

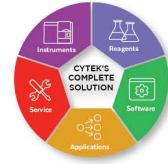


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Eotaxin-2 Generation Is Differentially Regulated by Lipopolysaccharide and IL-4 in Monocytes and Macrophages¹

Kimiko Watanabe, Peter J. Jose, and Sara M. Rankin²

The eotaxins are a family of CC chemokines that coordinate the recruitment of inflammatory cells, in particular eosinophils, to sites of allergic inflammation. The cDNA for eotaxin-2 (CC chemokine ligand 24) was originally isolated from an activated monocyte library. In this study, we show for the first time that peripheral blood monocytes generate bioactive eotaxin-2 protein constitutively. Eotaxin-2 production was significantly up-regulated when monocytes were stimulated with the proinflammatory cytokine IL-1 β and the microbial stimuli, LPS and zymosan. In contrast, the Th2 cytokines, IL-4 and IL-13, and the proinflammatory cytokine, TNF- α , acting alone or in combination, did not enhance the generation of eotaxin-2 by monocytes. Indeed, IL-4 suppressed the generation of eotaxin-2 by LPS-stimulated monocytes. Although other chemokines, including macrophage-inflammatory protein-1 α , monocyte chemoattractant protein-1, macrophage-derived chemokine, and IL-8 were generated by monocytes, eotaxin-1 (CC chemokine ligand 11) could not be detected in the supernatants of monocytes cultured in the presence or absence of any of the stimuli used in the above experiments. Furthermore, human dermal fibroblasts that produce eotaxin-1 did not generate eotaxin-2 under basal conditions or when stimulated with specific factors, including IL-4, IL-13, TNF- α , and LPS. When monocytes were differentiated into macrophages, their constitutive generation of eotaxin-2 was suppressed. Moreover, IL-4, but not LPS, up-regulated the production of eotaxin-2 by macrophages. Taken as a whole, these results support a role for macrophage-derived eotaxin-2 in adaptive immunity, with a Th2 bias. In contrast, a role for monocyte-derived eotaxin-2 is implicated in innate immunity. *The Journal of Immunology*, 2002, 168: 1911–1918.

The eotaxins are a family of CC chemokines that coordinate the recruitment of inflammatory cells, in particular eosinophils, to sites of allergic inflammation (1). To date, there are three members of this family, eotaxin-1 (CC chemokine ligand (CCL)³ 11), eotaxin-2 (CCL24), and eotaxin-3 (CCL26). Although there is low sequence homology between the eotaxins and eotaxin-1 is encoded on a different chromosome from that of eotaxin-2 and -3, in vitro, all the eotaxins have been shown to signal via the chemokine receptor, CCR3 (2–7). This receptor is highly expressed on eosinophils (3, 8, 9), and all three eotaxins have been shown to be potent eosinophil chemoattractants in vitro (2, 5–7, 10–12). More recently, CCR3 has been detected on other cell types, including basophils, mast cells, and a subpopulation of Th2 cells (13–15). As all of these cell types are involved in allergic inflammatory reactions, it is hypothesized that the eotaxins play an important role in coordinating the recruitment of all of these cell types to sites of allergic inflammation.

At present, it is not clear why there are multiple eotaxins. However, this pattern of redundancy is consistent with other chemokine/receptor pairs where multiple chemokines have been reported to bind to single chemokine receptors (16). It has been argued that this redundancy renders the chemokine system robust. As such, changes in the quantity or quality of chemokines or their receptors, for example, due to genetic variations, are unlikely to have dramatic effects. The existence of multiple ligands may also be a countermeasure to parasite host defense evasion mechanisms (17). However, although in vitro studies suggest that the eotaxins have redundant functions, the production of chemokines may occur in a coordinated manner such that they may function at distinct stages of disease progression (18). For instance, a study of late-phase allergic reactions in the skin of atopic subjects suggests that there is a temporal difference in the generation of eotaxin-1 and -2. Thus, up-regulation of eotaxin-1 was observed after 6 h, whereas an increase in eotaxin-2 was not evident until 24 h (19). Therefore, it was proposed that the generation of eotaxin-2 may be associated with a more sustained influx of eosinophils into these sites. Similarly, in another study, it was reported that there was a selective up-regulation of eotaxin-3 in bronchial biopsies of asthmatic subjects 24 h after allergen challenge (20). Therefore, it was proposed that eotaxin-3 rather than eotaxin-1 or -2 may account for the ongoing influx of eosinophils to the asthmatic lungs in the late phase of the allergic response. Taken together, these studies suggest that generation of the three eotaxins occurs at distinct times during the course of the allergic inflammatory reaction, hence they may be responsible for different phases of leukocyte recruitment.

In contrast to the idea that the eotaxins function in a coordinated manner, it is also possible that the three forms of eotaxin exhibit other distinct biological functions in vivo. It has been reported, for example, that eotaxin-2 inhibits the proliferation of myeloid progenitor cells, a function not apparently shared with the other eotaxins (4).

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³ Abbreviations used in this paper: CCL, CC chemokine ligand; HDF, human dermal fibroblasts; FSC, forward scatter; MCM, monocyte-conditioned media; MCP-1, monocyte chemoattractant protein-1; MDC, macrophage-derived chemokine; MIP-1 α , macrophage-inflammatory protein-1 α ; DiI-labeled acetyl-LDL, acetylated low-density lipoprotein labeled with 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate.

