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IL-9 and IL-13 Production by Activated Mast Cells Is Strongly Enhanced in the Presence of Lipopolysaccharide: NF-κB Is Decisively Involved in the Expression of IL-9[1]

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Mast cells, due to their ability to produce a large panel of mediators and cytokines, participate in a variety of processes in adaptive and innate immunity. Herein we report that in primary murine bone marrow-derived mast cells activated with ionomycin or IgE-Ag the bacterial endotoxin LPS strongly enhances the expression of IL-9 and IL-13, but not IL-4. This costimulatory effect of LPS is absent in activated mast cells derived from the LPS-hyporesponsive mouse strain BALB/c-*LPS*^{d}, although in these cells the proinflammatory cytokine IL-1 can still substitute for LPS. The enhanced production of mast cell-derived IL-13 in the presence of IL-1 is a novel observation. Coactivation of mast cells with LPS leads to a synergistic activation of NF-κB, which is shown by an NF-κB-driven reporter gene construct. In the presence of an inhibitor of NF-κB activation, the production of IL-9 is strongly decreased, whereas the expression of IL-13 is hardly reduced, and that of IL-4 is not affected at all. NF-κB drives the expression of IL-9 via three NF-κB binding sites within the IL-9 promoter, which we characterize using gel shift analyses and reporter gene assays. In the light of recent reports that strongly support critical roles for IL-9 and IL-13 in allergic lung inflammation, our results emphasize the potential clinical importance of LPS as an enhancer of mast cell-derived IL-9 and IL-13 production in the course of inflammatory reactions and allergic diseases. *The Journal of Immunology,* **2001, 166: 4391–4398.**

Mast cells have been shown to be important effector cells in Th2-dominated immune responses associated with IgE Ab production in the context of allergies and parasitic infections (1). Besides IgE-mediated activation, mast cells are able to perceive a variety of infectious agents either by opsonin-dependent mechanisms or via direct binding to parasites and bacteria (2, 3). Mast cells can also be activated by soluble factors such as LPS, an abundant glycolipid of the outer membrane of Gram-negative bacteria. LPS activation of mast cells has been shown to induce IL-6 production and cell surface expression of CD28 (4, 5). It was further reported that LPS is an enhancer of kit ligand (KL)[4]- or IgE-dependent expression of cyclo-oxygenase-2 and IL-1β mRNA (6).

Besides their ability to secrete mediators such as histamine, leukotrienes, and PGs, which directly mediate inflammatory reactions, mast cells can also produce a variety of cytokines that partly overlap with the cytokine pattern produced by Th cells of the Th2 subset (1).

IL-9, originally termed P40, T cell growth factor III, or mast cell growth-enhancing activity (7–9), is a multifunctional cytokine produced by activated Th cells (10) and activated mast cells (11). Although initially described as a T cell growth factor, naive T cells do not respond to IL-9, but recent data suggest that it is a critical factor for the early stages of intrathymic T cell maturation from precursor cells (12). It was also reported that IL-9 exerts growth-enhancing activity on bone marrow-derived murine mast cell (BMMC) lines (13, 14). Subsequently, it has been demonstrated that elevated levels of IL-9 in vivo lead to pronounced mastocytosis, which enhances the resistance to infections with nematodes (15–17).

Among other biological activities (10), it was recently suggested on the basis of genetic linkage analyses that IL-9 might play an important role in the pathogenesis of asthma (18, 19). This assumption was supported by the lung-specific expression of IL-9 in transgenic mice, which was accompanied by airway inflammation, bronchial hyper-responsiveness, and mast cell hyperplasia (20) as well as the increased expression of IL-9 and its receptor in human patients with atopic asthma (21).

IL-13, originally designated P600, was initially described as a cytokine, which is produced by activated Th2 cells, but its expression by activated mast cells has also been reported (22–25). By using the IL-4Rα chain and STAT6 for signaling, IL-13 shares some biological activities with IL-4, such as the promotion of human B cell growth and the switching of B cells to the IgE isotype (26). Furthermore, it has been shown that IL-13, like IL-9, is a critical mediator in experimental models of allergic asthma (27, 28). The selective neutralization of IL-13 ameliorated asthma symptoms, including airway hyper-responsiveness, eosinophil recruitment, and

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[4] Abbreviations used in this paper: KL, kit ligand; BMMC, bone marrow-derived murine mast cells; mIL-9, mouse IL-9; *LPS*^{d}, LPS nonresponder; PDTC, pyrrolidinedithiocarbamate; TLR, Toll-like receptor.

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mucus overproduction. These findings were further corroborated by the pulmonary expression of IL-13 (29).

In this paper we report that LPS acts as a strong costimulator for the production of IL-9 and IL-13 by primary BMMC activated with either ionomycin or cross-linked IgE, whereas the production of IL-4 remains unaffected. LPS treatment of BMMC leads to an increased activation of NF-κB, as demonstrated using an NF-κB-dependent reporter gene construct. Gel shift analyses and reporter gene assays reveal the presence of three binding sites for NF-κB within the IL-9 promoter.

Materials and Methods

Animals

BALB/cAnn mice were originally obtained from the Zentralinstitut für Versuchstierforschung (Hannover, Germany), bred in our animal facility, and used at 5–10 wk of age. BALB/c congenic mice, which express the *LPS*^{d} allele from the LPS hyporesponsive strain C3H/HeJ (30), were provided by Chris Galanos (Max Planck Institut für Immunbiologie, Freiburg, Germany).

Cytokines, cytokine assays, mAbs, and reagents

Mouse IL-9 (mIL-9) was assayed by a 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 (provided by J. Van Snick, Ludwig Institute for Cancer Research, Brussels, Belgium). This ELISA detects biologically active mIL-9, as confirmed using an IL-9-specific bioassay (8). The detection limit of this ELISA is 50–100 pg/ml. Human rIL-1β (lot 693-98) was provided by Seiler (Behringwerke, Marburg, Germany).

Murine IL-3 was isolated from supernatants of myelomonocytic WEHI-3B cells using DEAE chromatography. The cDNA of His-tagged murine KL (provided by G. W. Bornkamm (31)) was expressed in *Escherichia coli*, and KL was affinity purified using the QIAexpress system, according to the manufacturer's directions (Qiagen, Dusseldorf, Germany). The biological activity of KL was verified using a proliferation assay measuring [^{3}H]thymidine uptake by mast cells. Murine rIL-4 was a gift from W. Müller (Department of Experimental Immunology, Braunschweig, Germany). For the detection of IL-4 via ELISA, we used mAbs BVD4-1D11 and BVD6-24G2, which were gifts from A. O'Garra (DNAX Research Institute, Palo Alto, CA), and 1 U/ml of IL-4 corresponds to 100 pg/ml (standard from R&D Systems, Minneapolis, MN). IL-13 was detected by ELISA with MAP413 and BAF413 using known amounts of rmIL-13 (R&D Systems). Pyrrolidinedithiocarbamate (PDTC), an antioxidant inhibitor of NF-κB activation, LPS (*E. coli* serotype 055:B5), ionomycin, and polymyxin B were purchased from Sigma (Steinheim, Germany). LPS from *Salmonella abortusequi* was donated by Chris Galanos (Max Planck Institut für Immunbiologie).

Generation of BMMC

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 3×10^6 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, 20 U/ml mIL-3, and 50 U/ml mIL-4. Alternatively, cells were grown in the presence of IL-3 alone or in combination with additional KL (100 ng/ml). Nonadherent cells were transferred to fresh culture plates every 2–3 days for a total of at least 21 days to remove adherent macrophages and fibroblasts. FACS analyses using an anti-CD13 Ab (R3-242, PharMingen, San Diego, CA) (32) and IgE plus anti-IgE mAb (33–35) as well as May-Grünwald-Giemsa and toluidine blue staining revealed that the resulting cell population consisted of >99% BMMC (data not shown).

