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Regulation of CD8 Expression in Mast Cells by Exogenous or Endogenous Nitric Oxide

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We recently reported a novel CD8 molecule on rat alveolar macrophages and peritoneal mast cells (PMC). However, little is known about the regulation of CD8 expression and function on these cells. We investigated the regulation of CD8 expression on PMC by NO, because NO can regulate inflammatory responses and also because anti-CD8 Ab stimulates inducible NO synthase and NO production by PMC and alveolar macrophages. Ligation of CD8α on PMC with Ab (OX8) induced CD8α mRNA expression after 3–6 h, and flow cytometry demonstrated that OX8 treatment increased CD8α protein expression compared with PMC treated with isotype control IgG1. To test whether NO mediates the up-regulation of CD8α, we used the NO donor S-nitroso glutathione (500 μM) and NO synthase inhibitors (N\[^{4}\]-monomethyl-L-arginine and N\[^{G}\]-nitro-L-arginine methyl ester; 100 μM). S-nitroso glutathione up-regulated both mRNA and protein expression of CD8α in PMC compared with that in sham-treated cells, while NO synthase inhibitors down-regulated OX8 Ab-induced CD8α expression. To investigate how NO regulates CD8 expression on PMC, we examined the cGMP-dependent pathway using 8-bromo-cGMP (2 mM) and the guanylate cyclase inhibitor, 1H-oxadiazoloquinoxalin-1-one (20 μM). 8-Bromo-cGMP up-regulated CD8 expression, whereas 1H-oxadiazoloquinoxalin-1-one down-regulated its expression. Thus, ligation of CD8α up-regulates CD8 expression on PMC, a response mediated at least in part by NO through a cGMP-dependent pathway. The significance of this up-regulation of CD8α on mast cells (MC) is unclear, but since ligation of CD8α on MC with OX8 Ab can alter gene expression and mediator secretion, up-regulation of CD8α may enhance the MC response to natural ligation of this novel form of CD8.


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Abbreviations used in this paper: MC, mast cell; PMC, peritoneal MC; GSNO, S-nitroso glutathione; HBTS, HEPES-buffered Tyrode’s solution; NOS, NO synthase; sGC, soluble guanylyl cyclase; iNOS, inducible NOS; L-NMMA, L-nitroarginine methyl ester; MFI, mean fluorescence intensity; ODQ, 1H-(1,2,4)oxadiazolo[4,3-a]quinoxalin-1-one; sGC, soluble guanylyl cyclase.

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Materials and Methods

Animals and reagents

Male Sprague Dawley rats (7–400 g) were obtained from Charles River Breeding Laboratories (St. Constant, Canada) (6). Animals were maintained in an isolated room in filter-top cages to minimized unwanted infections. Most studies used normal rats, but in some experiments rats were infected with L3 larvae of *Nippostrongylus brasiliensis* 5–6 wk before MC isolation as previously described (26). Food and water were provided ad libitum, and animals were maintained on a 12-h dark, 12-h light (0700–1900) cycle. Experimental procedures were approved by the university animal care committee (Jikei University School of Medicine, Tokyo, Japan) and were in accordance with the guidelines of the Canadian Council on Animal Care (Ottawa, Canada).

Ab OX8 (anti-CD8α hinge region, IgG1) (29), OX8-FITC, and 341 (anti-CD8β, IgG1) (30) were purchased from Serotec (Toronto, Canada). 341g and IgG1 were purchased from BD Pharmingen (Mississauga, Canada). IgG1-FITC was purchased from Accurate Chemical and Scientific (New York, NY). Heparin I and N^δ^-nitro-arginine methyl ester (t-NAME) were purchased from Sigma-Aldrich (St. Louis, MO). RPMI 1640 medium was purchased from Life Technologies (Grand Island, NY). GSNO, N^δ^-monomethyl-l-arginine (t-NMMA), and 8-bromo-cGMP were purchased from Calbiochem (La Jolla, CA). 1H-(1,2,4)-oxidiazolo[4,3-a]quinoxalin-1-one (ODQ) was purchased from Tocris Neuramin (Ballwin, MO). The viability of PMC was not affected by in vitro treatment with any of these compounds at the doses used.