Eotaxin-1 was originally identified as the sole eosinophil chemoattractant activity in the bronchoalveolar lavage fluid of allergen-challenged sensitized guinea pigs (10, 21). Subsequently, human, mouse, and rat eotaxin-1 has been cloned (2, 11, 22, 23). Studies in man and animal models have repeatedly shown a strong correlation between the presence of eotaxin-1 in a tissue and a tissue eosinophilia (1, 16). Furthermore, in animal models, the use of mice with a genetic deficiency in eotaxin-1 (24) and neutralizing mAbs to eotaxin-1 (25) have confirmed the role of this chemokine in allergic inflammatory reactions.

In contrast to the vast amount of information regarding the *in vitro* and *in vivo* function of eotaxin-1, very little is known about the role of eotaxin-2 in specific disease pathologies. Murine eotaxin-2 has been identified and has been shown to share 59% sequence identity with human eotaxin-2 (26). Ag challenge of sensitized mice or expression of IL-4 under the lung-specific Clara cell promoter led to an up-regulation of eotaxin-2 expression in the lung (26). In both instances, a lung eosinophilia was reported. A role for this chemokine in allergic inflammatory reactions in man has been implied by its detection using *in situ* hybridization and immunohistochemical techniques in late-phase allergic reactions in the skin of atopic subjects and in the bronchial mucosa of atopic and nonatopic asthmatics (19, 27). Thus, although there is evidence to suggest a functional role for this chemokine in allergic inflammation, this has not, as yet, been experimentally proven.

Insight into the role of these three eotaxins in disease pathology may be gained by identification of their cellular source and the factors that regulate their production. *In situ* hybridization and immunohistochemical studies have been used to identify the cells that express eotaxin-1 and -2 mRNA and protein. Such studies indicate that in the asthmatic lungs of man and in the lungs of allergen-challenged sensitized animals, bronchial and small airway epithelium, endothelial cells, alveolar macrophages, and airway smooth muscle cells are all potential sources of eotaxin-1 (27–29). Eotaxin-2 mRNA and protein has been detected in endothelial cells, epithelial cells, and macrophages in the bronchial mucosa of atopic and nonatopic asthmatics (27). Similar studies examining the cellular expression of eotaxin-3 in tissues have not, as yet, been reported.

The Th2 cytokines IL-4 and IL-13 have been shown to play a functional role in the generation of eotaxin-1 in animal models of allergic airways inflammation (30). Furthermore, *in vitro* studies show that certain cell types, including human dermal fibroblasts (HDF) and the epithelial cell lines BEAS2B and A549, generate eotaxin-1 protein when stimulated with IL-4 and IL-13 (31–33). Therefore, these reports are consistent with a role for eotaxin-1 in allergic inflammatory reactions.

Although the cDNA for eotaxin-2 was originally isolated from a library of activated monocytes (4–6), to date there have been no studies examining the generation of eotaxin-2 protein by defined cells *in vitro*. Thus, the cellular source of eotaxin-2 and the factors that regulate its generation have not been determined.

To gain further insight into the potential role of eotaxin-2 *in vivo*, in this study we have sought to identify the cells that generate eotaxin-2 and to investigate the factors that regulate the generation of this chemokine. We have demonstrated that monocytes and macrophages have the capacity to generate eotaxin-2. Furthermore, we show that eotaxin-2 production is differentially regulated in monocytes and macrophages.

Materials and Methods

Cells and reagents

The culture medium used was RPMI 1640 to which 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin was added (Life Technolo-

gies, Paisley, U.K.). LPS (*Escherichia coli* 026:B6), zymosan (*Saccharomyces cerevisiae* (58856-93-2)), BSA, FBS, and Histopaque 1077 were purchased from Sigma-Aldrich (Poole, U.K.). Cycloheximide was purchased from Tocris (Baldwin, MO). HBSS and HEPES were both purchased from Life Technologies. Donkey anti-rabbit Ig HRP was purchased from Amersham Pharmacia Biotech (Bucks, U.K.), and neutravidin/HRP was from Perbio Science (Tattenhall, U.K.), K Blue Substrate was from SkyBio (Wyboston, Bedfordshire, U.K.). Acetylated low-density lipoprotein labeled with 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI-labeled acetyl-LDL) was purchased from Biomedical Technologies (Stoughton, MA). Primary cultures of HDF were a gift from Dr. F. Watt (Imperial Cancer Research Fund, London, U.K.).

Cytokines, chemokines, and Abs

The human recombinant cytokines used were M-CSF, IL-4, TNF- α , and IL-1 β (all used at 10 ng/ml and purchased from PeproTech (London, U.K.)). The human recombinant chemokines used were eotaxin-1, eotaxin-2, monocyte chemoattractant protein-1 (MCP-1), macrophage-inflammatory protein-1 α (MIP-1 α), macrophage-derived chemokine (MDC), and IL-8 purchased from PeproTech. Anti-CCR3 mAb, 7B11, was a kind gift from Millenium Pharmaceuticals (Cambridge, MA). Murine IgG2a (UPC-10) isotype control Ab was from Sigma-Aldrich. The neutralizing mAb to eotaxin-2 (2G12) was a kind gift from Dr. J. White (GlaxoSmith-Kline, King of Prussia, PA).