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 triplicate using 96-well plates with 10^5 cells/well in a final volume of 200 μl, including 0.5 μM ionomycin alone or in combination with LPS, IL-1, PDTC, or polymyxin B as indicated in the figure legends.

For stimulation of BMMC via their Fcϵ receptors, mast cells (5×10^5/well) were incubated with the IgE-anti-DNP Ab A2 (33, 34) for 48–72 h and subsequently cultured in 48-well plates previously coated with DNP-BSA (2 μg/ml in PBS). After 48 h the supernatants were tested for the presence of IL-9 by ELISA.

Plasmids, transfection, and reporter gene assays

The 5′-region of the murine IL-9 gene (36) 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′-GTG Taa gct tGA CGG GAG TCT GGA ACT C-3′ as primers, and this was verified by DNA sequencing. Lowercase letters indicate the authentic *Bam*HI site and the artificial *Hin*dIII site, which was introduced to allow forced cloning into the promoterless pGL3 Basic luciferase reporter gene vector (Promega, Mannheim, Germany). Mutageneses of the three potential NF-κB sites was performed using the QuickChange site-directed mutagenesis kit (Stratagene, Amsterdam Zuidoost, The Netherlands) and was verified by DNA sequencing (Table I).

pTATALUC$^+$(4×NF-κB), provided by Ralf Marienfeld (Institute of Molecular Pathology, Wurzburg, Germany), comprises four copies of an NF-κB binding site from the murine c-Myb intronic enhancer (37). BMMC (2×10^6 cells in 0.2 ml of serum-free IMDM) were transfected with 10 μg of pTATALUC$^+$(4×NF-κB) or the same amount of pTATALUC$^+$ as a control (38). For transfection of the mIL-9 promoter construct or the derived NF-κB mutants 20 μg of the respective plasmid was used. Transfections were performed by electroporation in 0.2-cm cuvettes at room temperature using a Bio-Rad Gene Pulser (Hercules, CA) set at 350 V, R = ∞, and 960 μF. Cells were allowed to recover for 2 h in IMDM supplemented with FCS, glutamine, and pyruvate (described above), then were harvested, washed with IMDM, and stimulated under different conditions as outlined in the figure legends. To exclude differences in transfection efficiency, cells were cotransfected with 200 ng of pRL-TK (Promega), 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, Germany) using the dual luciferase reporter assay system from Promega. Data were standardized according to the *Renilla* luciferase activity.

EMSA

EMSAs were performed as described previously (39) using 5 μg of nuclear protein and 0.7 μg of poly(dI-dC). For competition, 30 ng of an oligonucleotide containing the NF-κB site 5′-GGGAATTTCC-3′ from the mouse invariant chain MHC class II promoter was added (40). In supershift EMSAs, 1 μg of Abs specific for NF-κB p65, c-*Rel*, RelB, p50, and p52 were added to the incubation mixture (Santa Cruz Biotechnology, Santa Cruz, CA).

Table I. *Location of the three NF-κB binding sites of the mIL-9 promoter and sequences of the primers used for mutagenesis and EMSA*[a]

	Wild-Type	Mutant	Primer for Mutagenesis and EMSA
NF-κB 1 (−429 to −420)	5′-GGGGTTTTCC-3′	5′-GG**A**GTTTTCC-3′	5′-GCCAGAGGAAAAC(C/**T**)CCAATGAGTGAAAGG-3′
Antisense strand			3′-CGGTCTCCTTTTG(G/**A**)GGTTACTCACTTTCC-5′
NF-κB 2 (−167 to −158)	5′-TGGTATTTCC-3′	5′-TGGTAT**A**TCC-3′	5′-GAGGAATGGTAT(T/**A**)TCCTGGCATAAGACAG-3′
Sense strand			3′-CTCCTTACCATA(A/**T**)AGGACCGTATTCTGTC-5′
NF-κB 3 (−47 to −38)	5′-GGGTTTTTCC-3′	5′-GGGTTTT**AG**C-3′	5′-GATGTCAGGGTTTT(CC/**AG**)CCGGTTTGAAG-3′
Sense strand			3′-CTACAGTCCCAAAA(GG/**TC**)GGCCAAACTTCTC-5′

[a] Mutations are shown in bold.

Results

LPS acts as a strong costimulator of IL-9 and IL-13 production by activated mast cells

BMMC were generated using IL-3 in combination with IL-4 as described in *Materials and Methods* according to a recent paper (41). Activation of these BMMC by the Ca^{2+} ionophore ionomycin induced a low, but significant, production of IL-9 as described recently (11) as well as moderate levels of IL-13. Fig. 1 demonstrates that addition of LPS strongly augmented the production of both IL-9 and IL-13 in a dose-dependent fashion, with 10 ng/ml LPS already exhibiting a significant effect.[5] The addition of polymyxin B, a cationic peptide capable of neutralizing LPS (42), significantly reduced the costimulatory function of LPS. In contrast to IL-9 and IL-13, the production of IL-4, which was induced by ionomycin, remained unaffected regardless of whether LPS or LPS plus polymyxin B were present. Also, at lower doses of ionomycin, leading to a decreased production of IL-4, no effect of LPS on the expression of IL-4 could be observed (data not shown). With LPS alone no cytokine production was detected, indicating the requirement for Ca^{2+} signaling as a primary stimulus.

To further address the question of whether LPS acts as a costimulator for the production of IL-9 and IL-13, we used BMMC derived from LPS-hyporesponsive BALB/c-*LPS*^{d} mice. As shown in Fig. 2, these cells did not respond to costimulation by LPS with increased production of IL-9 and IL-13, but they responded to costimulation with IL-1. Augmentation of IL-9 production of mast cells by the proinflammatory cytokine IL-1 has been reported recently (11), but has not been found for IL-13.

The finding that the production of mast cell-derived IL-1β mRNA was stimulated by LPS (6) suggests that LPS acts on the production of IL-9 and/or IL-13 via endogenously induced IL-1β. To exclude this possibility, we stimulated BMMC with ionomycin plus LPS in the presence of either human rIL-1R antagonist as described recently (11) or a combination of neutralizing polyclonal anti-IL-1α and anti-IL-1β Abs. Under these conditions, compared with the respective controls, we could not detect any significant decrease in cytokine production (data not shown).

To further examine whether the costimulatory effect of LPS can also be observed under the conditions of a physiological stimulation via the high affinity Fcϵ receptor, BMMC were activated by cross-linked IgE. Without additional costimuli only low amounts of IL-9 and IL-13 were detectable, but the addition of LPS strongly enhanced the production of both cytokines (Fig. 3).

Throughout this study BMMC grown in the presence of IL-3 and IL-4 were used, but the costimulatory effect of LPS on the production of IL-9 and IL-13 was also observed in BMMC generated with IL-3 or a combination of IL-3 and KL (Fig. 4).

Because there are no reports of the expression of CD14 or Toll-like receptor protein (TLR4) by mast cells, we investigated the presence of the respective mRNAs using RT-PCR and found both mRNAs to be constitutively expressed, but we could not detect CD14-positive BMMC via FACS analysis using a PE-conjugated monoclonal anti-CD14 Ab (data not shown). Therefore, we conclude that mast cells express only low levels of CD14.

