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In Activated Mast Cells, IL-1 Up-Regulates the Production of Several Th2-Related Cytokines Including IL-9

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Mast cells can play detrimental roles in the pathophysiology and mortality observed in anaphylaxis and other Th2-dominated allergic diseases. In contrast, these cells contribute to protective host defense mechanisms against parasitic worm infections. After IgE/Ag activation, mast cells can produce multiple cytokines that may enhance allergic inflammations, while a similar panel of Th2-related cytokines may support immunological strategies against parasites. Here we report that in primary mouse bone marrow-derived mast cells activated by ionomycin or IgE/Ag, the proinflammatory mediator IL-1 (α or β) up-regulated production of IL-3, IL-5, IL-6, and IL-9 as well as TNF, i.e., cytokines implicated in many inflammatory processes including those associated with allergies and helminthic infections. IL-1 did not induce significant cytokine release in the absence of ionomycin or IgE/Ag, suggesting that Ca-dependent signaling was required. IL-1-mediated enhancement of cytokine expression was confirmed at the mRNA level by Northern blot and/or RT-PCR analysis. Our study reveals a role for IL-1 in the up-regulation of multiple mast cell-derived cytokines. Moreover, we identify mast cells as a novel source of IL-9. These results are of particular importance in the light of recent reports that strongly support a central role of IL-9 in allergic lung inflammation and in host defense against worm infections. The Journal of Immunology, 2000, 164: 5556–5563.

Materials and Methods

Mice

BALB/c mice were bred in our animal facilities under specific pathogen-free conditions until the age of 6 wk and then kept under conventional conditions (GSF, Munich, Germany). Mice of both sexes were used as bone marrow donors at ages of 8–28 wk.
Cytokines and Abs

Recombinant murine (mu) kit-ligand (KL) was expressed in Escherichia coli and purified by affinity chromatography as described by Reisbach et al. (43). The following cytokines were commercially obtained as listed: rmuIL-1α, rmuTNF, and rat anti-mouse IL-4 mAb (code 1688-01; Genzyme, Boston, MA), rmuIL-3 (Bachem Biochemica, Heidelberg, Germany), recombinant human (hu) IL-6 (Life Technologies, Grand Island, NY), rhuIL-1 receptor antagonist (IL-1RA), and rmuIL-9 (British Biotechnology, Oxford, U.K.). RmuIL-4 was provided by Dr. W. Müller (Institut für Genetik, Universität Köln, Köln, Germany). RmuIL-1α was a kind gift from Dr. R. Munker (Med. Klinik III, Universitätssklinikum Grosshadern, München, Germany) and originated from Hoffmann-La Roche (Basel, Switzerland). RmuIL-1β (commercial product from Genzyme) was pro- vided by Dr. R. Munker (Klinik and Poliklinik für Hals-, Nasen- und Ohren- kranke, Universitätssklinikum Grosshadern, München, Germany). The neu- tralizing rat anti-muIL-3 Ab 19B3.1 (44) was a kind gift of Dr. J. Abrams (DNAX, Palo Alto, CA). The rat anti-muIL-6 Ab 6B4 (45) was provided by Dr. J. Van Snick (Ludwig Institute for Cancer Research, Brussels, Belgium). As a source of rmuIL-3 or rmuIL-5, we used batches of supernatants derived from X63Ag8-653 myeloma cells transfected with a retroviral vec- tor carrying the mouse IL-3 or IL-5 genes (46). These transfected cell lines were kindly provided by Dr. F. Melchers (Basel Institute for Immunology, Basel, Switzerland). Hybridoma cells secreting DNP33-BSA-specific IgE Ab were originally developed at the Weizmann Institute of Science (Re- hovot, Israel) (47) and kindly provided by Dr. B. Reck (Max Planck Institut für Immunobiologie, Freiburg, Germany). Anti-DNP-BSA IgE was purified by separation on a protein G column.

Mast cell cultures

BALB/c bone marrow cells were suspended at 5 × 10^6/ml in RPMI 1640 medium including 20% FCS, 2 mM l-glutamine, 100 U/ml penicillin- streptomycin, 10–3 M 2-mercaptoethanol, and 1 ml/mL rmuIL-3 (pretested super- natant from IL-3 gene-transfected X63Ag8-653 cells (46) containing about 1000 U/ml IL-3) and then distributed into 96-well microplates (Nunc, Wiesbaden, Germany) (200 µl/well) and incubated at 37°C in a fully hu- manized atmosphere (10% CO2 in air). After 14 days, all nonadherent cells from two 96-well plates were pooled and transferred to culture flasks containing fresh culture medium (50 ml/flask). Following another 2 wk in culture, we regularly obtained suspensions of apparently homogeneous populations of BMMC (97–100% Alcian blue/5′Aminoflavin) as described previously (48).