Isolation and culture of human monocytes and macrophages

Whole blood was obtained from healthy volunteers after informed consent. Plasma was removed from citrated whole blood after centrifugation for 20 min at 250 \times g. The buffy coat was gently layered onto Histopaque 1077 and centrifuged for 30 min at 400 \times g. After separation, the mononuclear cells were collected and washed three times with HBSS with 10 mM HEPES and 0.1% BSA. Mononuclear cells (2×10^6 cells/well) were allowed to adhere in a 24-well tissue culture plate for 60 min at 37°C in serum-free RPMI 1640 medium. The nonadherent cells were subsequently gently washed off the plate, and the remaining adherent cells were cultured overnight in RPMI 1640 with 10% FBS. Surface marker (i.e., CD14 Ag) analysis revealed that the remaining adherent cell population consisted of >95% monocytes. The cells were then washed and incubated in RPMI 1640 with 0.1% BSA in the presence or absence of cytokines, LPS, and zymosan as indicated. For macrophage cultures, fresh RPMI 1640 plus 10% FBS was added to the monocyte culture after the overnight incubation and washing step. These cells were cultured for an additional 7 days in RPMI 1640 with 10% FBS supplemented with M-CSF (10 ng/ml). The latter culture conditions yielded macrophages as judged by the ability of >95% of these cells, and not monocytes, to take up DiI-labeled acetyl-LDL. Macrophages were then washed and incubated with RPMI 1640 plus 0.1% BSA with IL-4 and LPS, as indicated. For cycloheximide experiments, the cells were preincubated with 1 μ g/ml cycloheximide for 1 h before LPS stimulation.

Quantitation of chemokines by ELISA

Supernatants from cell cultures were harvested and stored at -20°C until assayed. Immunoreactive eotaxin-1 was measured by a specific sandwich ELISA as described previously (34). Immunoreactive eotaxin-2, MCP-1, MIP-1 α , MDC, and IL-8 were measured by ELISA, using matched Ab pairs purchased from R&D Systems (Abingdon, U.K.). Detection limits for the eotaxin-1, eotaxin-2, MIP-1 α , MCP-1, IL-8, and MDC ELISAs were 10, 5, 20, 5, 5, and 10 pM, respectively. Using an interassay standard, we determined that the coefficient of variation for the eotaxin-2 ELISA was <7%. Therefore, the variability in the absolute levels of eotaxin-2 produced was due to the inherent differences in monocytes prepared from different individuals.

Measurement of eosinophil shape change using flow cytometry

For isolation of mixed granulocytes for the leukocyte shape change bioassay, plasma was removed from whole blood as described above, and the erythrocytes were removed by dextran sedimentation. After centrifugation over Histopaque 1077, the mononuclear cells at the interface were discarded, and any erythrocyte contamination of the granulocyte pellet was removed by hypotonic shock lysis. Purified granulocytes were washed in HBSS containing 10 mM HEPES and 0.1% BSA. Aliquots of 5×10^5 cells were then mixed with agonists or buffer in a final volume of 100 μ l in polypropylene microtubes as previously described (35). In some experiments, Abs were added to 50 μ l of cell aliquots or agonists for 10 min before the addition of the other components of the assay. The tubes were

placed in a shaking water bath at 37°C for 4 min, then transferred immediately to an ice-water bath to stop the reaction. Cells were fixed with 250 μ l ice-cold cell fix (1/40 dilution of cell fix from BD Biosciences, Oxford, U.K.) and were analyzed on the FACSCalibur flow cytometer (BD Biosciences, Oxford, U.K.). Data was acquired on the FL-2 channel set on a sensitive setting to distinguish eosinophils from neutrophils based on their high autofluorescence. Forward scatter (FSC), side scatter, and FL-2 data were acquired and acquisition was terminated after 500 high-fluorescence (eosinophil) events. The basal eosinophil FSC was determined as the FSC exhibited by eosinophils incubated in the absence of agonists. The change in FSC was the value obtained by subtracting the basal FSC from the FSC detected after agonist stimulation of eosinophils.

Statistics

Statistical analysis was conducted using Student's *t* test, and significance was assigned at $p \leq 0.05$.

Results

Production of eotaxin-2 protein by monocytes stimulated with IL-1 β , LPS, and zymosan

The cDNA for eotaxin-2 was originally isolated from an activated monocyte library (4–6). Therefore, we examined whether human peripheral blood monocytes generated eotaxin-2 protein under basal conditions or when stimulated with specific cytokines, proinflammatory stimuli, and microbial agents. For comparison, we also monitored the generation of eotaxin-1 under these conditions. In these experiments, monocytes were incubated for 48 h at 37°C in assay medium (RPMI 1640 containing 0.1% BSA) in the presence or absence of the Th2 cytokine IL-4, the proinflammatory cytokines TNF- α and IL-1 β , and the microbial agents LPS and zymosan.

As shown in Fig. 1, there is a high basal level of eotaxin-2 detectable in the supernatant of unstimulated monocytes (0.74 nM). This suggests that monocytes generate eotaxin-2 constitutively. IL-1 β , LPS, and zymosan all stimulated a dramatic increase in the generation of eotaxin-2 by monocytes. In contrast, IL-4 and TNF- α acting alone or in combination had no significant effect on the generation of eotaxin-2 by these cells. Moreover, eotaxin-1 immunoreactivity was not detectable in the supernatant of monocytes cultured under any of these conditions (data not shown).

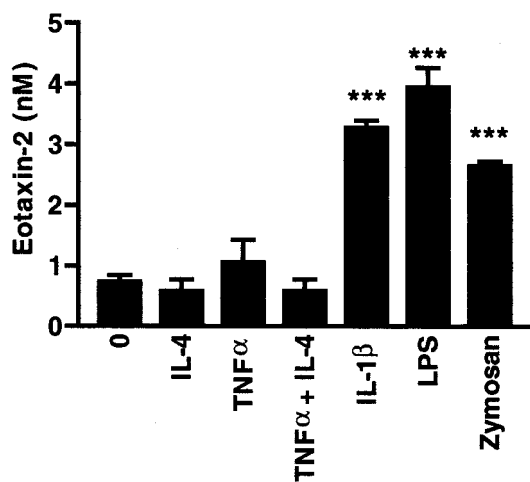


FIGURE 1. Effect of different stimuli on eotaxin-2 generation by monocytes. Monocytes were incubated in the presence or absence of IL-4, TNF- α , IL-1 β (all at 10 ng/ml), LPS (100 ng/ml), or zymosan (10⁶ particles/ml) for 48 h. Immunoreactive eotaxin-2 was assayed in the supernatants by ELISA. Bars represent the mean \pm SEM of triplicate samples. A representative graph of $n = 3$ independent experiments is shown. ***, $p < 0.001$; statistically significant increase compared with control (0) as determined by Student's *t* test.

