Murine Bone Marrow-Derived Mast Cells as Potent Producers of IL-9: Costimulatory Function of IL-10 and kit Ligand in the Presence of IL-1

Michael Stassen, Martina Arnold, Lothar Hültner, Christian Müller, Christine Neudörfl, Tanja Reineke and Edgar Schmitt

*J Immunol* 2000; 164:5549-5555; 
doi: 10.4049/jimmunol.164.11.5549
http://www.jimmunol.org/content/164/11/5549

References
This article cites 43 articles, 19 of which you can access for free at: http://www.jimmunol.org/content/164/11/5549.full#ref-list-1
Murine Bone Marrow-Derived Mast Cells as Potent Producers of IL-9: Costimulatory Function of IL-10 and kit Ligand in the Presence of IL-1

Michael Stassen,* Martina Arnold,* Lothar Hültner,† Christian Müller,* Christine Neudörfl,* Tanja Reineke,* and Edgar Schmitt2*

Recently, the Th2-type cytokine IL-9 was identified by genetic mapping analyses as a key mediator that determines the susceptibility to asthma. This has been further supported by data from IL-9-transgenic mice in which the overexpression of IL-9 in the lung causes airway inflammation, mast cell hyperplasia, and bronchial hyperresponsiveness. In an accompanying paper, we demonstrate that murine bone marrow-derived mast cells (BMMC) after stimulation with either ionomycin, a combination of ionomycin and IL-1, or via IgE-Ag complexes and IL-1 are very potent producers of IL-9. Herein we show that a dramatic increase of IL-9 production is observed when BMMC activated with ionomycin/IL-1 or with IgE-Ag complexes/IL-1 are treated with either additional kit ligand (KL) or IL-10. Both KL and IL-10 considerably enhance the production of IL-9 mRNA and protein. We were also able to demonstrate that the production of endogenous IL-10 by activated mast cells acts on the production of IL-9. Half-life measurements of IL-9 mRNA revealed no significant effect by KL, but a 2-fold increase of mRNA stability under the influence of IL-10. Reporter gene assays of transfected BMMC showed an enhanced transcriptional activity of the IL-9 promoter in the presence of either IL-10 or KL compared with cells stimulated only with a combination of IL-1 and ionomycin. The influence of KL and IL-10 might be of physiological importance, because it is known that both cytokines are produced by bronchial epithelial cells. The Journal of Immunology, 2000, 164: 5549–5555.

Mast cells are important effector cells in anaphylactic reactions and are involved in a variety of immunological processes (for review, see Refs. 1 and 2), including their potential influence on the differentiation of naive T cells toward Th2 cells (3).

Besides their ability to secrete mediators such as histamine, leukotrienes, and prostaglandins, which directly mediate inflammatory reactions, it has been shown that mast cells also produce a variety of cytokines that partly overlap with the cytokine pattern produced by Th cells of the Th2 subset (4, 5).

IL-9 is a multifunctional cytokine produced by activated T cells (6), which was originally termed P40, TCGF III, or MEA (7–9). Although initially described as a T cell growth factor, naive T cells do not respond to IL-9, but this cytokine induces the proliferation of murine T cell lymphomas in vitro and its overexpression in vivo leads to the development of thymic lymphomas (10). In this context, IL-9 is also expressed by primary and cultured Hodgkin lymphoma and Reed-Sternberg cells and acts as an autocrine growth factor for these tumor cells (11). In parallel, it was reported that IL-9 exerts growth-enhancing activity on murine bone marrow-derived mast cell lines (12, 13). Consequently, it has been demonstrated that elevated levels of IL-9 in vivo lead to pronounced mastocytosis, which enhances the resistance to infections with nematodes (14, 15).

On the basis of genetic linkage analyses, it was recently suggested that IL-9 might play an important role in the pathogenesis of asthma (16, 17). This assumption was supported by lung-specific expression of IL-9 in transgenic mice, which was accompanied by airway inflammation, bronchial hyperresponsiveness, and mast cell hyperplasia (18).