Cytokine induction experiments

If not otherwise indicated, primary BMMC (in vitro age, 4 wk) were grown in the presence of IL-3, BMMC were washed twice, suspended at 1 × 10^6 cells/ml in RPMI 1640 medium including 1 U/ml rmuIL-3 (a dose war- ranteeing cellular survival) and the other supplements described above, and then transferred into 24-well plates (Nunc) (0.5 ml/well) containing the Ca-ionophore ionomycin (Sigma, St. Louis, MO) and/or the potentially activating cytokines to be tested in 5–10 µl volumes (replicate wells/ group). Ionomycin and various cytokines were tested at the following range of concentrations (given in parentheses): ionomycin (0.25–4.0 µM), rmIL-1α (0.1–10 U/ml), rmIL-1β, rmIL-1β, and rmIL-6 (0.1–1.0 ng/ ml, respectively), rmTNF-α (0.2–20.0 ng/ml). In experiments with rmIL- 1α (20 ng/ml finally) or anti-IL-4R mAb (20 µg/ml finally), the mast cells had been preincubated (2 h; 37°C) with these agents before the addition of IL-1 and ionomycin. To study the effects of IL-1 in mast cells activated via cross-linking of their high-affinity FcεRI, 24-well plates were coated with 10 µg DNP-BSA/0.5 ml PBS/well and incubated overnight at 4°C. The coated wells were washed twice with PBS (1 ml/well). Mast cells (10 × 10^5/ml) were preincubated in a shaking water bath (1h; 37°C) with anti-DNP-BSA-specific IgE (10 µg/ml finally, diluted in Tyrode’s buffer containing 0.05% gelatin). Then the cells were washed twice in PBS and suspended at 1 × 10^6/ml in RPMI 1640 medium including the sup- plements described above but lacking IL-3. The cell suspension was plated (0.5 ml/well, 2 wells/group) in the absence or presence of IL-1 into Ag- coated wells and incubated at 37°C (10% CO2 in air). Cell-free superna- tants were usually harvested after 24 or 48 h and stored at −20°C until assayed.

Cell lines and cytokine bio-assays

The physiological activities of several murine cytokines were quantitated using specific indicator cell lines and purified recombinant reference cytokines in short-term proliferation assays (MTT test (49) or measurement of [3H]hy- midine uptake) defining 1 U/ml as a cytokine concentration provoking a half-maximum response in the respective assay (for details see the refer- ences cited). The following cell lines have been employed: 32Dcl.23 for IL-3 (22), 7TD1 for IL-6 (45), and TS1.C3 (19) or ST2/K8.42 (26) for IL-9 measurements. A standard cytokotoxicity assay for TNF bioactivity us- ing a TNF-sensitive L929 fibroblast cell line was used as described (50). The specificities of the biological assays were confirmed employing neu- tralizing doses of specific anti-mouse cytokine mAb. At the relevant con- centrations, the agents and cytokines tested in the cytokine induction assays did not interfere with cytokine activities in the different biological assays.

Murine cytokine-specific ELISA tests

The two-site ELISA tests employed in the present study have been de- scribed recently as described in the references cited below. The following anti-mouse cytokine Abs have been used: affinity-purified anti-mouse IL-4 mAb 11B11, rabbit anti-mouse IL-4 antiserum, as well as biotinylated swine anti-rabbit antiserum (Dakopatts, Hamburg, Germany) (51), affinity purified anti-muIL-5 mAb TRFK5 and biotinylated anti-muIL-5 mAb TRFK4 (26), hamster anti-muIL-9 mAb C12 (gift of Dr. J. Van Snick, Ludwig Institute, Brussels, Belgium), and biotinylated rat anti-muIL-9 mAb 229.4 (28).

Northern blot analysis

Total cellular RNA was prepared from BMMC by MMC with the single step acid guanidinium thiocyanate-phenol-chloroform extraction method (52). RNA was glyoxylated and electrophoresed through a 1.0% agarose gel and blotted by vacuum blotting onto nylon membranes (Hybond-N; Amersham, Braunschweig, Germany). Hybridization and stringency washes of blots were performed as previously described (53). The probe used for hybridization was a cDNA fragment of murine IL-9 (20) (0.36-kb NcoI-BamHI fragment; kindly provided by Dr. J. Van Snick), which had been labeled with [32P]dCTP by the random priming method (Megaprime DNA labeling system; Amersham). Transfer efficiency was controlled by an additional hybridization to a mouse 28S rRNA probe, kindly provided by Dr. I. Grummt (German Cancer Research Center, Heidelberg, Germany). Auto- radiographic analysis was performed with the Fuji digital imaging system (exposition on Fuji imaging plates and subsequent evaluation with a Fujix BAS1000 Bio-Imaging Analyzer; Fuji, Düsseldorf, Germany). The amounts of IL-9 mRNA were normalized based on 28S RNA levels.

