Lactoferrin Regulates an Axis Involving CD11b and CD49d Integrins and the Chemokines MIP-1 α and MCP-1 in GM-CSF-Treated Human Primary Eosinophils

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Eosinophils are multifunctional immune cells that contribute to innate and adaptive immune/repair responses. Lactoferrin (LF) is an iron-binding protein indicated to alter cell adhesion and immune function by receptor-mediated interactions or by participating in redox mechanisms. The eosinophil adhesion molecules, α M β 2 and α 4 β 1, are differentially expressed following exposure to the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) and various redox agents. We hypothesized that LF can alter the function and production of proteins involved in adhesion/migration. Utilizing eosinophil peroxidase activity or fluorescent labeling adhesion assays, LF reduced GM-CSF-induced eosinophil adhesion in the presence of fibronectin or vascular adhesion molecule-1 compared with GM-CSF treatment alone. Flow cytometric analysis of eosinophil α M (CD11b) and α 4 (CD49d) integrins revealed that cotreatments (24 h) with LF plus GM-CSF induced a significant increase in CD11b compared with control and GM-CSF treatments but a significant decrease in CD49d compared with control and GM-CSF treatments. These changes in CD11b and CD49d levels were significantly correlated with the increased production of chemokines (macrophage inflammatory Protein-1 α , monocyte chemoattractant protein-1) and an identified increase in S100A9 production. Thus, LF release at sites of inflammation may alter eosinophil recruitment/activation and possibly the progression of diseases such as cancer and asthma where significant eosinophil influx has been described.

Introduction

EOSINOPHILS ARE MULTIFUNCTIONAL immune cells that accumulate in various tissues in response to normal development (gastrointestinal, mammary, uterine, and thymic) and chronic inflammation (Kita 2011; Woodruff and others 2011). Eosinophils may participate in tissue homeostasis, wound/repair processes, and the innate and adaptive immune responses via the release of various granule proteins, growth factors, lipid mediators, cytokines, and chemokines (Kita 2011). Eosinophil migration from blood vessels into tissue has been proposed to involve mediator-induced activation of eosinophil cell adhesion molecules, eosinophil adhesion to endothelia, and subsequent eosinophil transmigration (Tachimoto and others 2002; Lampinen and others 2004). A functional role of certain cell adhesion molecules, that is, the integrins, has been identified in the progression of chronic inflammatory disease (Singh and others 2004; Garmy-Susini and others 2010).

The integrins α 4 β 1 (CD49d/CD29, very late antigen-4) and α M β 2 (CD11b/CD18, Mac-1, complement receptor type 3) are 2 particular integrins that have been extensively studied in eo-

sinophils (Barthel and others 2008). The α 4 β 1 integrin has been reported to mediate cell-cell and cell-extracellular matrix contacts to vascular adhesion molecule (VCAM)-1 and fibronectin, respectively (Johansson and others 1997). The α M β 2 integrin is thought to initiate phagocytosis via interaction with complement component iC3b and, analogous to α 4 β 1, α M β 2 is indicated to mediate cell-cell and cell-extracellular matrix contacts by binding interactions with intercellular adhesion molecule-1, VCAM-1, fibrinogen, and vitronectin (Ross 2002; Barthel and others 2008). In human primary blood eosinophils, increased cell surface expression of α M β 2, but not α 4 β 1, has been found to occur in response to the cytokines interleukin (IL)-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF; Wong and others 2003). Research has also indicated that expression of α M β 2 is increased, whereas, the α 4 β 1 is decreased in eosinophilia patients compared with normal controls (Kayaba and others 2001). Differential eosinophil expression of α M β 2 and α 4 β 1 has also been found to occur in response to reactive oxygen species (Nagata and others 2000; Chuang and others 2003), suggesting that redox reactions are one of the underlying mechanisms involved in modulating eosinophil activation and recruitment.