Expression of IL-9 depends on the activation of NF-κB

A well-documented signaling pathway for LPS leads to the activation of NF-κB. To examine whether mast cells also respond in this way to LPS, we measured the activity of an NF-κB-driven

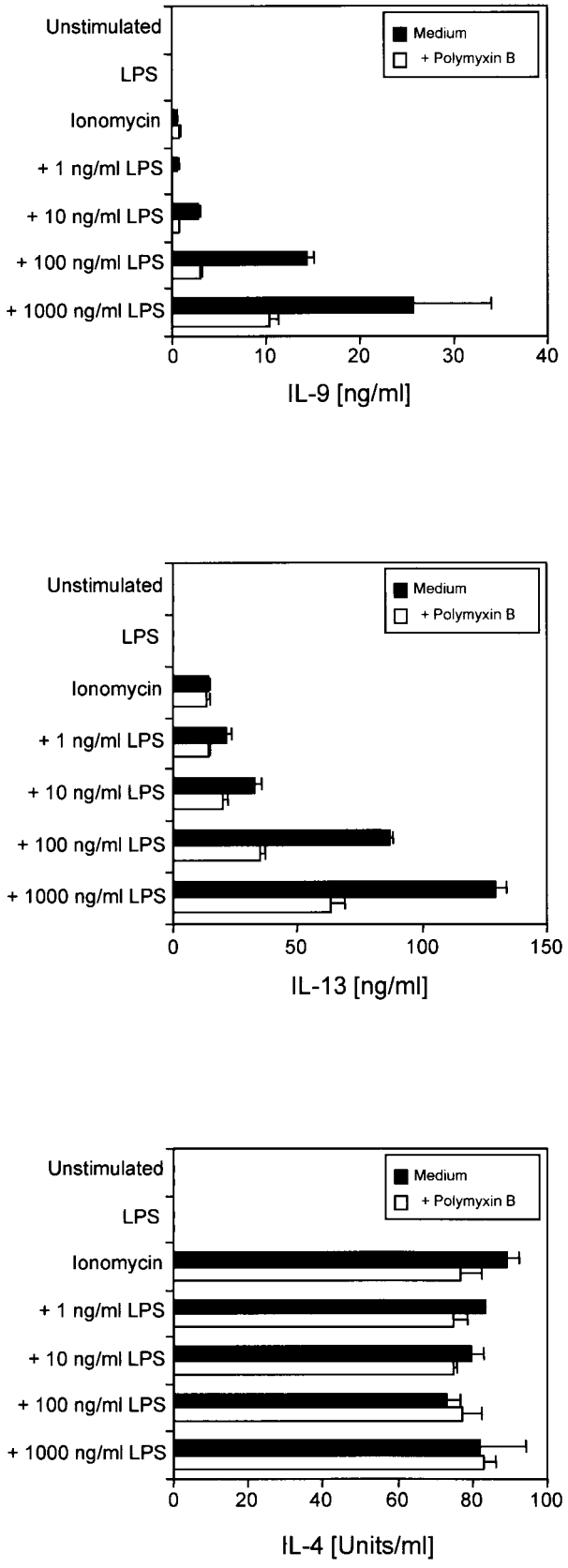

FIGURE 1. LPS specifically enhances mast cell-derived IL-9 and IL-13 production. BMMC were activated with ionomycin (0.5 μM), LPS (100 ng/ml), or a combination of both with increasing amounts of LPS as indicated. Neutralization of LPS was achieved by the addition of polymyxin B (20 μg/ml). After 48 h the levels of IL-9, IL-13, and IL-4 were determined by ELISA. Shown are the mean ± SEM of three experiments.

[5] LPS derived from *Escherichia coli* was used throughout this study, although LPS from *Salmonella abortusequi* proved to be equally effective.

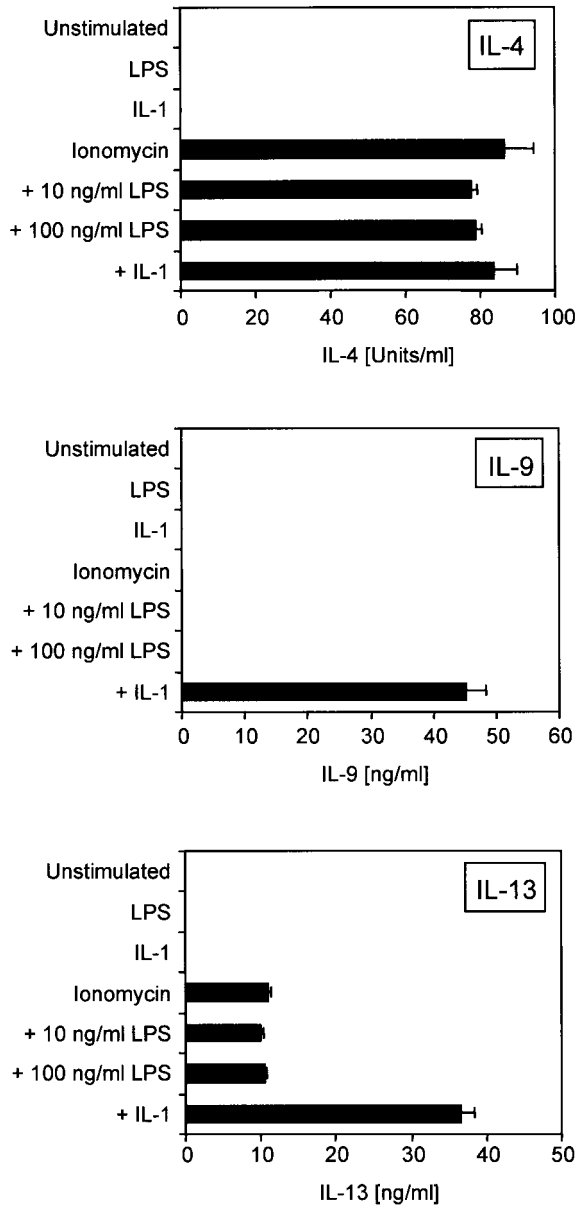

FIGURE 2. LPS does not exhibit costimulatory function on the production of IL-9 and IL-13 in mast cells derived from LPS nonresponder BALB/c-*LPS*^{d} mice. BMMC were activated as indicated, using ionomycin at 0.5 μM, IL-1 at 75 U/ml, and the LPS control at 100 ng/ml. IL-4, IL-9, and IL-13 were determined after 48 h by ELISA. Shown are the mean ± SEM of three experiments.

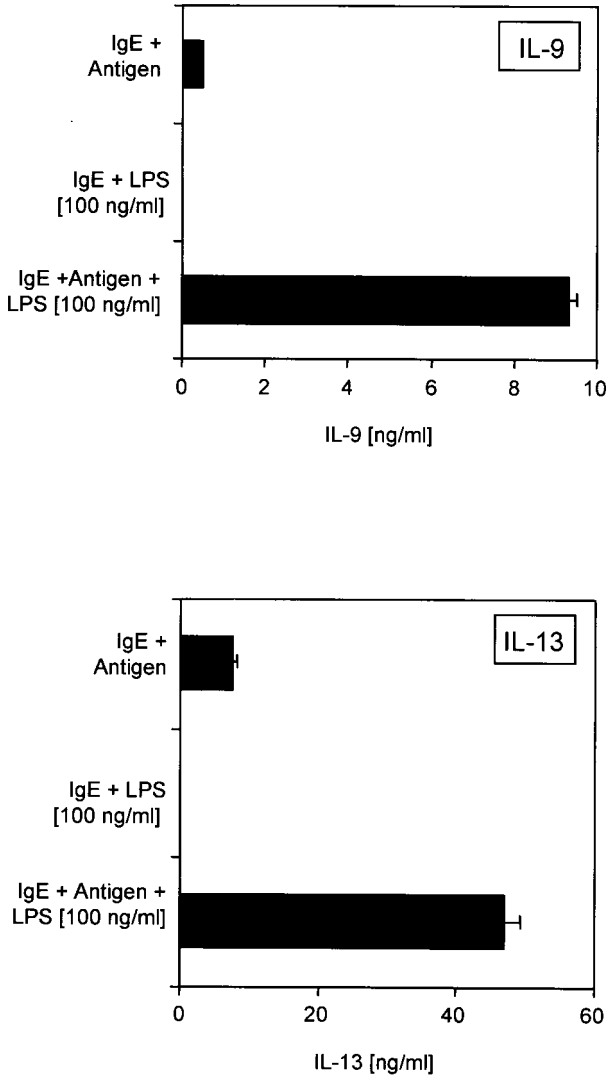

FIGURE 3. LPS augments the IL-9 and IL-13 production of mast cells activated by cross-linked IgE. BMMC were preincubated with an IgE-anti-DNP Ab and stimulated under various conditions. IL-9 and IL-13 were determined after 48 h by ELISA. Shown are the mean ± SEM of three experiments.