In this work, we describe the influence of IL-10 and kit ligand (KL)3 on the production of IL-9 by BMMC. Both IL-10 and KL considerably enhance the expression of IL-9 mRNA and protein by mast cells activated by a combination of IL-1 and ionomycin or via cross-linked IgE and IL-1. Reporter gene assays indicate that both factors enhance the transcription of the IL-9 gene, and RNA t1/2 measurements reveal an additional stabilizing effect of IL-10 on the IL-9 message. Furthermore, we demonstrate that BMMC activated by IL-1 and ionomycin also produce IL-10, which enhances the production of IL-9 by an autocrine mechanism.

Materials and Methods

Cytokines, cytokine assays, and mAbs

Mouse IL-9 (mIL-9) was assayed by specific sandwich ELISA with reference standard curves using known amounts of mIL-9. To detect mIL-9, we used mAb 229.4 and biotinylated mAb D9302.C12 (kindly provided by Dr. J. Van Snick, Ludwig Institute for Cancer Research, Brussels, Belgium). The detection limit of this ELISA is 50–100 pg/ml. This ELISA detects biologically active mIL-9, as confirmed by using an IL-9-specific bioassay (8).

The cDNA of his-tagged murine rKL (kindly provided by Dr. G. W. Bornkamm (19)) was expressed in Escherichia coli and the KL affinity purified using the QIAexpress system, according to the manufacturer (QIAGEN, Düsseldorf, Germany). Biological activity of KL was verified using a proliferation assay measuring [3H]thymidine uptake by mast cells. mIL-3

Received for publication October 4, 1999. Accepted for publication February 7, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the Deutsche Forschungsgemeinschaft, Grant SFB 548.

2 Address correspondence and reprint requests to Dr. Edgar Schmitt, Institut für Immunologie, Hochhaus am Augustusplatz, D-55131 Mainz, Germany. E-mail address: eschmitt@mail.uni-mainz.de

3 Abbreviations used in this paper: KL, kit ligand; BMMC, bone marrow-derived mast cell; HGPRT, hypoxanthine-guanine phosphoribosyl transferase; mIL-3, mouse IL-3.
was isolated from supernatants of myelomonocytic WEHI-3B cells using DEAE chromatography. Murine rIL-4 was a gift of W. Müller (Institute of Genetics, Cologne, Germany). Human recombinant IL-1α (Lot 719–27) and IL-1β (Lot 693–99) were kindly provided by Dr. Seller (Behringwerke, Germany), and titration analyses for optimal biological activity were performed in the range of 0.005–150 U/ml. Baculo-derived murine rIL-10 was a gift from Dr. J. Schlaak (I. Med. Klinik, University of Mainz, Mainz, Germany) and titrated from 1 to 100 U/ml. Units were defined based on the mast cell stimulatory activity of IL-10 (20). Anti-IL-10 Ab JES5.2A5 was kindly provided by Dr. Anne O’Garra (DNAX, Palo Alto, CA), and anti-IFN-γ Ab R46A2 (21) was used as isotype control as well as total rat Ig, purified from serum by ammonium sulfate precipitation and hydrophobic interaction chromatography. Ionomycin (Sigma-Aldrich Chemie, Steinheim, Germany) was titrated within the range of 0.1–0.75 μM.

**Generation of BMMC**

BMMC were cultured from BALB/cAnn mice. The animals were originally obtained from the Zentralinstitut für Versuchstierforschung (Hannover, Germany), bred in our animal facility, and used at the age of 5–10 wk. The mice were sacrificed by cervical dislocation, intact femurs and tibias were removed, and bone marrow cells were harvested by repeated flushing with MEM.

The cell culture was established at a density of 3 × 10⁶ cells/ml in IMDM, supplemented with 10% FCS (inactivated at 56°C), 2 mM L-glutamine, 1 mM pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 U/ml IL-4, and 20 U/ml mIL-3. Nonadherent cells were transferred to fresh medium every 2–3 days for a total of at least 21 days to remove adherent macrophages and fibroblasts.