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Low affinity lactoferrin (LF) receptors have been identified on eosinophils (Spik and others 1994). LF is an iron-binding glycoprotein released from neutrophils at the site of inflammation and secreted by epithelial cells that contribute to the composition of bodily fluids (milk, tears, saliva, bile, and pancreatic fluid; Legrand and Mazurier 2010). Studies have indicated that either bovine or human LF is able to modulate the proliferation of various cell types (eg, tumor cells, osteoblasts, and fibroblasts) and alter the host immune responses to allergens and cancer (Brock 2002; Kruzel and others 2006; Chodaczek and others 2007; Nadolska and others 2010; Li and others 2011; Naot and others 2011). The functions of LF have been attributed to signaling events arising from the binding interactions of the protein with host (CD14, heparan sulfate proteoglycans, intelectin, and LDL receptor-related protein) or pathogen (lipopolysaccharide and bacterial unmethylated CpG DNA) cell surface molecules (Brock 2002; Suzuki and others 2005; Legrand and Mazurier 2010) and from the iron-binding properties of the molecule (Klebanoff and Waltersdorph 1990; Sandomirsky and others 2003; Chodaczek and others 2007). These various processes have been reported to inhibit tumorigenesis (Brock 2002) and to reduce allergic inflammation (Kruzel and others 2006; Chodaczek and others 2007). In part, these biological effects may be a response to the reduced eosinophil migration found to occur after LF treatment (Bournazou and others 2010).

Based on this prior information regarding the biological functions of LF, we tested the hypothesis that LF can alter the function and production of proteins involved in migration by examining eosinophil adhesion, integrin expression, and the production of chemokines and calgranulins (S100A9). The data herein support the idea of LF as an important regulator of eosinophil responses and provide insight into the inflammatory reactions observed in cases of allergy and asthma in addition to certain cancers.

Materials and Methods

Isolation of peripheral blood eosinophils

Peripheral blood was obtained from human allergic subjects under informed consent. The study was approved by the University of Wisconsin-Madison Center for Health Sciences Human Subjects Committee. Eosinophils were purified from heparinized blood that was diluted with Hank's buffered salt solution without Ca^{2+} , Mg^{2+} , and phenol red (HBSS; Mediatech) and centrifuged for 20 min at $700\times g$ over 1.090 g/mL Percoll. A granulocyte fraction was obtained after removal of the plasma, mononuclear cell band, and Percoll. Granulocytes were then subjected to red blood cell lysis via hypotonic shock, washed with 4°C HBSS supplemented with 2% new born calf serum (Life Technologies), and incubated 40 min with magnetic beads coated with anti-CD16, anti-CD14, and anti-CD3 (Miltenyi Biotechnology) prior to negative selection with an AutoMACS separator (Miltenyi Biotechnology). The recovered mixture ($>97\%$ purity, $>98\%$ viability) was evaluated via Giemsa's-based Diff-Quik stain (Baxter Scientific Products) and trypan blue exclusion respectively.

Assay of eosinophil peroxidase activity

Cellular eosinophil peroxidase (EPO) activity was determined using adherent cells in a manner similar to that detailed in previous studies (Strath and others 1985; Nagata

and others 2000; Barthel and others 2006; Bates and others 2010). Purified human blood eosinophils were added to protein-coated surfaces that were prepared by applying $10\mu\text{g/mL}$ of either soluble recombinant human VCAM-1, purified human fibronectin (both kindly provided by Dr. Deane Mosher, University of Wisconsin-Madison), or bovine serum albumin (BSA, A-6003; Sigma Chemical Co.) in TBS (pH 8) to triplicate wells of a 96-well plate (Sarstedt) for 4 h at 37°C . Coating buffers were aspirated and eosinophils ($1\times 10^5/\text{mL}$) suspended in 0.1% human serum albumin (HSA; Irvine Scientific) HBSS with Ca^{2+} , Mg^{2+} , but without phenol red (Mediatech) and treated with buffer control, 100 pM GM-CSF (R&D Systems) or $0.3\text{--}10\mu\text{g/mL}$ bovine LF (Morinaga Milk Industry Co., Ltd.) for 1 h at 37°C . Additional eosinophils ($1\times 10^5/\text{mL}$) were saved on ice for use in a standard curve. After 1 h at 37°C , the plate was washed thrice with $200\mu\text{L}$ of 37°C TBS and $100\mu\text{L}$ of 37°C HBSS with Ca^{2+} , Mg^{2+} , but without phenol red, was added to each well. Additional eosinophils were plated in triplicate ($100\mu\text{L}/\text{well}$) and serially diluted for use as a standard curve. To identify the presence of eosinophils in the assay, $100\mu\text{L}$ of reaction buffer containing 0.1% Triton[®] X-100 (Sigma Chemical Co.), 50 mM Tris (pH 8), 1 mM H_2O_2 , and 1 mM O-phenylenediamine dihydrochloride (OPD; Sigma Chemical Co.) was added to each well for 30 min to allow for oxidation of OPD and the consequent production of OPD diimine colored derivatives via the released EPO. After 30 min, the reaction was stopped with 4 M H_2SO_4 ($50\mu\text{L}$) and optical density (OD) was measured at 490 nm. The absorbance levels of EPO activity were used as an indirect measurement of eosinophil adherence by examining the OD means of the samples relative to the standard curve.