reporter gene, pTATALUC$^+$(4×NF-κB). It comprises the luciferase gene under the control of a core promoter encompassing a TATA box and four NF-κB binding sites. BMMC were transfected with this construct via electroporation and stimulated as detailed in Fig. 5. Activation solely with LPS had only a marginal effect on the luciferase gene expression, whereas stimulation with ionomycin led to a significant activity of the reporter gene. Activation of BMMC with ionomycin and LPS further increased luciferase activity ~5-fold, indicating a strong coactivation of NF-κB by LPS in mast cells. These results could be confirmed using cells stimulated with IgE-Ag complexes after transfection with the NF-κB-dependent reporter gene (Fig. 6). To elucidate whether the enhanced production of IL-9 and/or IL-13 depends on the activation of NF-κB, BMMC were stimulated with ionomycin and ionomycin plus LPS in the presence of PDTC, an inhibitor of IκB-α phosphorylation (43), and the levels of IL-9, IL-13, and IL-4 were determined by ELISA. Fig. 7 depicts the strong dose-dependent inhibition of IL-9 production by PDTC, suggesting an important role for NF-κB regarding activation of the IL-9 gene. The fact that PDTC also inhibits the ionomycin-induced production of IL-9 might be explained by a low level activation of NF-κB by ionomycin, which is also obvious in Fig. 5. In contrast to IL-9, the production of IL-13 is hardly reduced, and the level of IL-4 remains unaffected in the presence of the inhibitor. These data are in good agreement with the recent finding that the expression of mast cell-derived IL-4 is NF-κB independent (44). It should be noted that PDTC exhibited toxic effects, leading to impaired cell viability at concentrations >100 nM.

The IL-9 promoter comprises three active binding sites for NF-κB

We have recently shown that the 5′ region of the IL-9 gene from −610 to +32 is sufficient for the inducible activation of the IL-9 gene (41). In the human T cell line C5 MJ2, transformed with leukemia

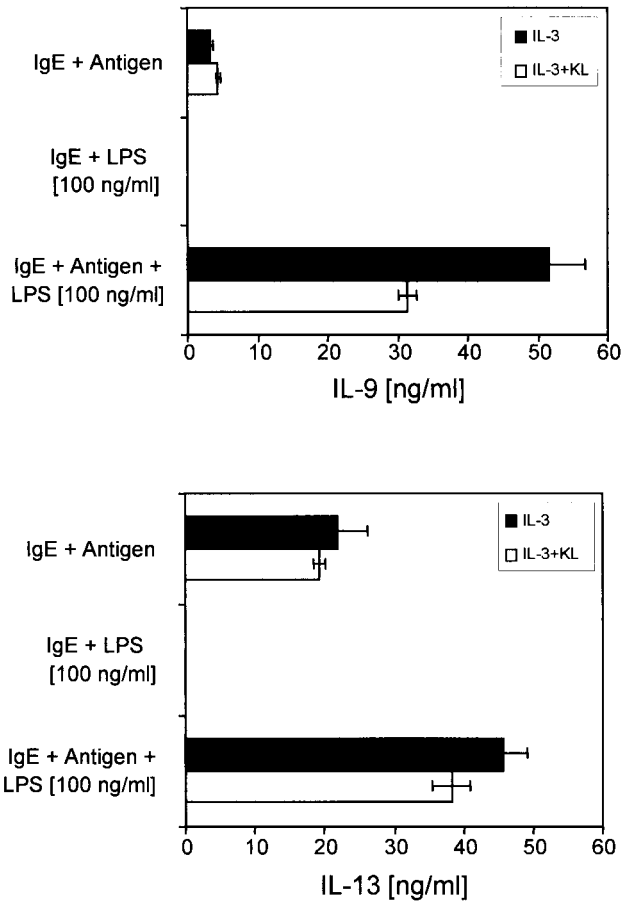

FIGURE 4. LPS enhances the IL-9 and IL-13 production of mast cells grown in the presence of IL-3 and IL-3 plus KL. BMMC were preincubated with an IgE-anti-DNP Ab and stimulated under various conditions. IL-9 and IL-13 were determined after 48 h by ELISA. Shown are the mean ± SEM of three experiments.

virus type I, an NF-κB site at −59 to −50 was shown to be involved in basal and PHA-induced expression of the IL-9 gene (45). Using the TRANSFAC database (www.transfac.gbf-braunschweig.de), we identified three potential binding sites for NF-κB within the murine IL-9 promoter, termed NF-κB1–3. To examine whether all potential NF-κB sites contribute to transcription of the IL-9 gene, we introduced point mutations into each site and compared the activities of these mutants with that of the wild-type sequence using reporter gene assays of transfected BMMC stimulated with ionomycin and LPS. Point mutations were carefully chosen to avoid the generation of potential binding sites for other transcription factors, based on the TRANSFAC database. Fig. 8 demonstrates that mutation of NF-κB1 (−429 to −420, antisense strand) and NF-κB3 (−47 to −38, sense strand) reduced reporter gene activity ~50% in each case, which fits with the observation that both binding sites comprise identical sequences. Mutation of NF-κB2 (−167 to −158, sense strand) also resulted in significant and reproducibly lower luciferase activity, although the reduction was only in the range of 25% compared with that in the wild type. NF-κB2 represents a predicted high score binding site for NF-κB, which differs in sequence from the identical sites NF-κB1 and NF-κB3 (Table I). Mutation of all three NF-κB binding sites does not ablate the activity of the IL-9 promoter, indicating that other factors must be involved in the inducible expression of the IL-9 gene.

The binding of NF-κB family members to the IL-9 promoter could be confirmed by EMSAs using double-stranded oligonucleotides corresponding to the three putative binding sites (Table I and Fig. 9*A*). This binding was abolished upon competition with a defined high affinity NF-κB binding site and using the mutated sequences of all three NF-κB sites. Interestingly, the sequence element NF-κB2 displays the lowest affinity for NF-κB, which is in good agreement with the finding that the corresponding mutant reduced the activity of the IL-9 promoter to a lesser extent compared with the mutations of NF-κB1 and NF-κB3 (see Fig. 8). Supershift analyses using the sequence element NF-κB1 from the IL-9 promoter indicate the binding of p50, p65, and c-*Rel*; the binding of p65 is only detectable upon strong stimulation with ionomycin and LPS (Fig. 9*B*).