RT-PCR analysis

The primer pairs and the method used to detect transcripts for the house-keeping gene GAPDH and for specific mouse cytokine genes (IL-3, IL-6, IL-9) have been described previously (54, 55).

Results

IL-1 acts as a potent costimulator of cytokine production in mast cells activated by ionomycin

Because mast cells are regarded as important cellular regulators and effectors of many immunological and inflammatory reactions, which also involve the action of various proinflammatory media- tors (e.g., IL-1, IL-6, TNF), we were interested in potential influ- ences of these mediators on mast cell cytokine production. BMMC generated in the presence of IL-3 were activated following a stand- ard protocol (ionomycin (1 µM), 24 h) in the absence or presence of rmIL-1α (2 U/ml). Mast cell supernatants were then tested for a panel of different cytokines (i.e., IL-3, IL-4, IL-5, IL-6, IL-9, and TNF) employing specific ELISAs or biological assays. As exemplified for IL-3, IL-6, IL-9, and TNF in Fig. 1, there was no sub- stantial constitutive cytokine production and only moderate cyto- kine production after activation with ionomycin alone. However, when in addition to the Ca-ionophore also rmIL-1α (2 U/ml) was provided during the induction period, substantially higher concen- trations of IL-3, IL-6, IL-9, and TNF were measured in 24-h super- nutants (Fig. 1). In contrast, no significant differences were found between ionomycin-induced IL-4 levels of mast cell superna- tants in the presence or absence of rmIL-1α as a coactivating agent (data not shown). In the absence of the Ca-ionophore, IL-1 induced only small amounts of IL-6 but no other cytokines (Fig. 1), indicating that Ca-dependent signaling was required for cyto- kine induction by IL-1. This IL-1 effect was dose dependent, as

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illustrated for IL-3, IL-5, IL-6, and IL-9 induction with a maximum at 1–10 U/ml IL-1α (Fig. 2), and highly specific, as preincubation (1 h) of BMMC with 20 ng/ml rhuIL-1Ra abolished the action of a saturating dose of rhuIL-1α (2.5 U/ml), an effect that could be efficiently counteracted by increasing the IL-1 dose (demonstrated for IL-9 production in Fig. 3). A similar enhancement of cytokine production in ionomycin-activated BMMC was noted with rhuIL-1β or rmuIL-1β (saturating maximum effects at 1.0–10 ng/ml) but not with rhuIL-6 (0.1–10.0 ng/ml) or rmuTNF (0.2–20.0 ng/ml) (data not shown).

To exclude a contribution of non-mast cells to cytokine production in our system, we developed a panel of primary BMMC clones from limiting dilution cultures of BALB/c bone marrow cells in 96-well microtiter plates under conditions (500 cells seeded per well) warranting a statistical probability of 95% that the mast cell populations were of clonal origin. In pilot limiting dilution experiments, the frequency of IL-3-responsive mast cell progenitors in BALB/c bone marrow cells was found to be about 1/5000, slightly lower than in our earlier reports when PWM-stimulated spleen cell-conditioned medium had been used as a source of mast cell growth factors (48, 54). As shown in Fig. 4, most of the 20 primary mast cell clones analyzed were able to produce variable amounts of IL-3, IL-6, and IL-9 upon activation with ionomycin plus IL-1, while some of them produced exceptionally high cytokine levels. All these ionomycin-activated BMMC clones produced substantially lower levels in the absence of IL-1 (data not shown).

Kinetics of cytokine production after activation of mast cells with ionomycin/IL-1

When ionomycin/IL-1-activated BMMC were followed over an induction period of 6–72 h, a remarkable difference in the kinetics of IL-9 production was observed compared with all other cytokines analyzed. As shown for IL-3, IL-5, and IL-6, these bio-activities increased almost constantly over time without a significant time delay after activation, whereas IL-9 production was clearly delayed with a very moderate increase from 6 to 24 h and a dramatic increase from 24 to 48 h (Fig. 5).