MC isolation and in vitro stimulation

Isolation and purification of rat PMC were performed as previously described (31). Briefly, 20 ml of cold HEPES-buffered Tyrode’s solution (HBTS) was used to lavage the peritoneal cavity of each rat. The recovered cells were collected on a 30.80% discontinuous Percoll gradient and centrifuged at 600 × g for 20 min at 4°C. The purity of PMC from SD rat was ≥98% (32). PMC were incubated for 3–20 h with OX8 (2.5–10 μg/ml) or IgG1 isotype (10 μg/ml) with or without t-NMMA (100 μM), 8-bromo-cGMP (2 mM), GSNO (500 μM), or ODQ (20 μM) in RPMI 1640 medium (Life Technologies) and used for RT-PCR. For analysis of CD8 protein expression, PMC were incubated for 20 h with OX8 (10 μg/ml), 341 (10 μg/ml) or IgG1 isotype (10 μg/ml) with or without NO inhibitors (t-NMMA, t-NAME; 100 μM, 8-bromo-cGMP (2 mM), GSNO (500 μM), or ODQ (20 μM) in RPMI 1640 medium and used for flow cytometric analysis.

RT-PCR

Total RNA extraction from PMC was performed by the method of Chomczynski and Sacchi (33, 34) with some modifications. After isolation, a 1–μg aliquot of RNA was incubated in 15 μl of 333 U/ml pancreatic RNase (Sigma-Aldrich) in a reaction mixture of 5 mM Tris (pH 7.5), 1 mM CaCl_2, and 7.5 U RNase inhibitor (Life Technologies) for 2 h at 22°C to remove contaminating heparin (34). To synthesize a first-strand cDNA, each RNA sample (1 μg) was mixed with 1 μl of oligo(dT)$_{12-18}$ primer (500 μg/ml; Life Technologies), mRNA was reverse transcribed by Moloney murine leukemia virus RT (Life Technologies) using a PTC-100 Programmable Thermal Controller (Fisher Scientific, Nepean, Canada) according to the manufacturer’s protocols.

PCR was conducted using a hot start method by adding 2 μl of cDNA product to 18 μl of PCR buffer containing 67 mM Tris (pH 8.8), 1.5 mM MgCl$_2$, 16.6 mM (NH$_4$)$_2$SO$_4$, mixed dNTPs at 200 μM, 125 U/ml Taq polymerase (Life Technologies), and 0.3 μM sense and antisense primers. The primers used were rat β-actin: sense primer 5'–GTGAGTGAAGGCTCCTCCGA–3' and antisense primer 5'–GCTGTTGCTCCACATGCTAC–3'; and rat CD8α: sense primer 5'-CATGTTACAGTTGTCAACAA-3' and antisense primer 5'-CAGCAATTTTCCGAGCTGTC-3'. The PCR products for β-actin and CD8α were 812 and 630 bp, respectively. After denaturing at 94°C for 2 min, the reaction was conducted for 30 cycles at 94°C for 1 min, at 60°C (β-actin) or 55°C (CD8α) for 1 min, and at 72°C for 1 min. Products were electrophoresed on a 2% agarose gel and visualized by staining with ethidium bromide. Care was taken in optimizing conditions for semiquantitative analysis of RT-PCR results, including loading controls with β-actin. Complementary studies of protein expression were also conducted as outlined below. All PCR data shown are from one representative experiment of four performed.

Flow cytometric analysis

In 96-well U-bottom plates, cells (5 × 10^5 cells/well) were preincubated in RPMI 1640 medium (5% FBS) and 10% normal mouse serum (for conjugate primary Ab only) for 1 h before incubation with Ab for 1 h at 4°C. Cells were washed three times (with HBTS) and resuspended in 400 μl of HBTS, and 10,000 cells were analyzed on a FACSscan (BD Biosciences, Mountain View, CA). The mean fluorescence intensity (MFI; logarithmic scale) of cell populations was determined, and the results presented are net values of MFI following subtraction of values for isotype-matched controls. The percentage of CD8α-positive cells is indicated for both responding and nonresponding populations for each stimulus used.

Measurement of NO production

PMC were incubated (2 × 10^5 cells/well) with 5–10 μg of Ab for 48 h. Cell-free supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)ethylendiamine dihydrochloride, and 2.5% H_3PO_4) and incubated for 10 min at room temperature (35). The concentration of NO$_2$ was determined by measuring the absorbance at 540 nm with a Vmax Kinetic Microplate Reader ( Molecular Devices, Menlo Park, CA). NaNO2 was used as a standard. In experiments with t-NMMA, inhibitor was added 10 min before addition of Ab.

Statistical analysis

For flow cytometric data, statistical analysis was performed using two-tailed Student’s *t* test. For PCR data, scanned images are one representative experiment of four experiments conducted.