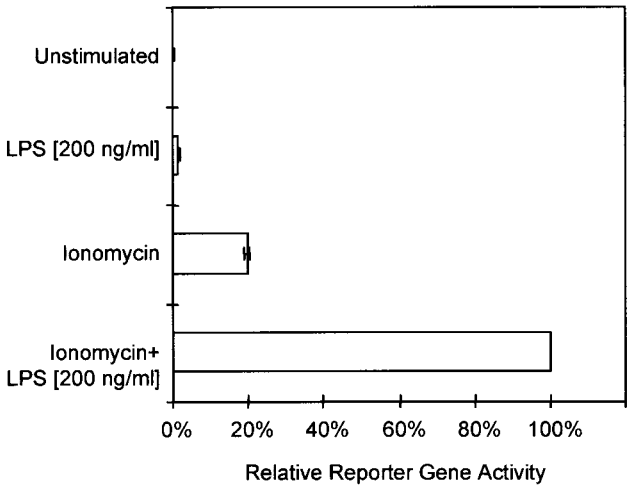

FIGURE 5. Activation of an NF-κB-dependent reporter gene by ionomycin and LPS. Electroporated BMMC were stimulated with ionomycin (0.5 μM) and/or LPS (200 ng/ml) for 24 h, and luminescence was measured. The activity of the pTATALUC$^+$ control vector was subtracted from the indicated values, and data were standardized according to the *Renilla* luciferase activity. Shown are the mean ± SEM of three experiments.

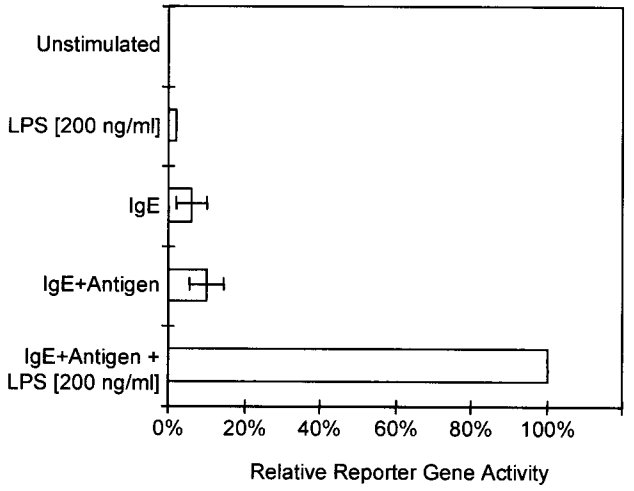

FIGURE 6. Activation of an NF-κB-dependent reporter gene by cross-linked IgE and LPS. Electroporated BMMC were stimulated as indicated for 24 h, and luminescence was measured. The activity of the pTATA LUC$^+$ control vector was subtracted from the indicated values, and data were standardized according to the *Renilla* luciferase activity. Shown are the mean ± SEM of three experiments.

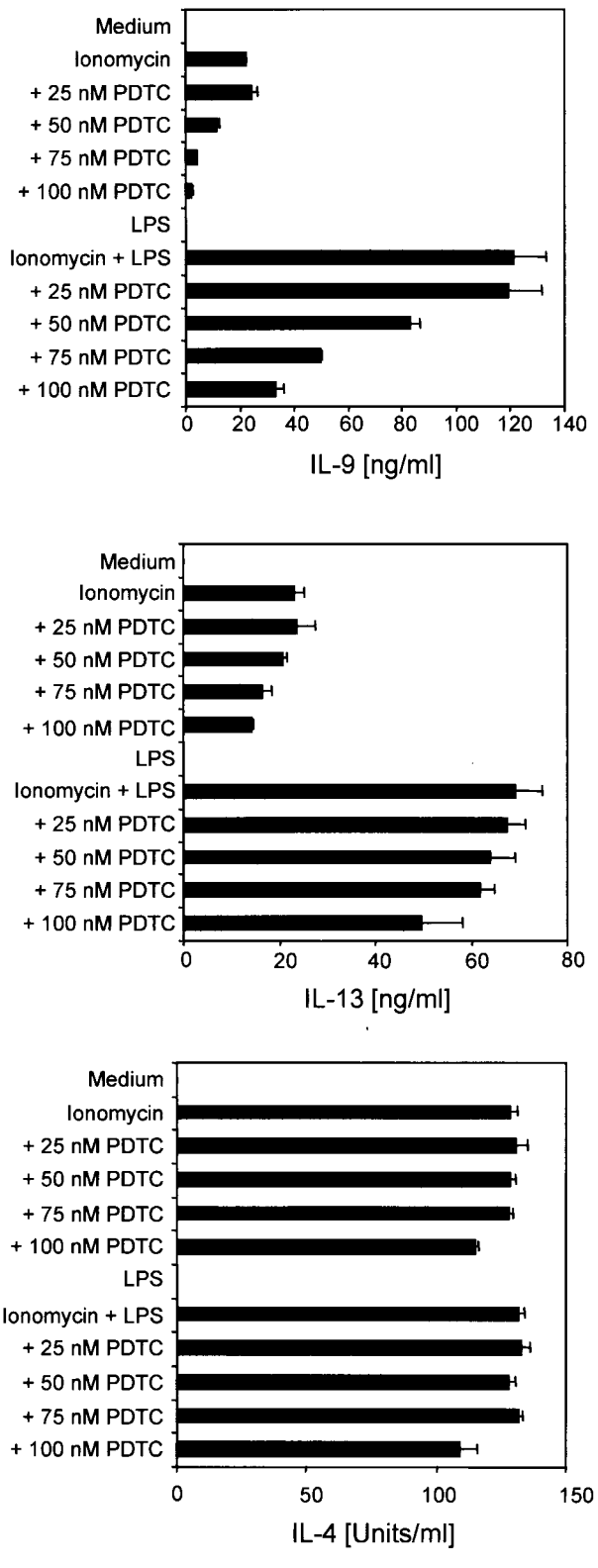

FIGURE 7. Production of mast cell-derived IL-9 is strongly decreased by inhibition of NF-κB activation with PDTC. BMMC were activated with ionomycin (0.5 μM) and/or LPS (100 ng/ml) with or without addition of PDTC as indicated. IL-9, IL-13, and IL-4 were determined after 48 h by ELISA. Shown are the mean ± SEM of three experiments.

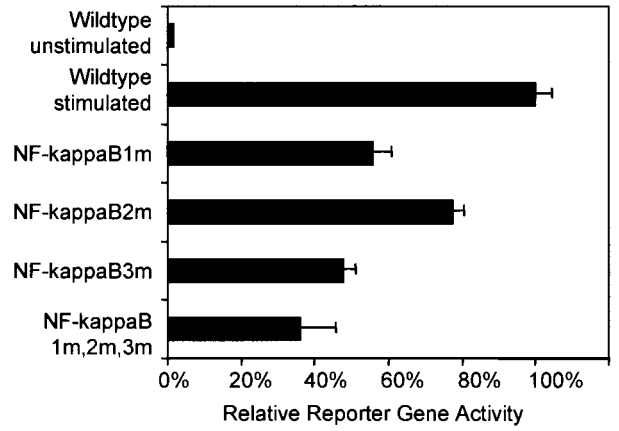

FIGURE 8. The IL-9 promoter comprises three active binding sites for NF-κB. BMMC were electroporated with an IL-9 promoter-luciferase construct or constructs with mutated NF-κB binding sites and stimulated with ionomycin (0.5 μM) and LPS (200 ng/ml). After 24 h cell extracts were prepared, and luminescence was measured. Data were normalized according to the *Renilla* luciferase activity. Shown are the mean ± SEM of three experiments.