Kinetics of IL-9 mRNA expression in mast cells activated by ionomycin or ionomycin plus IL-1

Fig. 6 shows a typical result of a Northern blot analysis of IL-9 expression in mast cells activated for 12, 24, or 48 h with either
Ionomycin alone or with ionomycin plus IL-1. In mast cells activated by ionomycin alone, IL-9 mRNA expression could not be detected before 48 h. In contrast, after activation with ionomycin plus IL-1, a faint IL-9-specific signal was already observed after 12 h and strong additional increases of IL-9 mRNA expression were seen after 24 and 48 h (Fig. 6). An IL-1-mediated enhanced expression of IL-9- as well as IL-3- and IL-6-specific mRNAs was also observed by RT-PCR analysis of activated BMMC (data not shown).

**Autocrine role of IL-4 for cytokine production in ionomycin/IL-1-activated mast cells**

The delayed kinetics of IL-9 mRNA expression (Fig. 6) and IL-9 protein secretion (Fig. 5) in mast cells activated by ionomycin plus IL-1 and the known enhancing effects of IL-4 on IL-9 production by activated murine and human T cells (28, 29) prompted us to investigate a possible autocrine role of IL-4 in IL-9 production by mast cells. As shown in Fig. 7, significantly reduced IL-9 levels were measured 48 h after activation of BMMC with ionomycin/IL-1 when the cells were treated with an Ab blocking the mouse IL-4 receptor (anti-IL-4R mAb). In contrast, the decline of the IL-4 levels observed in the absence of anti-IL-4R mAb from 24 to 48 h after mast cell activation could be completely prevented in its presence, indicating that substantial amounts of endogenously produced IL-4 were consumed by the mast cells 24 to 48 h after activation (Fig. 7). Moreover, this anti-IL-4R mAb also reduced the levels of IL-3 and IL-6, revealing the autocrine potential of endogeneous IL-4 to further enhance the production of these cytokines in activated mast cells (Fig. 7).

**Following maturation toward a connective tissue-type phenotype in vitro, activated BMMC have the capacity to produce higher amounts of cytokines in response to IL-1**

It is generally accepted that BMMC produced and maintained in vitro in the presence of IL-3 represent a population of relatively immature c-kit-expressing mast cells with the potential to further differentiate either along the mucosal or the connective tissue-type

**FIGURE 4.** Analysis of cytokine production (IL-3, IL-6, and IL-9) in a panel of primary mast cell clones derived from limiting dilution cultures. Individual clones (numbered 1–20) and a polyclonal BMMC population (P) (2 × 10⁶ cells/ml) were stimulated with ionomycin (1 μM) plus rhuIL-1α (10 U/ml) for 48 h. Replicate (n = 3) mast cell supernatants were analyzed for the indicated cytokines using bio-assays. Demonstrated are mean values, SD < 10% (not shown).

**FIGURE 5.** Kinetics of cytokine production in mast cells activated with ionomycin (1 μM) plus rhuIL-1α (10 U/ml). Shown are mean values ± SD (n = 3) of cytokine concentrations measured in bio-assays (IL-3, IL-6, and IL-9) or by ELISA (IL-5).

**FIGURE 6.** Northern blot analysis of the kinetics of IL-9 mRNA expression in mast cells following activation with ionomycin (1 μM) in the absence or presence of rhuIL-1α (10 U/ml). For details, see Materials and Methods.
mast cell lineage both in vitro and in vivo (1). Therefore, we tested the cytokine-producing capacity of BMMC cultured for 2 wk with IL-3 and then switched for another 2 wk to different cytokine conditions (IL-3 or IL-3/IL-4 or IL-3/IL-4/KL). Compared with BMMC grown in IL-3 alone, BMMC generated in the additional presence of IL-4 or IL-4 plus KL are known to contain significantly higher concentrations of histamine (56, 57), and a substantial proportion of them stained positively with Safranine (Table I), indicating the presence of phenotypically more mature cells with some characteristics of connective tissue-type or serosal mast cells. Compared with activated BMMC grown in IL-3 alone, those BMMC grown with IL-3/IL-4 and more pronounced with IL-3/IL-4/KL displayed a strikingly increased capacity to secrete IL-3, IL-6, and IL-9 in response to ionomycin/IL-1, roughly correlating with the grade of Safranine positivity of the different mast cell populations tested (BMMC_IL-3/IL-4/KL > BMMC_IL-3/IL-4 > BMMC_IL-3) (Fig. 8 and Table I). However, rather low and quite comparable amounts of these cytokines were produced by these different groups of BMMC populations after activation with ionomycin alone (Fig. 8).