Production of eotaxin-1 but not eotaxin-2 protein by HDF stimulated with cytokines

HDF have previously been reported to generate eotaxin-1 (31, 36, 37). To determine whether HDF also had the capacity to generate eotaxin-2, we incubated HDF with IL-4, IL-13, TNF- α , and LPS, alone or in combination, as indicated. After 48 h, the supernatant was collected and the levels of eotaxin-1 and -2 were determined by specific ELISA. From Table I, it is clear that HDF do not generate detectable levels of eotaxin-1 or -2 constitutively. We found that stimulation of HDF with IL-4, IL-13, or TNF- α induced the production of eotaxin-1, but not eotaxin-2. In addition, IL-4 and IL-13 acted synergistically with TNF- α to stimulate eotaxin-1 production, but, again, no eotaxin-2 was detected. Furthermore, LPS, a factor shown to induce eotaxin-2 generation by monocytes, did not stimulate eotaxin-1 or -2 generation by HDF (Table I). The results of these experiments indicate that eotaxin-1 and -2 are generated by distinct cell types.

Characterization of eotaxin-2 production by monocytes stimulated with LPS

To establish the kinetics of eotaxin-2 generation by monocytes, the cells were incubated in the presence or absence of LPS (100 ng/ml) for 2–72 h. The supernatant was harvested at different time points and was assayed for eotaxin-2 immunoreactivity by ELISA. The results presented in Fig. 2A indicate that monocytes generate eotaxin-2 constitutively. Stimulation of the cells with LPS resulted in an increased production of eotaxin-2 that was delayed in onset, and hence evident at 24 h but not 8 or 2 h, and continued thereafter for up to 72 h. In contrast, as previously reported (38), IL-8 was rapidly up-regulated by exposure of monocytes to LPS, with a significant increase observed at 2 h (data not shown).

To determine whether the LPS-stimulated production of eotaxin-2 was dependent on the dose of LPS, monocytes were incubated with LPS (10–10,000 ng/ml as indicated) for 96 h at 37°C. The supernatant was then harvested and assayed for eotaxin-2 immunoreactivity by ELISA. As shown in Fig. 2B, LPS induced a dose-dependent generation of eotaxin-2 by monocytes.

To establish whether the LPS-stimulated release of eotaxin-2 is from preformed stores or is dependent on de novo protein synthesis, monocytes were pretreated with 1 μ g/ml cycloheximide for 1 h and were then incubated for an additional 48 h in the presence or absence of LPS (100 ng/ml). As shown in Fig. 2C, preincubation of monocytes with cycloheximide completely abrogated the LPS-stimulated up-regulation of eotaxin-2 production by these cells and additionally reduced the constitutive generation of eotaxin-2. Thus, it appears that the generation of eotaxin-2 by monocytes is dependent on de novo protein synthesis.

Table I. Eotaxin-1 and -2 generation by HDF in response to different mediators at 48 h^a

Treatment	Eotaxin-1 (pM)	Eotaxin-2 (pM)
None	N/D ^b	N/D
IL-4	36 \pm 19	N/D
IL-13	26 \pm 15	N/D
TNF- α	17 \pm 2	N/D
TNF- α + IL-4	517 \pm 85	N/D
TNF- α + IL-13	385 \pm 121	N/D
LPS	N/D	N/D

^a HDF were incubated in DMEM with 0.1% BSA in the presence or absence of TNF- α , IL-4, IL-13 (all at 10 ng/ml), or LPS (100 ng/ml). Supernatants were harvested at 48 h and analyzed for chemokine levels by ELISA. The mean \pm SEM of three independent experiments is shown.

^b N/D, Immunoreactive protein below the level of detection.