Discussion

It is well established that LPS provides a key signal leading to activation of the immune system, in particular via the stimulation of macrophages. Stimulation of cells by LPS encompasses the binding of LPS to the soluble LPS binding protein and the recognition of this complex by CD14, a protein that is either anchored in the cell membrane via a glycosylphoshotidyl moiety or secreted as a soluble factor. More recently, it has been recognized that LPS signaling depends on a member of the evolutionary ancient TLRs, the TLR4 (46). Evidence was based on the finding that hypo- or nonresponsiveness of certain mouse strains to LPS is due to genetic defects in the TLR4 gene (47, 48). The most prominent outcome of cellular stimulation by LPS mediated by the TLR4-dependent signaling cascade, which is related to that induced by the IL-1R, is the activation of NF-κB (49). Moreover, activation of mitogen-activated protein kinases and AP-1 has been reported (50–52). The latter might account for our observation that LPS enhances the expression of IL-13 independently of NF-κB.

The strong up-regulation of mast cell-derived IL-9 and IL-13 by LPS suggests a potential role for these cytokines at sites of inflammation. Of special interest might be the earlier finding that IL-9 serves as a growth factor for mast cells, thereby possibly supporting their local proliferation via an autocrine loop. The growth-promoting activity of IL-9 has also been reported for the B-1 subpopulation of B cells, which are able to produce Abs against common bacterial Ags without Ag-specific T cell help (53). In addition, mast cells might be decisively involved in the recruitment of eosinophils at sites of inflammation through the secretion of IL-9, IL-13, and IL-5. In this context it has been shown that IL-9 induces the expression of the IL-5R α-chain on eosinophils (54) and that IL-13 induces eotaxin expression by several cell types, including airway epithelial cells (55–58). Furthermore, it has been demonstrated that IL-9 induces the expression of CC chemokines, also acting as eosinophil chemotactic factors, by epithelial cells (59). We propose that LPS could be a powerful coactivating stimulus for mast cells in the course of inflammatory reactions and infectious diseases caused by Gram-negative bacteria, often following viral infections. Interestingly, inflammatory and bronchial obstructive responses to inhalation of endotoxin in asthmatic patients have been described, and it has been supposed that bacterial Ags can potentiate the action of inhalant Ags (60–62). In asthmatics, extravasation of LPS binding protein and soluble CD14 into the bronchoalveolar compartment after allergen

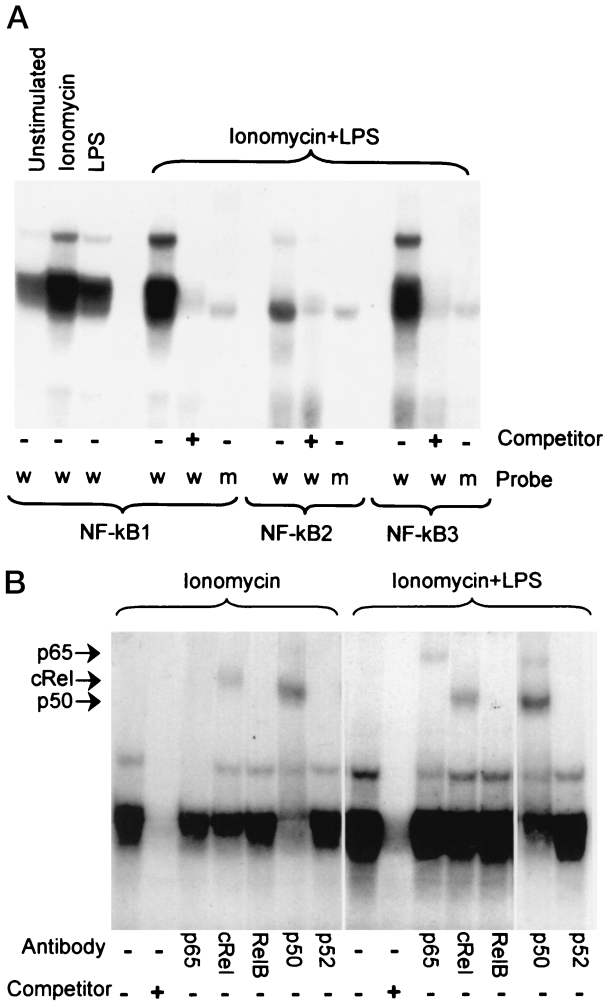

FIGURE 9. EMSAs confirm the binding of NF-κB family members to the sequence elements NF-κB1–3 from the IL-9 promoter. *A*, Cells were stimulated with LPS (100 ng/ml) or ionomycin (0.5 μM) with or without LPS for 1 h or were left untreated before the preparation of nuclear extracts. Radiolabeled NF-κB1–3 (w) with or without an unlabeled NF-κB competitor or the respective radiolabeled mutants (m) were used as probes. *B*, Supershift analyses were performed with nuclear extracts from cells that had previously been stimulated with ionomycin (0.5 μM) or ionomycin plus LPS (100 ng/ml) for 1 h. Radiolabeled NF-κB1 was used as a probe in combination with an NF-κB competitor or Abs specific for members of the NF-κB family. Representative data from two experiments with identical results are shown.

inhalation has also been reported, implying a role for LPS in amplification of the inflammatory response (63–65). During late asthmatic reactions, an accumulation of mast cells occurs in lung in parallel with an increased IgE level in serum that promotes IgE-mediated activation of mast cells.

In the light of these findings, our observation that LPS enhances the production of IL-9 and IL-13 by mast cells activated with cross-linked IgE might be of pathophysiological importance, because it has been shown that both cytokines are central mediators of allergic asthma. Furthermore, the strong dependence of IL-9 expression on the activation of NF-κB might partly account for the inability of NF-κB-deficient mice to develop airway hyper-responsiveness and allergic pulmonary inflammation (66).