Results

Anti-CD8α Ab and NO donor up-regulate CD8α mRNA expression in PMC

RT-PCR was used to examine the regulation of CD8α mRNA expression by anti-CD8α and the NO donor, GSNO (Fig. 1). cDNA from PMC stimulated with OX8 (2.5–10 μg/ml) or GSNO (100–500 μM) were used in the PCR reaction to detect CD8α mRNA. In the time-course study (Fig. 1A) OX8 (5 μg/ml) up-regulated CD8α mRNA after 3–6 h of stimulation, and mRNA levels returned toward baseline by 12 h. By contrast, the expression of CD8α mRNA in cells stimulated with GSNO was maximal.
after 12–20 h of stimulation (Fig. 1B). CD8α mRNA expression was up-regulated by OX8 (3-h stimulation) and GSNO (12-h stimulation) in a dose-dependent manner (Fig. 1B). IgG1 isotype (10 μg/ml) was used as a control for OX8, and no up-regulation of CD8 mRNA expression was found (data not shown).

To test whether endogenous NO is involved in the up-regulation of CD8 mRNA by OX8, the NOS inhibitor L-NMMA (100 μM) was used (Fig. 1C). PMC were stimulated by OX8 (10 μg/ml) with or without L-NMMA for 3 h. L-NMMA ablated OX8-induced CD8 mRNA expression.

Regulation of CD8 protein expression on PMC

Flow cytometry was used to examine the regulation of CD8 protein on PMC (Figs. 2 and 3). PMC were stimulated with GSNO (500 μM), IgG1 isotype (10 μg/ml), or OX8 (10 μg/ml) with or without NOS inhibitors (L-NMMA and L-NAME; 100 μM) for 20 h. The majority of PMC were CD8α positive (64.6 ± 15.3%), and some (24.5 ± 8.5%) were 341 (CD8β) positive as we reported previously (10). No significant difference in the proportion of positive cells was seen between preincubation and after 20 h of incubation (data not shown).

OX8 ligation induced a second peak of CD8α-positive cells (responding cells; MFI = 102.3) that was not seen using treatment with IgG1 isotype control Ab. For OX8 treatment, the populations expressing low (MFI = 34) and high (MFI = 102.3) levels of CD8α comprised 56.6 and 42.5% of the total population, respectively. Treatment of PMC with 500 μM of GSNO for 20 h also induced a second peak of CD8α-positive cells (Fig. 2A). For GSNO treatment, the two CD8α-positive cell populations were unevenly distributed, with 19.3% expressing low levels of CD8α (MFI = 28.3; nonresponding cells) and 76.7% expressing high levels of CD8α (MFI = 92.3; responding cells). Statistical analysis of MFI values showed that GSNO and OX8 significantly (p < 0.05) up-regulated CD8α protein expression compared with unstimulated PMC. One representative experiment is shown in Fig. 2B. By contrast, treatment of PMC with 341 (20 μg/ml, 20 h) or allergen (PMC from rats infected with N. brasiliensis) had no effect on CD8α protein expression (data not shown). Finally, neither OX8 (10 μg/ml, 20 h) nor GSNO (500 μM, 20 h) modified the expression of CD8β protein on PMC (data not shown).

Inhibitory effect of NOS inhibitor on CD8 expression by PMC

To test whether endogenous NO regulated CD8α protein expression as it had with CD8α mRNA (Fig. 1C), NOS inhibitor (t-NAME, 100 μM) was used (Fig. 3). Flow cytometry showed that OX8 increased CD8α expression in 46.4% of the cells (MFI = 115; responding cells). Only 19.8% of L-NMMA- and OX8-treated cells showed an increase in CD8α expression, suggesting that L-NMMA treatment inhibited the OX8 effect (MFI of the responding population = 41.2; Fig. 3A). t-NAME and L-NAME (data not shown) treatment significantly (p < 0.01) decreased MFI for CD8α in the responding population compared with OX8 treatment alone (Fig. 3B). t-NAME alone had no effect on the MFI or the number of CD8α-positive cells in either the responding or nonresponding population (data not shown).

Regulation of CD8 expression by NO involves a cGMP-dependent pathway

To determine how NO regulates CD8α expression, PMC were treated for 12 h with a membrane-permeable cGMP analog, 8-bromo-cGMP (2 mM). Time-course treatment indicated that 12 h was optimal for cGMP-induced expression of CD8α (data not shown). RT-PCR analysis showed that both 8-bromo-cGMP and GSNO...
(500 μM) up-regulated CD8α mRNA expression (Fig. 4). In addition, the mRNA expression evoked by GSNO was down-regulated by a selective inhibitor of NO-stimulated soluble guanylyl cyclase (sGC), ODQ (20 μM). Accordingly, to determine whether similar effects occurred with CD8α protein expression, flow cytometric analysis was conducted (Fig. 5). With 8-bromo-cGMP (20 h), 40.8% of the cells responded, and their MFI was 54.0. Similarly with GSNO, 55.9% of the cells responded, and their MFI was 85.1.