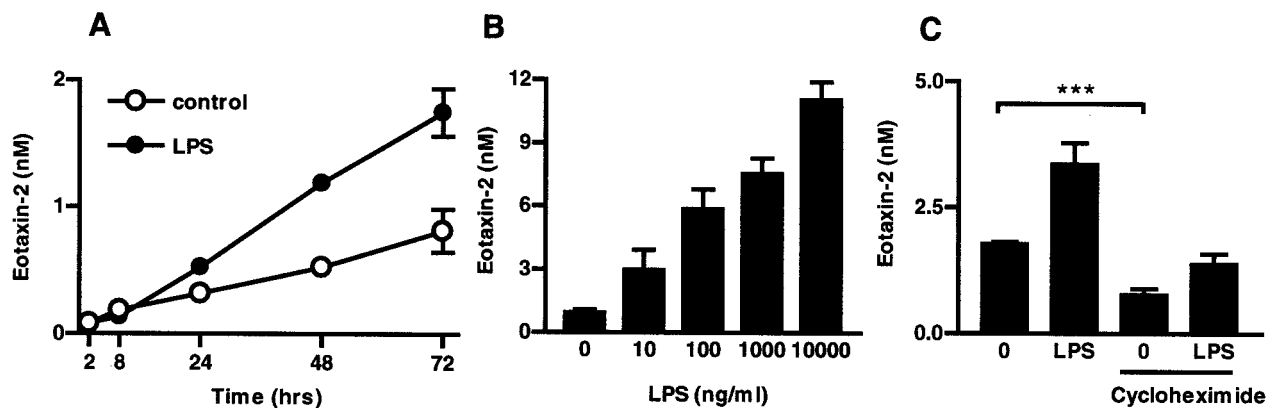


FIGURE 2. Characterization of eotaxin-2 production by monocytes stimulated with LPS. *A*, Monocytes were incubated in the presence or absence of 100 ng/ml LPS for 2–72 h. *B*, Monocytes were incubated in the presence or absence of different concentrations of LPS (0–10,000 ng/ml) for 96 h. *C*, Monocytes were preincubated with 1 µg/ml cycloheximide or medium alone for 1 h and were then incubated in the presence or absence of LPS (100 ng/ml) for 48 h. Immunoreactive eotaxin-2 in the culture supernatants was assayed by ELISA. Each point represents the mean \pm SEM of triplicate samples. A representative graph of $n = 3$ independent experiments is shown. ***, $p < 0.001$; statistically significant inhibition compared with control as determined by Student's t test.

The eotaxin-2 produced by monocytes is biologically active

In the next experiments, we set out to investigate whether the eotaxin-2 protein detected by ELISA in the supernatant of monocytes was biologically active. Monocytes were incubated for 48 h in assay medium in the absence of any stimuli. The supernatant was then harvested and stored in aliquots at -20°C for use in the bioassay. The level of eotaxin-2 immunoreactivity present in this supernatant was 1 nM as determined by ELISA. Freshly isolated mixed granulocytes were incubated at 37°C for 4 min, with either eotaxin-2 (0–0.5 nM) or dilutions of the supernatant in a FACS-based bioassay that detects changes in eosinophil shape as an increase in FSC (35). A dose-dependent shape change of eosinophils was observed in response to both eotaxin-2 and the monocyte-conditioned medium (MCM; Fig. 3, *A* and *B*). A shape change of 82 ± 1 was observed in response to 0.5 nM eotaxin-2 (Fig. 3*A*), and a shape change of 79 ± 1 was observed in response to 50% MCM (Fig. 3*B*). To determine whether the eosinophil-stimulating bioactivity present in the MCM was indeed eotaxin-2, the experiment was repeated with a single concentration of either eotaxin-2 (0.25 nM) or MCM (25%) assayed in the presence or absence of mAbs to eotaxin-1, eotaxin-2, or CCR3. The results of this experiment, shown in Fig. 3*C*, indicate that the eosinophil bioactivity present in the MCM was virtually abrogated in the presence of neutralizing mAbs to either CCR3 or eotaxin-2, whereas a neutralizing mAb to eotaxin-1 had no effect on the eosinophil-stimulating activity in the MCM. Thus, we conclude that the eotaxin-2 is bioactive and accounts for all the eosinophil-stimulating activity in the MCM.

Differential generation of eotaxin-2 by monocytes and macrophages stimulated with IL-4 and LPS

The next experiments were designed to investigate whether eotaxin-2 production is differentially regulated in monocytes and macrophages. Monocytes were cultured either overnight in RPMI containing 10% FBS or for 7 days in RPMI containing 10% FBS and 10 ng/ml M-CSF. The latter culture conditions yielded macrophages, as judged by the ability of these cells, and not monocytes, to take up DiI-labeled acetyl-LDL (data not shown). Separate cultures of monocytes and macrophages were washed and subsequently incubated in assay buffer in the presence or absence of LPS (100 ng/ml), IL-4 (10 ng/ml), or LPS plus IL-4 (at 100 and

10 ng/ml, respectively). After 48 h, the supernatant was harvested and assayed for eotaxin-1 and -2.

Eotaxin-1 immunoreactivity was not detected in the supernatants of monocytes or macrophages under basal conditions or when these cells were stimulated with either IL-4 or LPS (data not shown). In contrast, as shown above, there was a high basal generation of eotaxin-2 by monocytes that was markedly up-regulated by LPS, but not IL-4. Indeed, IL-4 inhibited the LPS-stimulated generation of eotaxin-2 (Fig. 4). The basal level of eotaxin-2 production was significantly reduced in macrophages as compared with monocytes (0.08 vs 0.27 nM, respectively). In macrophages, LPS did not enhance the generation of eotaxin-2. In contrast, stimulation of macrophages with IL-4 significantly increased their production of eotaxin-2. Furthermore, IL-4-induced eotaxin-2 production by macrophages was significantly attenuated in the presence of LPS (Fig. 4). These results indicate that there is a fundamental difference in the regulation of eotaxin-2 generation by monocytes and macrophages (Fig. 4).

Differential generation of MCP-1, MIP-1 α , IL-8, and MDC by monocytes and macrophages stimulated with IL-4 and LPS

To investigate whether the production of other chemokines was differentially regulated in monocytes and macrophages, we determined the levels of MCP-1, MIP-1 α , IL-8, and MDC in the supernatants derived from the monocyte and macrophage cultures described above. It was found that a similar pattern of chemokine generation was observed for MIP-1 α , MCP-1, and IL-8 in the monocyte and macrophage cultures (Fig. 5). Thus, LPS up-regulated the generation of these chemokines by monocytes, whereas IL-4 stimulated chemokine production by macrophages. Furthermore, IL-4 attenuated the LPS-driven production of MCP-1 and IL-8 by monocytes, whereas, conversely, LPS attenuated the IL-4-stimulated production of all three chemokines in the macrophage cultures (Fig. 5).