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References

1. Metcalfe, D. D., D. Baram, and Y. A. Mekori. 1997. Mast cells. *Physiol. Rev. 77:1033.*
2. Galli, S. J., M. Maurer, and C. S. Lantz. 1999. Mast cells as sentinels of innate immunity. *Curr. Opin. Immunol. 11:53.*
3. Abraham, S. N., and M. Arock. 1998. Mast cells and basophils in innate immunity. *Semin. Immunol. 10:373.*
4. Leal Berumen, I., P. Conlon, and J. S. Marshall. 1994. IL-6 production by rat peritoneal mast cells is not necessarily preceded by histamine release and can be induced by bacterial lipopolysaccharide. *J. Immunol. 152:5468.*
5. Marietta, E. V., J. J. Weis, and J. H. Weis. 1997. CD28 expression by mouse mast cells is modulated by lipopolysaccharide and outer surface protein A lipoprotein from *Borrelia burgdorferi*. *J. Immunol. 159:2840.*
6. Moon, T. C., M. Murakami, M. D. Ashraf, I. Kudo, and H. W. Chang. 1998. Regulation of cyclooxygenase-2 and endogenous cytokine expression by bacterial lipopolysaccharide that acts in synergy with c-kit ligand and Fcε receptor I crosslinking in cultured mast cells. *Cell. Immunol. 185:146.*
7. Hultner, L., C. Druez, J. Moeller, C. Uyttenhove, E. Schmitt, E. Rude, P. Dormer, and J. Van Snick. 1990. Mast cell growth-enhancing activity (MEA) is structurally related and functionally identical to the novel mouse T cell growth factor P40/TCGFIII (interleukin 9). *Eur. J. Immunol. 20:1413.*
8. Schmitt, E., R. van Brandwijk, J. Van Snick, B. Siebold, and E. Rüde. 1989. TCGF III/P40 is produced by naive murine $CD4^+$ T cells but is not a general T cell growth factor. *Eur. J. Immunol. 19:2167.*
9. Van Snick, J., A. Goethals, J. C. Renauld, E. Van Roost, C. Uyttenhove, M. R. Rubira, R. L. Moritz, and R. J. Simpson. 1989. Cloning and characterization of a cDNA for a new mouse T cell growth factor (P40). *J. Exp. Med. 169: 363.*
10. Demoulin, J. B., and J. C. Renauld. 1998. Interleukin 9 and its receptor: an overview of structure and function. *Int. Rev. Immunol. 16:345.*
11. Hultner, L., S. Kolsch, M. Stassen, U. Kaspers, J. P. Kremer, R. Mailhammer, J. Moeller, H. Broszeit, and E. Schmitt. 2000. In activated mast cells, IL-1 up-regulates the production of several Th2-related cytokines including IL-9. *J. Immunol. 164:5556.*
12. De Smedt, M., B. Verhasselt, T. Kerre, D. Vanhecke, E. Naessens, G. Leclercq, J. C. Renauld, J. Van Snick, and J. Plum. 2000. Signals from the IL-9 receptor are critical for the early stages of human intrathymic T cell development. *J. Immunol. 164:1761.*
13. Hultner, L., J. Moeller, E. Schmitt, G. Jager, G. Reisbach, J. Ring, and P. Dormer. 1989. Thiol-sensitive mast cell lines derived from mouse bone marrow respond to a mast cell growth-enhancing activity different from both IL-3 and IL-4. *J. Immunol. 142:3440.*
14. Moeller, J., L. Hültner, E. Schmitt, and P. Dörmer. 1989. Partial purification of a mast cell growth-enhancing activity and its separation from IL-3 and IL-4. *J. Immunol. 142:3447.*
15. Faulkner, H., N. Humphreys, J. C. Renauld, J. Van Snick, and R. Grencis. 1997. Interleukin-9 is involved in host protective immunity to intestinal nematode infection. *Eur. J. Immunol. 27:2536.*
16. Faulkner, H., J. C. Renauld, J. Van Snick, and R. K. Grencis. 1998. Interleukin-9 enhances resistance to the intestinal nematode *Trichuris muris*. *Infect. Immun. 66:3832.*
17. Richard, M., R. K. Grencis, N. E. Humphreys, J. C. Renauld, and J. Van Snick. 2000. Anti-IL-9 vaccination prevents worm expulsion and blood eosinophilia in *Trichuris muris*-infected mice. *Proc. Natl. Acad. Sci. USA 97:767.*
18. Nicolaides, N. C., K. J. Holroyd, S. L. Ewart, S. M. Eleff, M. B. Kiser, C. R. Dragwa, C. D. Sullivan, L. Grasso, L. Y. Zhang, C. J. Messler, et al. 1997. Interleukin 9: a candidate gene for asthma. *Proc. Natl. Acad. Sci. USA 94:13175.*
19. Noguchi, E., M. Shibasaki, T. Arinami, K. Takeda, T. Maki, T. Miyamoto, T. Kawashima, K. Kobayashi, and H. Hamaguchi. 1997. Evidence for linkage between asthma/atopy in childhood and chromosome 5q31–q33 in a Japanese population. *Am. J. Respir. Crit. Care Med. 156:1390.*
20. Temann, U. A., G. P. Geba, J. A. Rankin, and R. A. Flavell. 1998. Expression of interleukin 9 in the lungs of transgenic mice causes airway inflammation, mast cell hyperplasia, and bronchial hyperresponsiveness. *J. Exp. Med. 188:1307.*
21. Shimbara, A., P. Christodoulopoulos, A. Soussi-Gounni, R. Olivenstein, Y. Nakamura, R. C. Levitt, N. C. Nicolaides, K. J. Holroyd, A. Tsicopoulos, J. J. Lafitte, et al. 2000. IL-9 and its receptor in allergic and nonallergic lung disease: increased expression in asthma. *J. Allergy Clin. Immunol. 105:108.*
22. Cherwinski, H. M., J. H. Schumacher, K. D. Brown, and T. R. Mosmann. 1987. Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. *J. Exp. Med. 166:1229.*
23. Brown, K. D., S. M. Zurawski, T. R. Mosmann, and G. Zurawski. 1989. A family of small inducible proteins secreted by leukocytes are members of a new superfamily that includes leukocyte and fibroblast-derived inflammatory agents, growth factors, and indicators of various activation processes. *J. Immunol. 142: 679.*
24. McKenzie, A. N., J. A. Culpepper, M. de Waal, F. Briere, J. Punnonen, G. Aversa, A. Sato, W. Dang, B. G. Cocks, and S. Menon. 1993. Interleukin 13, a T-cell-derived cytokine that regulates human monocyte and B-cell function. *Proc. Natl. Acad. Sci. USA 90:3735.*
25. Burd, P. R., W. C. Thompson, E. E. Max, and F. C. Mills. 1995. Activated mast cells produce interleukin 13. *J. Exp. Med. 181:1373.*
26. Zurawski, G., and J. E. de Vries. 1994. Interleukin 13, an interleukin 4-like cytokine that acts on monocytes and B cells, but not on T cells. *Immunol. Today 15:19.*