**In vitro cell stimulation**

Culture medium was IMDM supplemented with 5% FCS (previously inactivated at 56°C), 1 mM pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Stimulations were conducted in triplicates using 96-well plates with 10⁵ cells/well in a final volume of 200 μl, including 0.25 μM ionomycin and 10 U/ml IL-1 alone or in combination with either additional IL-10 (50 U/ml) or KL (400 ng/ml). For stimulation of BMMCs, 48-well plates were coated with DNP-BSA (2 μg/ml in PBS) and used to activate mast cells (5 × 10⁶/well) that previously had been incubated with the IgE anti-DNP Ab A2 for 72 h (22, 23). Cytokine combinations were used as above, except that ionomycin was omitted. After 24 h, the supernatants were tested for the presence of IL-9 by ELISA.

**RNA purification, PCR amplification, and Northern blotting**

RNA was isolated using a modification of the protocol of Chomczynski et al. (24), as detailed previously (25). RNA electrophoresis under denaturing conditions was conducted as described (26). RNA was transferred to positively charged nylon membranes (Boehringer Mannheim, Indianapolis, IN) and UV fixed. Mast cell RNA isolated after stimulation with ionomycin and IL-1 for 24 h was used for reverse transcription with SUPERSCRIPT II reverse transcriptase following the recommendations of the supplier (Life Technologies, Karlsruhe, Germany). A fragment (bases 196–433) of the mIL-9 cDNA (7) was amplified via PCR using IL-9 forward (CTG ATG ATT GTA CCA CAC CGT GC) and IL-9 reverse primers (GCC TTC GTA TCT CTG TG). After electrophoresis, the cDNA was eluted from the agarose gel, and an additional phenol/chloroform extraction was conducted. For use as a probe, the cDNA was labeled with [α-32P]dCTP (27) to a sp. act. of 2–5 × 10⁶ cpm/μg DNA. Hybridizations were performed in 5× SSC, 0.1% SDS, 1× Denhardt’s solution, 100 μg/ml salmon testes DNA at 65°C with 2 × 10⁶ cpm/ml. After washing with 0.1× SSC, 0.1% SDS at 65°C, blots were exposed to a Kodak X-AR-5 film using a Kodak BioMax MS intensifying screen or directly quantified with a phosphor imager (Fujifilm BAS 2500; Fuji, Tokyo, Japan).

To investigate the expression of endogenous IL-10 after stimulation of BMMC with IL-1 and ionomycin, RNA was isolated at the indicated time points and used for RT-PCR with primers encompassing the entire mIL-10 cDNA (28): IL-10 forward, GGG GTA CCG CCG CCA TCA TCT GCT GCT CAG CA; IL-10 reverse, GGGA CCTA TCC TAG TTG TTG ATC ATC ATA A. HPRT was amplified using HGPRF forward, GTT GGA TAC AGG CCA GAC TTT GTT G; HGPRF reverse, GAG GGT AGG CTT GGC TAT AGG CT. cDNA was serially diluted 1/5, and a semi-quantitative PCR was conducted for both IL-10 and HPRT as endogenous reference. After electrophoresis of the PCR products and visualization with ethidium bromide, gels were photographed (Gel Print 2000i; MWG-Biotech, Ebersberg, Germany), digitally saved, and processed using the program ZERO-Dscan (MWG Biotech, Ebersberg, Germany). The IL-10 mRNA data were standardized according to the HPRT expression.

**Treatment of cells with actinomycin D**

Treatment with 5 μg/ml actinomycin D (29, 30) was conducted at a density of 3–4 × 10⁶ cells/ml after 24-h stimulation (described above), and cells were used at given time points for the isolation of RNA and Northern blotting. After hybridization, signal intensities were determined using a phosphor imager.