By contrast, following treatment with GSNO and ODQ together, the MFI of the responding population (45.9%) was only 60.7 compared with 85.1 after treatment with GSNO alone. ODQ alone had no effect on CD8α protein expression.

**Discussion**

This study confirms and extends our previous finding that freshly isolated rat PMC and rat basophilic leukemia cells (RBL 2H3) express a novel form of CD8α as well as CD8β (10). A mouse M1 line has also been reported to express CD8α (36). Importantly, in the current investigations we have established that ligation of this CD8α with OX8 Ab to its hinge region up-regulates CD8α mRNA and surface protein expression on MC. This is the first report on factors that regulate CD8α expression on MC, and to our knowledge it is the first report that NO regulates CD8α expression on any cell type (5). Interestingly, about half the MC up-regulate CD8α protein in response to OX8, GSNO, or 8-bromo-cGMP. Therefore, although most MC express CD8α, only half the cell population (responding cells) is capable of increasing protein expression of CD8α in response to the stimuli used.

We have also shown that OX8-mediated increases in CD8α expression are mediated by NO, since the NOS inhibitor L-NMMA abrogates the OX8 effect. The NO donor (GSNO) increases CD8α mRNA expression as early as 3 h and maximally at 12–20 h. This enhanced expression of CD8α is associated with an earlier increase in NO production, which in our previous studies of both alveolar macrophages (6, 7) and PMC (37) involves the induction of iNOS mRNA by OX8 Ab within 6 h of stimulation (7). Treatment with OX8 Ab increases the expression of CD8α mRNA within 3 h, whereas the maximal effect of GSNO on CD8α mRNA is at 12–20 h. L-NMMA, an NOS inhibitor, blocks the OX8 effect in 3 h. This is an interesting observation that most likely reflects a major difference between the effects of exogenously administered NO (GSNO) and endogenously produced NO. The latter could arise from either constitutive NOS or iNOS in MC and could probably involve compartmentalization of production of NO in selected sites within the cell (37).

Moreover, flow cytometric data showed that GSNO and 8-bromo-cGMP increased CD8α expression by a pathway dependent upon sGC. NO can initiate its biological effects through activation of sGC, resulting in the production of cGMP and affecting pathways with other signaling systems, such as phosphoinositides, eicosanoids, cAMP, and Ca2+ (38). Therefore, our results suggest that OX8-mediated up-regulation of MC CD8α is mediated via the NO-sGC pathway and results in cell activation. We have previously shown that both TNF and NO production are induced by OX8 and 341 Ab in a dose-dependent manner (10).

Up-regulation of CD8α expression has been demonstrated in other cell types. Ligation of CD40 on Langerhans cells is associated with acquisition of CD8 and a dendritic cell phenotype by these cells (39). It is possible that when CD40 ligand on MC interacts with CD40 on B lymphocytes or other cells, MC expression of CD8 is modulated, in turn influencing selected functions of the MC such as Ag presentation (15–17). By contrast to this enhanced expression of CD8α, TGF-β1, TGF-β2, and PGE2 down-regulate CD8α expression on human peripheral blood lymphocytes (40). This could involve calcineurin, as has been shown in double-positive lymphocytes where PMA is known to inhibit CD8 expression (41).

It is attractive to postulate that through endogenously or exogenously produced NO, MC expression of CD8 is altered in a manner that influences its competence to respond to various signals and, in turn, selectively channels its functions in various microenvironments. For example, NO inhibits IgE-dependent histamine and serotonin secretion (21, 22, 25, 42, 43), platelet-activating factor production (24), and adhesion to fibronectin (26), but potentiates TNF-mediated cytotoxicity (23). How the up-regulation of CD8α expression by NO fits in with this spectrum of other effects of NO on MC is not currently clear. It is intriguing that ligation of
CD8a on the MC surface with OX8 Ab induces NO and TNF production directly, but does not modulate IgE Ag-dependent mediator secretion. Taken together with the inhibitory effects of NO on MC secretion of stored mediators such as histamine and serotonin, it is tempting to speculate that NO depresses the functions of the MC associated with the immediate phase of allergic reactions, but enhances other components of MC function such as innate immunity and regulation of immune responses.

The ligand for the novel form of CD8 on MC is currently unknown. It might be classical (1, 5) or nonclassical (44) MHC class I. Alternatively, given that the novelty of MC and macrophage CD8 lies in the N-terminal Ig-like domain that on T cell CD8s binds MHC class I, it is possible that MC CDS8a binds another ligand, such as gp180 (45) from epithelial or other cells, or a ligand that has yet to be identified. The identity of the ligand(s) for CD8 on MC is an important challenge that must be addressed if we are to fully understand the functional significance of the expression of CD8 on MC and other cells and the value of knowledge about the controls on its expression by NO and other factors.

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