27. Wills-Karp, M., J. Luyimbazi, X. Xu, B. Schofield, T. Y. Neben, C. L. Karp, and D. D. Donaldson. 1998. Interleukin-13: central mediator of allergic asthma. *Science 282:2258.*
28. Grunig, G., M. Warnock, A. E. Wakil, R. Venkayya, F. Brombacher, D. M. Rennick, D. Sheppard, M. Mohrs, D. D. Donaldson, R. M. Locksley, et al. 1998. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science 282:2261.*
29. Zhu, Z., R. J. Homer, Z. Wang, Q. Chen, G. P. Geba, J. Wang, Y. Zhang, and J. A. Elias. 1999. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J. Clin. Invest. 103:779.*
30. Takakuwa, T., H. P. Knopf, A. Sing, R. Carsetti, C. Galanos, and M. A. Freudenberg. 1996. Induction of CD14 expression in Lpsn, Lpsd and tumor necrosis factor receptor-deficient mice. *Eur. J. Immunol. 26:2686.*
31. Reisbach, G., I. Bartke, B. Kempkes, G. Kostka, J. Ellwart, A. Birner, K. Thalmeier, R. Mailhammer, G. W. Bornkamm, and A. Ullrich. 1993. Characterization of hemopoietic cell populations from human cord blood expressing c-kit. *Exp. Hematol. 21:74.*
32. Hansen, A. S., O. Noren, H. Sjostrom, and O. Werdelin. 1993. A mouse aminopeptidase N is a marker for antigen-presenting cells and appears to be co-expressed with major histocompatibility complex class II molecules. *Eur. J. Immunol. 23:2358.*
33. Mazurek, N., H. Schindler, T. Schurholz, and I. Pecht. 1984. The cromolyn binding protein constitutes the Ca^{2+} channel of basophils opening upon immunological stimulus. *Proc. Natl. Acad. Sci. USA 81:6841.*
34. Eshhar, Z., M. Ofarim, and T. Waks. 1980. Generation of hybridomas secreting murine reaginic antibodies of anti- DNP specificity. *J. Immunol. 124:775.*
35. Baniyash, M., and Z. Eshhar. 1984. Inhibition of IgE binding to mast cells and basophils by monoclonal antibodies to murine IgE. *Eur. J. Immunol. 14:799.*
36. Renauld, J. C., A. Goethals, F. Houssiau, H. Merz, E. Van Roost, and J. Van Snick. 1990. Human P40/IL-9. Expression in activated $CD4^+$ T cells, genomic organization, and comparison with the mouse gene. *J. Immunol. 144: 4235.*
37. Suhasini, M., C. D. Reddy, E. P. Reddy, J. A. DiDonato, and R. B. Pilz. 1997. cAMP-induced NF-κB (p50/relB) binding to a c-myb intronic enhancer correlates with c-myb up-regulation and inhibition of erythroleukemia cell differentiation. *Oncogene 15:1859.*
38. Altschmied, J., and J. Duschl. 1997. Set of optimized luciferase reporter gene plasmids compatible with widely used CAT vectors. *BioTechniques 23:436.*
39. Klein-Hessling, S., G. Schneider, A. Heinfling, S. Chuvpilo, and E. Serfling. 1996. HMG I(Y) interferes with the DNA binding of NF-AT factors and the induction of the interleukin 4 promoter in T cells. *Proc. Natl. Acad. Sci. USA 93:15311.*
40. Pessara, U., and N. Koch. 1990. Tumor necrosis factor alpha regulates expression of the major histocompatibility complex class II-associated invariant chain by binding of an NF-κB-like factor to a promoter element. *Mol. Cell Biol. 10:4146.*
41. Stassen, M., M. Arnold, L. Hultner, C. Muller, C. Neudorfl, T. Reineke, and E. Schmitt. 2000. 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. *J. Immunol. 164:5549.*
42. Scott, M. G., A. C. Vreugdenhil, W. A. Buurman, R. E. Hancock, and M. R. Gold. 2000. Cutting edge: cationic antimicrobial peptides block the binding of lipopolysaccharide (LPS) to LPS binding protein. *J. Immunol. 164:549.*
43. Traenckner, E. B., S. Wilk, and P. A. Baeuerle. 1994. A proteasome inhibitor prevents activation of NF-κB and stabilizes a newly phosphorylated form of IκB-α that is still bound to NF-κB. *EMBO J. 13:5433.*
44. Marquardt, D. L., and L. L. Walker. 2000. Dependence of mast cell IgE-mediated cytokine production on nuclear factor-κB activity. *J. Allergy Clin. Immunol. 105: 500.*
45. Zhu, Y. X., L. Y. Kang, W. Luo, C. C. H. Li, L. Yang, and Y. C. Yang. 1996. Multiple transcription factors are required for activation of human interleukin 9 gene in T cells. *J. Biol. Chem. 271:15815.*
46. Anderson, K. V. 2000. Toll signaling pathways in the innate immune response. *Curr. Opin. Immunol. 12:13.*
47. Qureshi, S. T., L. Lariviere, G. Leveque, S. Clermont, K. J. Moore, P. Gros, and D. Malo. 1999. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J. Exp. Med. 189:615.*
48. Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J. Immunol. 162:3749.*
49. O'Neill, L. A., and C. A. Dinarello. 2000. The IL-1 receptor/toll-like receptor superfamily: crucial receptors for inflammation and host defense. *Immunol. Today 21:206.*
50. Tanaka, C., H. Kamata, H. Takeshita, H. Yagisawa, and H. Hirata. 1997. Redox regulation of lipopolysaccharide (LPS)-induced interleukin-8 (IL-8) gene expression mediated by NFκB and AP-1 in human astrocytoma U373 cells. *Biochem. Biophys. Acta 232:568.*
51. Dokter, W. H. A., S. B. Koopmans, and E. Vellenga. 1996. Effects of IL-10 and IL-4 on LPS-induced transcription factors (AP-1, NF-IL6 and NF-κB) which are involved in IL-6 regulation. *Leukemia 10:1308.*
52. Meng, F., and C. A. Lowell. 1997. Lipopolysaccharide (LPS)-induced macrophage activation and signal transduction in the absence of Src-family kinases Hck, Fgr, and Lyn. *J. Exp. Med. 185:1661.*
53. Vink, A., G. Warnier, F. Brombacher, and J. C. Renauld. 1999. Interleukin 9-induced in vivo expansion of the B-1 lymphocyte population. *J. Exp. Med. 189: 1413.*
54. Levitt, R. C., M. P. McLane, D. MacDonald, V. Ferrante, C. Weiss, T. Zhou, K. J. Holroyd, and N. C. Nicolaides. 1999. IL-9 pathway in asthma: new therapeutic targets for allergic inflammatory disorders. *J. Allergy Clin. Immunol. 103: S485.*
55. Shinkai, A., H. Yoshisue, M. Koike, E. Shoji, S. Nakagawa, A. Saito, T. Takeda, S. Imabeppu, Y. Kato, N. Hanai, et al. 1999. A novel human CC chemokine, eotaxin-3, which is expressed in IL-4-stimulated vascular endothelial cells, exhibits potent activity toward eosinophils. *J. Immunol. 163:1602.*
56. Kumagai, N., K. Fukuda, Y. Ishimura, and T. Nishida. 2000. Synergistic induction of eotaxin expression in human keratocytes by TNF-α and IL-4 or IL-13. *Invest. Ophthalmol. Vis. Sci. 41:1448.*
57. Terada, N., N. Hamano, T. Nomura, T. Numata, K. Hirai, T. Nakajima, H. Yamada, O. Yoshie, T. Ikeda-Ito, and A. Konno. 2000. Interleukin-13 and tumour necrosis factor-α synergistically induce eotaxin production in human nasal fibroblasts. *Clin. Exp. Allergy 30:348.*
58. Li, L., Y. Xia, A. Nguyen, Y. H. Lai, L. Feng, T. R. Mosmann, and D. Lo. 1999. Effects of Th2 cytokines on chemokine expression in the lung: IL-13 potently induces eotaxin expression by airway epithelial cells. *J. Immunol. 162:2477.*
59. Dong, Q., J. Louahed, A. Vink, C. D. Sullivan, C. J. Messler, Y. Zhou, A. Haczku, F. Huaux, M. Arras, K. J. Holroyd, et al. 1999. IL-9 induces chemokine expression in lung epithelial cells and baseline airway eosinophilia in transgenic mice. *Eur. J. Immunol. 29:2130.*
60. Michel, O., R. Ginanni, B. Le Bon, J. Content, J. Duchateau, and R. Sergysels. 1992. Inflammatory response to acute inhalation of endotoxin in asthmatic patients. *Am. Rev. Respir. Dis. 146:352.*
61. Michel, O., R. Ginanni, and R. Sergysels. 1995. Protective effect of sodium cromoglycate on lipopolysaccharide-induced bronchial obstruction in asthmatics. *Int. Arch. Allergy Immunol. 108:298.*
62. Oehling, A. K. 1999. Bacterial infection as an important triggering factor in bronchial asthma. *J. Invest. Allergol. Clin. Immunol. 9:6.*
63. Dubin, W., T. R. Martin, P. Swoveland, D. J. Leturcq, A. M. Moriarty, P. S. Tobias, E. R. Bleecker, S. E. Goldblum, and J. D. Hasday. 1996. Asthma and endotoxin: lipopolysaccharide-binding protein and soluble CD14 in bronchoalveolar compartment. *Am. J. Physiol. 270:L736.*
64. Virchow, J. C. J., P. Julius, H. Matthys, C. Kroegel, and W. Luttmann. 1998. CD14 expression and soluble CD14 after segmental allergen provocation in atopic asthma. *Eur. Respir. J. 11:317.*
65. Garty, B. Z., Y. Monselise, and M. Nitzan. 2000. Soluble CD14 in children with status asthmaticus. *Isr. Med. Assoc. J. 2:104.*
66. Donovan, C. E., D. A. Mark, H. Z. He, H. C. Liou, L. Kobzik, Y. Wang, G. T. De Sanctis, D. L. Perkins, and P. W. Finn. 1999. NF-κB/Rel transcription factors: c-Rel promotes airway hyperresponsiveness and allergic pulmonary inflammation. *J. Immunol. 163:6827.*