**Transfection and luciferase assay**

The 5′ region of the mIL-9 gene (31) encompassing nucleotides −610 to +32 was amplified from genomic DNA by PCR with 5′-CCg gat ccT CAA GGC CAA TGC TAG C-3′ and 5′-GTT Gaa gGt CA GCG TACT GGA ACT C-3′ as primers and verified by DNA sequencing. Small letters indicate the authentic BamHI and artificial HindIII site that was introduced to allow forced cloning into the pXP1 promoterless luciferase reporter gene vector (32). BMMC (2 × 10⁶ cells in 0.2 ml serum-free IMDM) were transfected with 10 μg plasmid by electroporation in 0.2-cm cuvettes at room temperature using a Bio-Rad Gene Pulser (Richmond, CA) set at 350 V, R = 180 and 960 μF. Cells were allowed to recover for 3 h in IMDM supplemented with FCS, glutamine, pyruvate, IL-4, and IL-3 (described above); harvested; washed with IMDM; and stimulated under the different conditions, as outlined above. To exclude differences in transfection efficiency, cells were cotransfected with 100 ng pRL-TK (Promega, Madison, WI), which contains the thymidine kinase promoter region upstream of the Renilla reniformis luciferase. Cells were lysed after 24 h, and luciferase activity was measured by a luminometer (Berthold, Bad Wildbad, Germany) using the dual-luciferase reporter assay system from Promega. Data were normalized according to the Renilla luciferase activity.

**Results**

**IL-10 and KL act as costimulators of IL-9 and IL-9 mRNA production by activated mast cells**

BMMC were generated using IL-3 in combination with IL-4, as described in Materials and Methods. Stimulation of such mast cells by the Ca²⁺-ionophore ionomycin induced a low but significant production of IL-9 after 24 h (Fig. 1). The addition of IL-1 resulted in an increased production of IL-9 that was further augmented in the presence of KL. Maximal amounts of IL-9 were obtained when BMMC were stimulated by ionomycin in the presence of a mixture of IL-1 and IL-10. In the absence of ionomycin, no IL-9 production could be observed irrespective of whether KL, IL-1, or IL-10 was present, indicating its dependence on Ca²⁺ signaling. Furthermore, KL as well as IL-10 exerted only a minimal positive effect on ionomycin-induced IL-9 production in the absence of IL-1 (data not shown).
In time course experiments, BMMC were stimulated as described above and IL-9 production of such cells was determined over a period of 72 h (Fig. 2). Detectable concentrations of IL-9 were found after 12 h under all conditions of stimulation. Only a moderate increase was observed until 24 h of stimulation, followed by a strong increase between 24 and 72 h, leading to very high amounts of IL-9 in the range of 1 µg/ml. In concordance with the results outlined in Fig. 1, the addition of KL or IL-10 enhanced the ionomycin/IL-1-induced production of IL-9 at all time points, with IL-10 having the strongest effect.

To examine whether the costimulating effects of KL and IL-10 on the production of IL-9 can also be observed under the conditions of a physiological stimulation, we activated BMMC via cross-linked IgE in the absence of ionomycin (Fig. 3). Upon activation without any exogenous cytokine, low amounts of IL-9 were measurable. The addition of IL-1 resulted in an about 3.5-fold increased production of IL-9, which was further augmented 6-fold by adding KL and 10-fold in the presence of IL-10 compared with cells stimulated without additional cytokines.

IL-9 mRNA is stabilized by IL-10

The comparatively large amounts of IL-9 mRNA from BMMC that were stimulated in the presence of KL or IL-10 might be the result of enhanced transcription and/or might be due to posttranscriptional stabilization of the mRNA that leads to its accumulation.

To test the latter assumption, we blocked transcription by actinomycin D after 24 h of stimulation either using solely a combination of ionomycin and IL-1 or in addition KL or IL-10. RNA was prepared on several time points after the actinomycin D block, and the decay of IL-9 RNA was determined by Northern blotting. Based on the assumption of a first order kinetics, the log of signal intensities was plotted against time. The value at time point 0 (e.g., addition of actinomycin D) is set 100%.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Ionomycin</th>
<th>Ionomycin + IL-1</th>
<th>Ionomycin + IL-1 + KL</th>
<th>Ionomycin + IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>92 ± 12.8</td>
<td>97.3 ± 11.6</td>
<td>102 ± 21</td>
<td>116 ± 22</td>
</tr>
<tr>
<td>48</td>
<td>100.1 ± 8.3</td>
<td>111.3 ± 15.7</td>
<td>104 ± 3.6</td>
<td>120.6 ± 7.5</td>
</tr>
</tbody>
</table>

* Percentage of viable cells ± SEM determined by trypan blue exclusion upon stimulation with ionomycin (0.25 µM), ionomycin plus IL-1 (10 U/ml) alone or in combination with either additional KL (400 ng/ml) or IL-10 (50 U/ml). Results of three experiments are shown, and the initial cell number (5 × 10⁶) is set at 100%.
experiments suggest that IL-10 prolongs the IL-9 mRNA about 2-fold (Table III).