Interestingly, the CC chemokine MDC was regulated in a distinct manner to the other chemokines. The constitutive generation of MDC was dramatically up-regulated as monocytes were differentiated into macrophages, resulting in a 26-fold increase in the basal production of this chemokine. As shown in Fig. 5, LPS stimulated MDC production by monocytes and macrophages, whereas

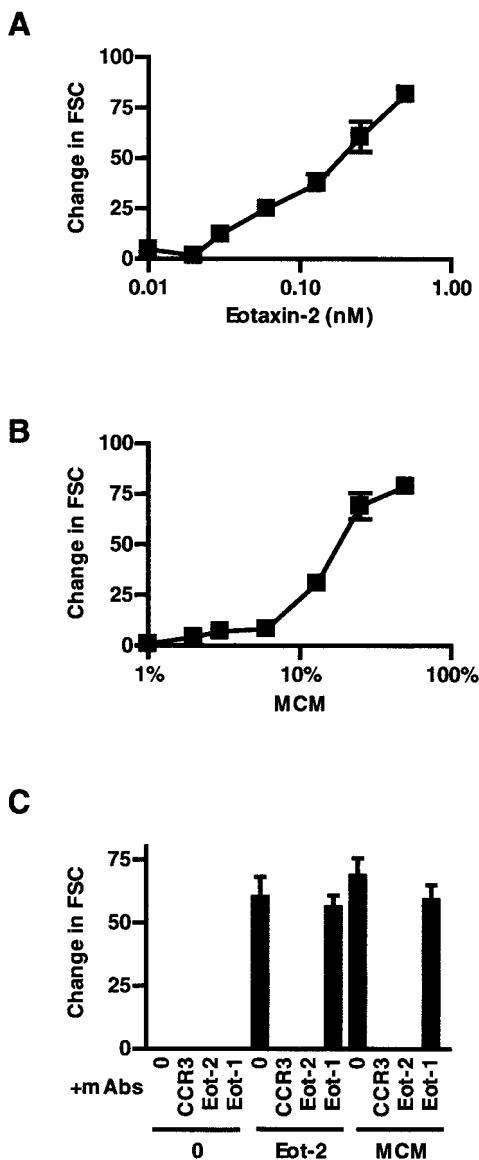


FIGURE 3. MCM contains an eosinophil-activating factor. MCM was generated by culturing monocytes with medium alone for 48 h. Mixed granulocytes were prepared as described in *Materials and Methods* and were incubated for 4 min at 37°C with either eotaxin (Eot-2) (0–0.5 nM; A) or MCM (at the dilutions specified; B), or eotaxin-2 (0.25 nM) and MCM (25%; C) in the presence or absence anti-CCR3 (3 μg/ml), anti-eotaxin-2 (10 μg/ml), anti-eotaxin-1 (10 μg/ml), or an isotype-matched control mAb (10 μg/ml) as indicated. The eosinophils were gated according to their autofluorescence and their FSC was determined by FACS analysis. Each point represents the change in FSC from cells incubated in the absence of agonist, and is the mean ± SEM of quadruplicate samples. Where error bars are not shown, they fall within the size of the symbols. A representative graph of *n* = 4 independent experiments is shown.

IL-4 stimulated MDC generation by monocytes but not macrophages. These findings are in agreement with previously reported data (39–43).

Discussion

There is evidence to suggest that eotaxin-2 has similar biological functions to eotaxin-1 and thus plays an important role in coordinating the recruitment of inflammatory cells (in particular eosinophils) to sites of allergic inflammation (1). However, the relatively high circulating plasma levels of eotaxin-2 and its ability to

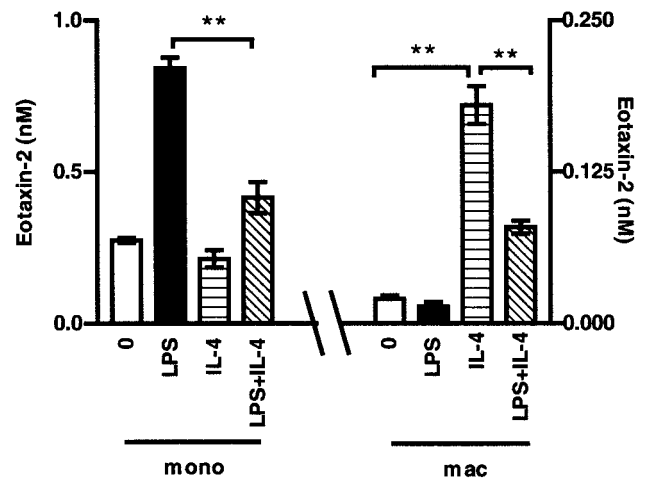


FIGURE 4. Effect of LPS and IL-4 on eotaxin-2 generation by monocytes and M-CSF-induced monocyte-derived macrophages (mac). Monocytes and macrophages from the same donors were isolated and cultured as described in *Materials and Methods*. The cells were then incubated in the presence or absence of IL-4 (10 ng/ml), LPS (100 ng/ml), or IL-4 plus LPS as indicated for 48 h. Immunoreactive eotaxin-2 was assayed in the supernatants by ELISA. Each bar represents the mean ± SEM of triplicate samples. A representative graph of *n* = 3 independent experiments is shown. **, *p* < 0.01; statistical difference as determined by Student’s *t* test.

suppress myeloid cell proliferation (4, 44) suggest that it may also exhibit biological functions distinct from those of eotaxin-1. Therefore, we reasoned that identification of the cells that generate eotaxin-2 and the factors that regulate the production of this chemokine may provide important clues as to the role of eotaxin-2 in vivo.