Carboxyfluorescein diacetate succinimidyl ester labeling as a measure of adherence

Eosinophils (2×10^6) were stained with Cell Trace[™] carboxyfluorescein diacetate succinimidyl ester (CFSE) ($2\mu\text{M}$, C34554; Invitrogen-Molecular Probes) per manufacturer's instructions. CFSE-labeled eosinophils were suspended in 10 mL of 0.1% fetal bovine serum (FBS; Hyclone) HBSS with Ca^{2+} , Mg^{2+} , but without phenol red. In a 96-well plate (Sarstedt) coated with $10\mu\text{g/mL}$ of fibronectin or VCAM-1, cells were plated in triplicate ($100\mu\text{L}$) and treated with vehicle control, 100 pM GM-CSF, $10\mu\text{g/mL}$ bovine LF (Morinaga) or $10\mu\text{g/mL}$ human LF (Cell Sciences) for 1 h at 37°C . Additional eosinophils ($2\times 10^5/\text{mL}$) were saved on ice for use in a standard curve. After 1 h at 37°C , the plate was washed thrice with $200\mu\text{L}$ of 37°C TBS and $100\mu\text{L}$ of 37°C HBSS with Ca^{2+} , Mg^{2+} , but without phenol red, was added to each well. Additional eosinophils were plated in triplicate ($100\mu\text{L}/\text{well}$) and serially diluted for use as a standard curve. The plate was immediately read on a Synergy[™] 2 Multi-Detection Microplate Reader (BioTEK[®] Instruments, Inc.) at an excitation of 485/20 and emission of 528/20. The CFSE fluorescence levels were used as a measure of eosinophil adherence by examining the average fluorescence of the samples relative to the standard curve.

Flow cytometric analysis of cell surface molecules

Eosinophils ($2\times 10^6/\text{mL}$) were suspended in 0.5 mL of 0.1% HSA (Irvine Scientific) RPMI (Mediatech) and placed in 24-well plates (Sarstedt) coated with $10\mu\text{g/mL}$ fibronectin.

Cells were treated with buffer control, 100 pM GM-CSF (R&D Systems), 10 mM N-acetyl-L-cysteine (NAC; Sigma Chemical Co.), or bovine LF (Morinaga) for 24 h. Eosinophils were then aspirated from the wells and treated with 5 μ L of phycoerythrin-conjugated anti-CD11b (Clone ICRF44, 555388; BD Biosciences Pharmingen), 5 μ L of allophycocyanin-conjugated anti-CD49d (Clone 9F10, 304307; Biolegend), or antibody isotype control for 30 min at 4°C. Cells were washed with 1 mL of 1% FBS RPMI (Mediatech) and suspended in 300 μ L PBS. To exclude dead cells, propidium iodide (3 μ g/mL) was added to each sample. A total of 10,000 events were assessed on a FACScan flow cytometer (Becton-Dickinson) at the University of Wisconsin Carbone Cancer Center Flow Cytometry Core Facility. Data were analyzed with FlowJo data analysis software (TreeStar, Ashland, OR).