The 5’ region of the IL-9 gene allows inducible expression that is enhanced by KL and IL-10

To investigate whether KL or IL-10 stimulates the transcription of the IL-9 gene, we used the 5’ part of the published IL-9 gene sequence from -610 to +32 (31) to direct the expression of a luciferase reporter gene in BMMC (Fig. 6). As expected, no significant constitutive transcription was observed, but activation with ionomycin and IL-1 induced the expression of the luciferase reporter. The addition of IL-10 or KL augmented the transcriptional activity of the IL-9 promoter, with IL-10 exerting the strongest effect.

From these data, we conclude that the increased production of IL-9 under the influence of KL or IL-10 is at least partially due to enhanced transcriptional activity of the IL-9 promoter.

Endogenous IL-10 stimulates the production of IL-9

It has been shown that rat peritoneal mast cells constitutively produce IL-10 (33). Because peritoneal mast cells represent connective tissue mast cells, whereas BMMC are the in vitro correlate of mucosal mast cells, the question arose whether and under which conditions such BMMC could produce IL-10 and whether this endogenous IL-10 influences the production of IL-9.

Kinetic studies using a semiquantitative RT-PCR approach revealed that IL-10 mRNA is not expressed by nonstimulated mast

FIGURE 4. Time course of IL-9 mRNA production by activated mast cells. Mast cells were stimulated for the indicated time in the presence of ionomycin (0.25 μM) and IL-1 (10 U/ml) alone or in combination with either additional KL (400 ng/ml) or IL-10 (50 U/ml). A, 28S rRNA represents the ethidium bromide staining of the ribosomal RNA from equalized RNA samples used for Northern blotting of IL-9 mRNA. B, Graphical representation after scanning densitometry of the Northern blot. Representative of three experiments with similar results.

FIGURE 5. Half-life of IL-9 mRNA. Mast cells were activated for 24 h in the presence of ionomycin (0.25 μM) and IL-1 (10 U/ml) alone or in combination with either additional KL (400 ng/ml) or IL-10 (50 U/ml), followed by addition of actinomycin D (5 μg/ml) to prevent further transcription. Cells were harvested at the indicated time points after the addition of actinomycin D, Northern blots were prepared, and the amount of mRNA was determined by phosphor imaging (left panel). EtBr represents ethidium bromide staining from equalized RNA samples used to prepare the Northern blots. The data of one of three experiments are shown (see Table III).
cells, but by mast cells activated with ionomycin and IL-1, however with a considerable delay of several hours after activation. Fig. 7 demonstrates that IL-10 mRNA could not be detected within the first 8 h of activation, whereas after 24 h, IL-10 mRNA was found in large amounts.

To test whether this endogenous IL-10 affects the production of IL-9, we stimulated BMMC in the presence of neutralizing anti-IL-10 mAb. Fig. 8A shows that the production of IL-9, which was determined after 72 h, was reduced to about 50% if the mast cells were stimulated in the presence of anti-mouse IL-10 mAb. By addition of human IL-10 in combination with the anti-mouse IL-10 mAb, the inhibitory effect of the mAb was overcompensated by a factor of about 4. Fig. 8B depicts the IL-9 production in the presence of either an isotype control (R46A2) or total rat Ig compared with the effect of the anti-IL-10 mAb. Thus, these data demonstrate the specificity of the mAb-mediated inhibition and indicate that the endogenously produced IL-10 is not sufficient to induce optimal costimulation for IL-9 production.

