk of age) were chosen for this study, as at this age the autoimmune-related alterations have not yet occurred. This allowed us to analyze the pathogenic events preceding the outcome of the disease. Experimental data indicate that LN cells of *lpr/lpr* mice are more sensitive to IL-18 stimulation both in terms of proliferation and IFN-γ production as compared with lymphocytes from age-matched control mice.

To explain the basis for this hyperreactivity, expression of the two chains of the IL-18R has been assessed. Analysis of the IL-18 binding chain expression revealed that IL-18Rα was constitutively detectable in LN cells from all strains. Addition of IL-12 could not

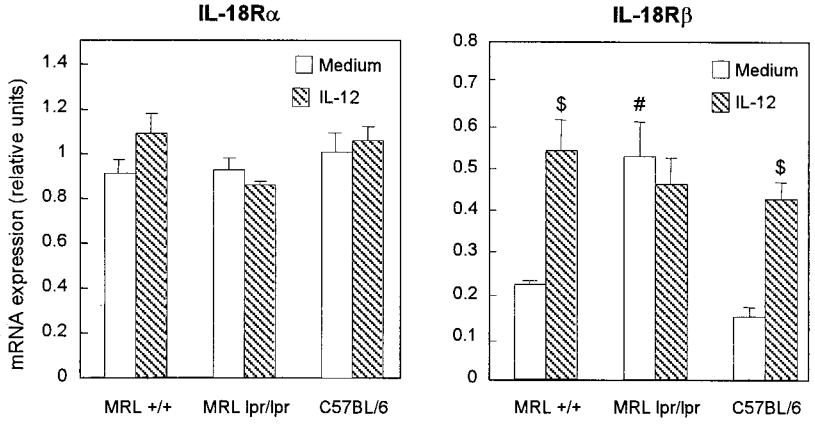

FIGURE 5. LN cells of *lpr/lpr* mice constitutively express IL-18Rβ. Semiquantitative RT-PCR analysis of IL-18R chain expression in LN cells from MRL +/+, *lpr/lpr*, and C57BL/6 mice exposed for 24 h to medium alone or containing 10 ng/ml IL-12. Expression of mRNA levels for the IL-18R chains IL-18Rα (*left*) and IL-18Rβ (*right*) is calculated as relative units vs expression of the housekeeping gene HPRT and reported as the mean ± SEM of data obtained from LN preparations of 3–11 mice. #, $p < 0.001$ vs unstimulated MRL +/+ and C57BL/6 cells. $, $p < 0.05$ vs medium control.

further increase IL-18Rα expression. Several reports have addressed the issue of regulation of the IL-18R expression by IL-12 (23–25, 38, 39). In some of these instances, the IL-12-driven increase of IL-18R expression was assessed in terms of IL-18 binding on the cell surface (23–25), thus without distinguishing between the relative expression of the two chains (IL-18Rα and IL-18Rβ) forming the IL-18R complex. Other results have shown that IL-12, together with Ag and APC, can drive Th1 polarization of splenic naive T or $CD4^+$ cells, thus also expression of IL-18Rα mRNA, whereas it cannot further increase IL-18Rα expression on polarized Th1 cells (38). However, in another report, IL-12 failed to up-regulate IL-18Rα expression during Th1 polarization of spleen naive T cells together with anti-CD3 unless IL-2 was present (25). Eventually, expression of IL-18Rα on human PBMC, detected with a specific Ab, could be up-regulated by IL-12 only on NK cells, not on $CD4^+$ or $CD8^+$ cells, in the absence of costimulation (39). These data may be only apparently contrasting, as it should be taken into account that different cell populations in different stages of polarization/activation were considered. It must be remembered that functional IL-18R are a complex of the two chains IL-18Rα and IL-18Rβ and that conceivably the rate-limiting chain for the complex formation is the accessory chain IL-18Rβ rather than the binding chain IL-18Rα, as in the case of the IL-1R complex. Indeed, a single class of low-affinity IL-18 binding sites (K_D 10^{-8}–10^{-7} M) can be measured on leukemia cells, on unstimulated Th1 cells, and on cells transfected with the cDNA coding for IL-18Rα (28, 40). In contrast, a second high-affinity class of IL-18 binding sites (K_D 10^{-10}–10^{-9} M) can be detected in murine T or B cells stimulated with IL-12 and in in vitro-polarized Th1 cells stimulated with anti-CD3 and IL-12 (25, 41). Thus, it is possible to speculate that in these circumstances IL-12 may induce expression of the accessory chain IL-18Rβ, which together with the low-affinity IL-18Rα may form high-affinity IL-18R complexes. It can be concluded that the effect of IL-12 in the up-regulation of IL-18R might be directed both at the IL-18Rα and at the IL-18Rβ-chains, depending on the cell type and state of activation/differentiation.