In this paper, we have shown for the first time that human peripheral blood monocytes generate eotaxin-2 constitutively (Figs. 1, 2, and 4). Moreover, we provide evidence that this eotaxin-2 is biologically active (Fig. 3). Thus, we propose that circulating monocytes may generate sufficient eotaxin-2 constitutively to account for plasma levels of this chemokine. However, the biological function of this circulating eotaxin-2 is still not clear. We show in this study that stimulation of monocytes with the proinflammatory cytokine IL-1β and the microbial agents LPS and zymosan led to a significant up-regulation of their production of eotaxin-2. Interestingly, the Th2 cytokines IL-4 and IL-13 and the proinflammatory cytokine TNF-α, acting alone or in combination, did not enhance the generation of eotaxin-2 by monocytes (Fig. 1 and data not shown).

LPS is an integral outer membrane component of Gram-negative bacteria that has been implicated in triggering the inflammatory cascade associated with sepsis (45, 46). Systemic exposure to LPS is associated with an influx of inflammatory leukocytes into host tissues, which is mediated in part by the localized production of chemokines. Thus, in vitro, it has previously been reported that monocytes generate IL-8, MCP-1, RANTES, MIP-1α, and MDC when stimulated with LPS, which could promote the recruitment of monocytes, lymphocytes, and neutrophils to sites of infection (40, 43, 47–49). In this study, we have shown that LPS also stimulates the generation of eotaxin-2 by monocytes (Figs. 1, 2, and 4). Similarly, we report in this study that zymosan, a preparation of yeast cell walls, stimulates eotaxin-2 generation by monocytes (Fig. 1). Given these results, we speculate that in conditions such as sepsis or bacteremia (i.e., microbial infection in the blood), circulating monocytes or monocytes recruited to the site of infection generate large quantities of eotaxin-2. At present it is not

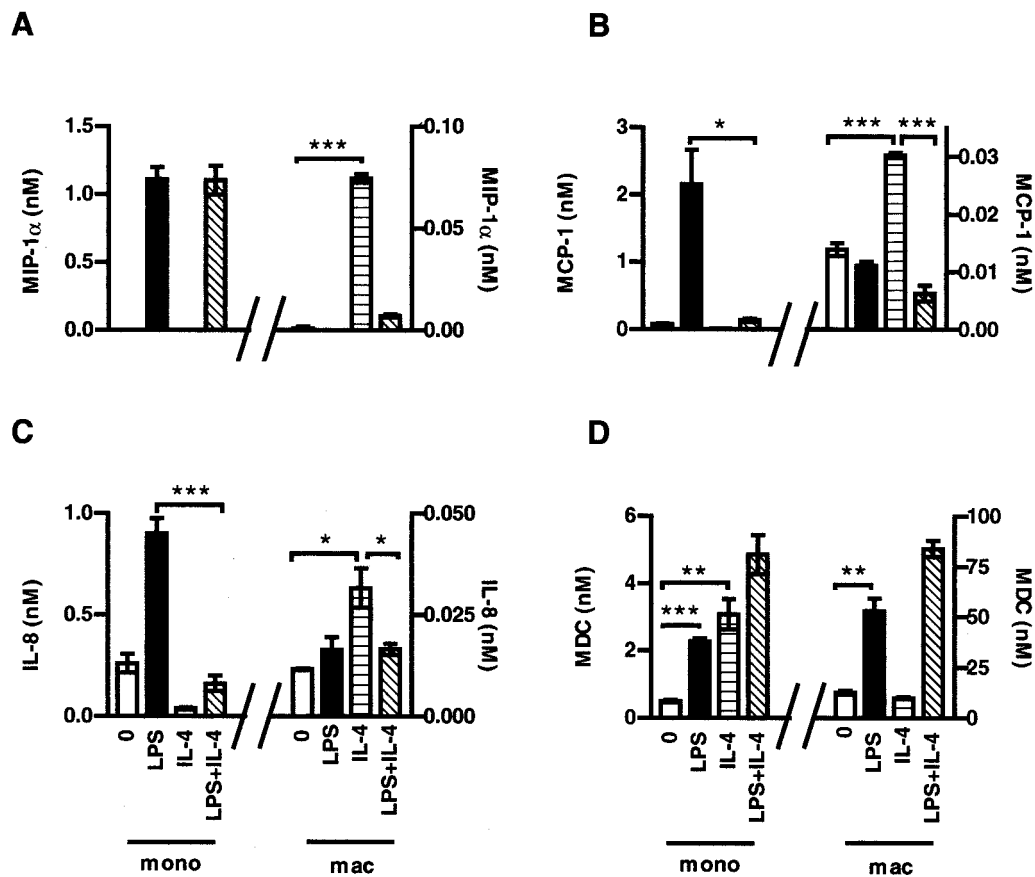


FIGURE 5. Effect of LPS and IL-4 on chemokine generation by monocytes and M-CSF-induced macrophages. Monocytes (mono) and macrophages (mac) were isolated as described in *Materials and Methods*. The cells were then incubated in the presence or absence of IL-4 (10 ng/ml), LPS (100 ng/ml), or IL-4 plus LPS as indicated for 48 h. Immunoreactive MIP-1 α (A), MCP-1 (B), IL-8 (C), and MDC (D) were assayed in the supernatants by ELISA. Each bar represents the mean \pm SEM of triplicate samples. A representative graph of $n = 3$ independent experiments is shown. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; statistical difference as determined by Student's t test.

known what the functional consequences of such an elevation of circulating or tissue eotaxin-2 levels may be. However, there are several reports of a delayed tissue eosinophilia in rats and mice that peaks 48 h after an intrapleural injection of LPS (50, 51). The delayed onset of this tissue eosinophilia is consistent with the time course for eotaxin-2 production by monocytes stimulated with LPS reported in this study (Fig. 2A).