Discussion

Many immune responses are orchestrated by Th cell-derived cytokines. Thus, the resolution of the molecular principles that regulate the expression of such cytokines is essential for the understanding of how immune reactions are controlled in health and, even more important for therapeutic interventions, in disease. With respect to the Th2-derived cytokine IL-9, it has recently been shown that it is decisively involved in the pathogenesis of asthma (18). Furthermore, we have reported in an accompanying paper that IL-9 cannot only be produced by Th2 cells, but also by mast cells. This finding implies a new regulatory function for mast cells, which have been shown to accumulate in lung tissue in the course of asthmatic reactions (18, 34).

Consequently, we have been focusing in this study on the regulation of IL-9 expression by mast cells. Originally, the production of IL-9 has been found to be restricted to naive CD4+ T cells and established Th2 cells. Further studies revealed that the regulation of IL-9 production differs between these cell populations. In naive Th cells, production of IL-9 is strongly enhanced by a combination of TGF-β and IL-4 (35), whereas Th2 cells require the presence of IL-1 as a cofactor to promote the synthesis of IL-9 (36). In this respect, mast cells, which also respond to IL-1 with enhanced IL-9 synthesis, resemble Th2 cells. By contrast, IL-4 and TGF-β exerted only a limited or no effect on the production of IL-9 by Th2 cells as well as mast cells. (data not shown). However, the inefficiency of IL-4 may only be apparent because for mast cells it was shown in a parallel report that endogenously produced IL-4 contributes significantly to the IL-1-enhanced production of IL-9. If this response to endogenous IL-4 is already near optimal, additional exogenous IL-4 will have no or only a limited effect.

IL-10 and KL are known as mast cell growth and differentiation factors that might exert at least some of their biological activities via the induction of mast cell-derived cytokines (20, 37, 38). Therefore, we tested whether these cytokines can stimulate the production of IL-9. IL-10 or KL alone had no or only a marginal effect, but both cytokines strongly enhanced the production of IL-9 by mast cells in the presence of IL-1. Detailed analyses revealed that IL-10 as well as KL increased the IL-9 promoter activity, with IL-10 having the strongest effect. In addition, our data suggest that IL-10 induced a doubling of the 1/2 of IL-9 mRNA, whereas KL had only a minor positive effect. However, the experimental setting does not allow to rule out that in the presence of IL-10 as a strong transcriptional enhancer, the IL-9 mRNA synthesis is not completely blocked by actinomycin D. Taken together, these data indicate that the costimulatory capacity of IL-10 for the production of IL-9 is a result of transcriptional activation and might also be partially based on increased IL-9 mRNA stability, whereas the effect of KL seems to be mainly based on enhanced transcriptional activity of the IL-9 gene. Preliminary data indicate that IL-10 and KL also act on different elements of the IL-9 promoter (data not shown). Compared with mast cells, IL-10 and KL could not stimulate the IL-9 production of Th2 cells neither alone nor in the presence of IL-1 (data not shown). Thus, the IL-9 promoter appears to be differentially regulated in mast cells and Th2 cells.
The IL-9 production by mast cells can be augmented by IL-1, which can be detected in the serum that favors an IgE-mediated activation of lung mast cells. Our data imply that the resulting production of IL-9 by mast cells can be augmented by IL-1, which can be detected in the airway walls during late asthmatic reactions (41), and that this IL-1-mediated effect can be further enhanced by IL-9 and KL, which were both found to be produced by bronchial epithelial cells (42, 43). Interestingly, it has recently been published that intratracheal injections of KL increase airway hyperreactivity in allergic and normal mice (44) by unknown mechanisms. Since it has been shown in addition that IL-9, IL-10, and KL are potent stimulators of mast cell proliferation, it is conceivable that mast cells accumulate in the lung under the influence of these autocrine (IL-9, IL-10)- and paracrine (IL-10, KL)-acting cytokines. In addition, mast cells might be decisively involved in the recruitment of eosinophils through the secretion of IL-9, IL-4, and IL-5. Regarding IL-9, it has been demonstrated that this cytokine induces the expression of IL-5 in T cells but is not a general T cell growth factor. Eur. J. Immunol. 19:2167.