In the present study, it is shown that the accessory chain IL-18Rβ is constitutively expressed in *lpr/lpr* LN cells. In control cells, from either MRL +/+ mice or C57BL/6 mice, IL-18Rβ expression is low, but it can be up-regulated by IL-12. The need for IL-12 in the up-regulation of IL-18Rβ expression is in line with previous data showing that IL-18 always needs synergism with IL-12 to be active (20, 21). The high constitutive expression of IL-18Rβ in *lpr/lpr* LN cells could be attributable, at least in part, to sustained endogenous stimulation by IL-12, which in fact is overproduced in *lpr/lpr* mice (12).

Thus, the constitutive expression of IL-18Rβ might be among the determinants of the hyperresponsiveness to IL-18 of LN cells from *lpr/lpr* mice. However, hyperresponsiveness to IL-18 apparently requires other factors beyond IL-18Rβ up-regulation. In fact, IFN-γ production by *lpr/lpr* LN cells in response to IL-18 in the absence of IL-12 is much lower than that of either +/+ or *lpr/lpr* cells in the presence of IL-12, despite comparable IL-18Rβ expression. A deeper quantitative and kinetical analysis of functional synergism between IL-18 and IL-12 will be required to clarify the relative role of the two cytokines in the *lpr/lpr* hyperreactivity and to identify the possible involvement of other still unidentified factors. As in the case of the homologous cytokine IL-1, it is possible that other regulatory molecules belonging to the IL-18 system itself could concur to the fine modulation of the IL-18 response. Indeed, as in the case of the decoy soluble $IL\text{-}1R_{II}$, a soluble IL-18 binding protein has been recently identified that can bind IL-18 with high affinity (K_D 10^{-10} M), thus inhibiting its binding to membrane receptors and the consequent cell activation and biological effects (42–44).

A preliminary study for the identification of the cell population responsible for hyperresponsiveness to IL-18 in LN and spleen of *lpr/lpr* mice was performed. Both in the LN and in the spleen of normal +/+ and autoimmune *lpr/lpr* mice the IL-18-responsive population is mainly represented by $CD4^-/CD8^-$ cells, in agreement with the notion that NK cells might be a major source of IFN-γ in response to IL-18 (19), even though the contribution of other cells (B cells, for example) cannot be excluded. However, a significant response to IL-18 also could be detected in NK-depleted and CD4/CD8-enriched populations, suggesting that some T cells also are responsible for IFN-γ production in response to IL-18. In particular, in *lpr/lpr* mice a highly significant fraction of responsiveness (27.6 $\pm$ 6.7 and 29.0 $\pm$ 5.0% of the value of unfractionated LN and spleen cells, respectively) could be found in the T cell-enriched fraction ($NK^-/CD4^+/CD8^+$ cells; data not shown). This fraction may contribute the autoreactive T cells that are characteristic of the *lpr/lpr* syndrome. The notion that Th1 autoreactive cell lines generated from *lpr/lpr* LN are hyperresponsive to IL-18 strongly suggests that the enhanced reactivity to IL-18 of *lpr/lpr* LN cells could be at least in part attributed to the autoreactive T lymphocytes normally present in the LN of these mice. In fact, in vitro-generated autoreactive Th1 cell lines are much more reactive to IL-18 stimulation than control alloreactive cell lines obtained from either *lpr/lpr* or +/+ mice. As expected, the in vitro-generated autoreactive Th1 lymphocytes showed high levels of both IL-18Rα and IL-18Rβ (data not shown). However, a significant expression of both receptor chains was observed also in Th1 alloreactive cells (although the accessory chain was clearly expressed in a minor extent in the +/+ cells; data not shown). The presence of both IL-18R chains on Th1 alloreactive cells is not surprising and can be explained by the activation state of the cells after in vitro generation. Therefore, the increased responsiveness of autoreactive cells to IL-18 can be in part attributed to enhanced expression of IL-18R chains, although additional factors are likely to be involved in the modulation of IL-18 effects. From data here reported, it is not possible to define whether hyperresponsiveness to IL-18 in vivo by autoreactive *lpr/lpr* LN cells indeed is a consequence of their abnormal activation, due both to defective T cell apoptosis and to the continuous presence of self-Ags. Likewise, the increased expression of the IL-18Rβ in *lpr/lpr* LN could be a consequence of autoreactive T cell activation.

In summary, LN cells of young *lpr/lpr* mice show enhanced sensitivity to IL-18 stimulation, both in terms of IFN-γ production and proliferation, long before the onset of the lymphoproliferative disease. This hypersensitivity, probably mediated by NK cells and also by autoreactive T cells, appears at least in part consequent to constitutive hyperexpression of the IL-18R accessory chain IL-18Rβ. Thus, in the deregulated immune system of *lpr/lpr* mice, hyperreactivity of LN cells to chronic stimulation by IL-18, which is constitutively overproduced in *lpr/lpr* LN (Bossù et al., manuscript in preparation), could be placed among the factors contributing to the excessive IFN-γ production and to the IFN-γ-driven subsequent lymphadenopathy and pathological derangement that characterize the disease progression.

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