To ascertain whether cell types other than monocytes have the capacity to generate eotaxin-2, we examined the ability of HDFs to generate eotaxin-1 and -2. HDFs were tested, as it has been well documented that these cells have the capacity to generate eotaxin-1 in culture (31, 36, 37). Therefore, it was of interest to determine whether a cell type known to generate eotaxin-1 also generated eotaxin-2. Moreover, because it has been reported that eotaxin-2 is expressed in late-phase allergic reactions in the skin of atopic subjects (19), HDFs seemed a likely source of this chemokine. Although our results confirm that HDF generate eotaxin-1 when stimulated with IL-4, IL-13, or TNF- α , we were not able to detect eotaxin-2 immunoreactivity in the supernatants from these cells (Table I), suggesting that distinct signaling pathways and transcription factors regulate the generation of these two forms of eotaxin. Moreover, our findings that eotaxin-1 and -2 are generated by different cell types in response to contrasting stimuli suggests that these two chemokines may exhibit distinct biological functions in vivo. In this regard, it is interesting that one of the first descriptions of eotaxin-2 documented its ability to inhibit the proliferation of myeloid progenitor cells (4), an activity not yet re-

ported for eotaxin-1. Therefore, it is possible that eotaxin-2 exhibits other biological activities that are distinct from those of eotaxin-1, a subject that warrants further investigation.

As monocytes migrate into tissues, they differentiate into macrophages, which are morphologically and phenotypically distinct from monocytes (52, 53). Thus, it has previously been reported that the profile of cytokines expressed by monocytes differs significantly from those expressed by macrophages (54). Furthermore, it has been demonstrated that the expression of chemokine receptors changes as monocytes differentiate into macrophages (55). In this study, we demonstrated a fundamental difference in the regulation of eotaxin-2 generation in monocytes and macrophages. Thus, although microbial agents and the proinflammatory cytokine IL-1 β stimulate a dramatic increase in the generation of eotaxin-2 by monocytes, they have no effect on the generation of this chemokine by macrophages (Fig. 4 and data not shown). In contrast, the Th2 cytokine IL-4 stimulates an enhanced eotaxin-2 generation in macrophages but not monocytes (Fig. 4).

IL-4 and IL-13 have been shown to be important regulators of monocyte and macrophage function (56). Several studies have reported that IL-4 suppresses the LPS-induced production of cytokines, including TNF- α , IL-1 β , IL-12, and IL-10, and chemokines, including MCP-1, MIP-1 α , and IL-8 by monocytes (48, 49, 57–62). Consistent with these reports in this study, we show that IL-4 suppresses the generation of MCP-1 and IL-8 by LPS-stimulated monocytes. Moreover, we show for the first time that IL-4 reduces

eotaxin-2 production by monocytes stimulated with LPS. In contrast to the IL-4-induced suppression of monocyte chemokine production, we have shown in this study that it enhances the generation of eotaxin-2, MIP-1 α , MCP-1, and IL-8 in monocyte-derived macrophages. This is a clear indication that IL-4 stimulates different signaling pathways in monocytes and macrophages. This finding is consistent with previous reports that the activation or differentiation of monocytes leads to altered IL-4 and IL-13 response profiles (56). It is thought that changes in the expression of specific receptor chains in turn results in the activation of distinct signaling pathways in monocytes and macrophages stimulated with IL-4 and IL-13. For example, it has been shown that the activation of STAT 6 by IL-4 and IL-13 is markedly reduced in macrophages as compared with monocytes (56). A reduction in the expression of the common γ -chain of the IL-4R and the α -1 chain of the IL-13R as monocytes differentiate into macrophages is thought to underlie this difference (56). Indeed, the identity of the IL-4R and IL-13R on macrophages is still unclear.

MDC was regulated in a distinct manner to the other chemokines. Consistent with previous reports, the constitutive expression of MDC is dramatically up-regulated as monocytes differentiate into macrophages (39–41). LPS stimulates an enhanced production of MDC by both monocytes and macrophages, and IL-4 stimulates monocytes, but not macrophages to generate MDC (39–43). The up-regulation of MDC in response to microbial products and Th2 cytokines is consistent with the role of the MDC/CCR4 chemokine/receptor axis in diverse pathologies, including allergic reactions and endotoxemia (63, 64).

These data highlight important differences in the responsiveness of monocytes and macrophages to specific stimuli with respect to their capacity to generate chemokines. Thus, it is clear that the array of chemokines generated by monocytes is dependent not only on the external stimuli, but also on their state of differentiation. With regard to eotaxin-2 production, it is clear that monocytes generate higher basal levels of eotaxin-2 than macrophages, which might account for the high plasma levels and low tissue levels detected. The up-regulation of eotaxin-2 production in monocytes by microbial agents suggests that it may play an as yet undefined role in the innate host defense system. In the context of tissue macrophages, we have shown that eotaxin-2 is up-regulated by Th2 cytokines; this is consistent with a role for eotaxin-2 in the local recruitment of eosinophils into tissues during allergic inflammatory reactions. These results are in keeping with previous papers that reported up-regulation of eotaxin-2 expression in the lung following Ag challenge of sensitized mice, in the lungs of asthmatics, and in late-phase allergic reactions in the skin of atopic subjects (19, 26, 27). Therefore, it is possible that tissue macrophages may contribute to eotaxin-2 generation and eosinophil recruitment during allergic inflammatory reactions.

This study highlights marked differences between eotaxin-1 and -2, namely their potential cellular sources and factors that stimulate their generation. It will be interesting in the future to determine how these differences are reflected by differential roles for these two chemokines in vivo.

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