Enzyme-linked immunosorbent assay

Monoclonal anti-macrophage inflammatory protein (MIP)-1 α antibodies (clone 14215, MAB670; R&D Systems, 1:250) or monoclonal anti-monocyte chemotactic protein (MCP)-1 antibodies (555055; BD Biosciences Pharmingen, 1:500) in 0.1 M sodium carbonate buffer (pH 9.6) were coated onto 96-well EIA/RIA plates (Costar). Blocking buffer containing 1% BSA (Sigma Chemical Co.) and 0.5% Tween[®]20 (Fischer Scientific) in PBS (pH 7.4) was added to wells for 2 h at room temperature. Serial dilutions of MIP-1 α standard (270-LD; R&D Systems), MCP-1 standard (554620; BD Biosciences Pharmingen), or cell-free supernatants were aliquoted and incubated at 4°C overnight. Eosinophil-secreted proteins were assayed using biotinylated MIP-1 α antibodies (BAF270; R&D Systems, 1:3,000) or biotinylated MCP-1 antibodies (554664; BD Biosciences Pharmingen, 1:1,000), and subsequent detection with streptavidin-horseradish peroxidase-40 (HRP-40; Fitzgerald Industries International). A colorimetric HRP substrate tetramethylbenzidine (Biofx Laboratories) was used to evaluate captured HRP activity and the enzymatic reaction was stopped by the addition of 0.18 M sulfuric acid. The OD was determined on an EL_x800 Universal Microplate Reader (BioTek Instruments, Inc.). Absorbance was quantified at 450 nm, using 600 nm as a reference wavelength. Concentrations were calculated by interpolation from a standard curve with an assay sensitivity of 12.5 pg/mL. All determinations were performed in triplicate.

Immunoblotting

Cells were solubilized with lysis buffer [1% sodium dodecyl sulfate (SDS)], 1 mM dithiothreitol, 0.5 mM Na₃VO₄, 1 mM EDTA, 10% glycerol, 10 mM Tris (pH 8.0), sonicated, and boiled (5 min). Concentrated cell lysis buffer (10 $\times$) was added to conditioned media (1:10 dilution) and boiled (5 min). Proteins were loaded onto a 15% SDS-polyacrylamide gel electrophoresis gel and transferred onto 0.45 μ m Immobilon-P polyvinylidene difluoride membranes (Millipore). Blots were cut for multiple detection and incubated with antibodies raised against human actin (BD Biosciences; 612656, 1:5,000) or S100A9 (Santa Cruz Biotechnology; 1:200). Blots were washed and subsequently incubated with HRP-conjugated secondary antibodies. Bound secondary antibody was visualized following incubation of the membrane with Super Signal West chemiluminescent HRP substrate (Thermo Scientific Pierce) and using an Epichemi II darkroom UVP equipped with a 12-bit cooled CCD camera. Luminescence was quantified and evaluated via the application of ImageJ software (National Institutes of Health).

Statistical analysis

Changes in CD49d, CD11b, MIP-1 α , and MCP-1 from cell incubations involving treatments with GM-CSF alone to GM-CSF plus LF (GM-CSF+LF), GM-CSF plus the antioxidant NAC (GM-CSF+NAC), or GM-CSF plus LF and NAC (GM-CSF+LF+NAC) were expressed as ratios. The associations between changes in CD49d or CD11b with MIP-1 α , and MCP-1 were evaluated using the Spearman rank correlation coefficient. All additional analyses were performed using a paired Student's *t*-test. A 2-sided *P* value of <0.05 was regarded as statistically significant.

Results

LF modulated human blood eosinophil adherence to fibronectin as measured by EPO activity

LF is found in airway lavage fluid (1–100 μ g/mL) and has been indicated to inhibit cell adhesion by interacting with RGD motifs required for fibronectin binding to certain integrins (Travis and others 1999; Sakamoto and others 2006). Conversely, the cytokine GM-CSF is known to induce