**Table I.** Proportion of Alcian blue<sup>+</sup> mast cells staining positively with Safranine in BMMC cultures generated in the presence of different cytokines (week 1 and 2, IL-3 only; week 3 and 4, IL-3 or IL-3/IL-4 or IL-3/IL-4/KL, or IL-3/IL-4/KL) at the following final cytokine concentrations: rmuIL-3 (100 U/ml), rmuIL-4 (10 ng/ml), rmuKL (200 ng/ml). After 4 wk, BMMC were stimulated for 48 h with ionomycin (1 μM) alone (□) or ionomycin plus rhuIL-1α (10 U/ml) (●). Shown are mean values ± SD of cytokine concentrations measured in duplicate mast cell supernatants derived from three separate cytokine induction experiments, each performed with BMMC generated independently with either IL-3, IL-3/IL-4, or IL-3/IL-4/KL. Representative aliquots of the cultured mast cells were phenotyped using Safranine/Alcian blue-stained cytoslides (see results in Table I).

**FIGURE 7.** Production of IL-3, IL-6, and IL-9 in IL-3-grown mast cells activated by ionomycin (1 μM) plus rhuIL-1α (10 U/ml) for 24 or 48 h in the presence of a mAb blocking the mouse IL-4 receptor (anti-IL-4R, 20 μg/ml) (●) or in the presence of a control Ab (□). Shown are mean cytokine values ± SD (n = 3) determined in bio-assays (IL-3 and IL-6) or by ELISA (IL-4 and IL-9). A dose of 1 ng/ml IL-9 in the ELISA corresponded to 10 U/ml IL-9 in the bio-assay. A second experiment with independently generated BMMC resulted in very similar results.

**FIGURE 8.** Analysis of IL-3, IL-6, and IL-9 production in BMMC generated in the presence of different cytokines (week 1 and 2, IL-3 only; week 3 and 4, IL-3 or IL-3/IL-4 or IL-3/IL-4/KL). In BMMC cultures, the cytokines were used at the final concentrations indicated in parentheses: rmuIL-3 (100 U/ml), rmuIL-4 (10 ng/ml), rmuKL (200 ng/ml). After 4 wk, BMMC were stimulated for 48 h with ionomycin (1 μM) alone (□) or ionomycin plus rhuIL-1α (10 U/ml) (●). Shown are mean values ± SD of cytokine concentrations measured in duplicate mast cell supernatants derived from three separate cytokine induction experiments, each performed with BMMC generated independently with either IL-3, IL-3/IL-4, or IL-3/IL-4/KL. Representative aliquots of the cultured mast cells were phenotyped using Safranine/Alcian blue-stained cytoslides (see results in Table I).

**IL-1 enhances production of IL-9 and other Th2-related cytokines in mast cells activated by IgE/Ag**

We next determined whether IL-1 would be able to augment cytokine production of mast cells under more physiological conditions in vitro, i.e., following activation via cross-linking of their...
Our finding that activated mast cells costimulated with IL-1 are able to secrete high amounts of IL-9 in vitro may have potential autocrine actions (e.g., IL-3 and IL-9). We think that IL-1 could provide a powerful coactivating stimulus to mast cells in the course of a variety of inflammatory reactions including allergic inflammation as well as host immune responses to worm parasites (1–3), while their life-saving role in bacterial infections was discovered only recently (67–69).

While we (22–25) and others (70) have previously described effects of IL-9 on the growth and functional activity of murine mast cells in vitro, recent reports on IL-9 transgenic mice confirmed the mastocytosis-inducing activity of this cytokine in vivo (71) and supported the idea that IL-9-driven mast cells can help to resolve experimental helminthic infections (72, 73). Moreover, recently IL-9 was suggested as a candidate gene for asthma (32), a hypothesis additionally strengthened by experimental results with IL-9 transgenic mice challenged with allergens in vivo (33, 34).

We think that IL-1 could provide a powerful coactivating stimulus to mast cells in the course of a variety of inflammatory reactions and infectious diseases, particularly including helminthic infections and allergic inflammations. Mast cells may then augment inflammatory cascades by the enhanced secretion of cytokines (e.g., IL-3, IL-5, IL-6, IL-9, and TNF) with paracrine and even autocrine actions (e.g., IL-3 and IL-9).

Our finding that activated mast cells costimulated with IL-1 are able to secrete high amounts of IL-9 in vivo may have potential clinical implications in the light of recent reports emphasizing important roles of IL-9 both in host defense against worm parasites (72, 73) and in allergic inflammation (32–34).
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