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

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

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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 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+CD20+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-γ receptor 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-1 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-18Ra (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-18Ra 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 insulinitis 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).
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 autoimmune 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 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 auxiliary and inguinal LN of three or more mice were cultured for the indicated times in complete medium 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 magnetic beads (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). For experiments in which NK cells were cultured, sterile fresh preparations of TNF-α (5 ng/ml; Genzyme, Sunnyvale, CA) were added to each well.

RT-PCR

Total RNA was prepared from up to 1.0 × 10⁷ 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 with 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 × PCR buffer (Stratagene) containing 2.5 μl of the first-strand cDNA, 50 μM of each dNTP, 1 μM of each primer (Endogen, Milan, Italy), and 1 U Taq DNA polymerase (Stratagene). The oligonucleotides used were: hypoxanthine phosphoribosyltransferase (HPRT) 5′-GGTGATAGCGCCAGACCTTTGTT-3′, HPRT 3′-GATTTCAACCTTCGTCACTATTGCAG-3′, IL-18Ra 5′-GGTCGCAACGGAATGACACAC-3′, IL-18Ra 3′-ATT TAAGGTCAAATTAGGCCAGAG-3′, IL-18RB 5′-GGATGGTGAGATAATGGAGTAT-3′, and IL-18RB 3′-CCGTCGCCAGAAGGAGATTT-3′. The PCR conditions were as follows: an initial denaturation step at 94°C for 2 min, followed by 30 cycles (94°C for 30 s, annealing temperature for 1 min, and 72°C for 1 min), and a final extension step at 72°C for 5 min. PCR products were separated in a 2% agarose gel and visualized by ethidium bromide staining. For semi-quantitative PCR, sequences of the housekeeping gene HPRT, IL-18Ra, and IL-18RB 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 C57BL6 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).
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 synergy 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 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 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− (77.7 ± 31.3 pg IFN-γ/10⁶ cells) but that a significant response could be attributed to CD4+/CD8+ cells (86.4 ± 4.5 pg IFN-γ/10⁶ cells) and in particular to NK-depleted CD4+/CD8+ cells (64.3 ± 8.6 pg IFN-γ/10⁶ 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 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
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-18Rβ 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 IgG2α 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-1g) 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
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 naïve T or CD4+ cells, thus also expression of IL-18Rα mRNA, whereas it cannot further increase IL-18R expression on polarized Th1 cells (38). However, in another report, IL-12 failed to up-regulate IL-18Rα expression during Th1 polarization of spleen naïve 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 co-stimulation (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 (Kd 10^{-10}–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 (Kd 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 kinetic 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-1Rα, a soluble IL-18 binding protein has been recently identified that can bind IL-18 with high affinity (Kd 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 ± 6.7 and 29.0 ± 5.0% of the value of un-depleted 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 (Bossi 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.

References


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