FIG. 1. LF-modulated adherence to fibronectin compared to BSA. Purified human blood eosinophils (1×10^5 cells/mL) were added to plates coated with 10 μ g/mL BSA or 10 μ g/mL FN. Cells were treated with 100 pM GM-CSF or increasing concentrations of bovine LF for 1 h. EPO activity was assessed at 490 nm as described under Materials and Methods. The average number of eosinophils adhered relative to a standard curve is displayed $\pm$ SEM, $n=5$, * $P < 0.02$ versus buffer control, † $P < 0.04$ versus GM-CSF alone. GM-CSF, granulocyte-macrophage colony-stimulating factor; EPO, eosinophil peroxidase; BSA, bovine serum albumin; LF, lactoferrin; FN, fibronectin.

eosinophil adherence to fibronectin (Anwar and others 1993; Bates and others 2010). To test the idea that LF can block GM-CSF-induced eosinophil adhesion to fibronectin, we performed EPO assays as an indirect measurement of eosinophil adherence using bovine LF. Plates were coated with a positive control of 10 μ g/mL BSA (a previously identified beta2 ligand; Zhu and others 2000) and 10 μ g/mL fibronectin as described in Materials and Methods. As shown in Fig. 1, GM-CSF treatment alone significantly ($P < 0.02$) enhanced eosinophil adherence/EPO activity to fibronectin compared with the control. Addition of increasing concentrations of LF to GM-CSF-treated wells significantly inhibited eosinophil adherence/EPO activity in the presence of fibronectin ($P < 0.02$) but not in the presence of BSA compared with GM-CSF treatment alone in experiments using eosinophils from 5 different donors (see Fig. 1).

LF modulation of eosinophil adherence to VCAM-1 as measured by EPO activity

To test whether LF also modulates eosinophil adherence/EPO activity when plated on VCAM-1, EPO assays were performed on cells treated with 100 pM GM-CSF $\pm$ 10 μ g/mL bovine LF and plated atop fibronectin or VCAM-1-coated wells as described in Materials and Methods. As shown in Fig. 2A, GM-CSF significantly enhanced eosinophil adherence/EPO activity to fibronectin ($P < 0.02$) but not VCAM-1 com-

pared with the control. In concurrence with previous data in Fig. 1, LF significantly reduced eosinophil adherence/EPO activity following GM-CSF treatment in the presence of fibronectin ($P = 0.03$) and VCAM-1 ($P = 0.01$) when compared with GM-CSF treatment alone. These assessments were performed using eosinophils from 5 different donors.

LF modulation of eosinophil adherence as measured by CFSE labeling

To further assess the potential functions of LF on eosinophil adhesion, and to validate the aforementioned cell adhesion measurements via EPO activity, we also labeled eosinophils with a fluorescent dye and assessed cell adhesion using fluorospectroscopic analyses. Specifically, fluorescently (CFSE) labeled eosinophils were plated in wells coated with 10 μ g/mL fibronectin or VCAM-1 and treated with 100 pM GM-CSF $\pm$ 10 μ g/mL bovine or human LF for 1 h. Residual fluorescence was examined after repeated plate washings as detailed in Materials and Methods. As shown in Fig. 2B and C, we observed that GM-CSF treatment significantly enhanced the relative fluorescence in the presence of fibronectin ($P < 0.03$) and VCAM-1 ($P < 0.04$) compared with the control. Similar to Fig. 2A, LF (bovine or human) treatment significantly reduced the relative fluorescence of GM-CSF cultures in the presence of fibronectin and VCAM-1 compared with GM-CSF treatment alone.

FIG. 2. LF-modulated adherence to FN and VCAM-1. Purified human blood eosinophils were cultured in plates coated with 10 μ g/mL FN or 10 μ g/mL VCAM-1. Cells were treated with 100 pM GM-CSF and 10 μ g/mL bovine or human (H) LF for 1 h and examined for eosinophil adherence via EPO activity or CFSE labeling. **(A)** The average number of eosinophils adhered relative to an EPO activity standard curve is displayed $\pm$ SEM, $n = 5$, * $P < 0.02$ versus buffer control, $^{\dagger}P < 0.03$ versus GM-CSF. **(B)** The average number of eosinophils adhered relative to a CFSE fluorescence standard curve is displayed $\pm$ SEM, $n = 8$, * $P < 0.03$ versus buffer control, $^{\dagger}P < 0.04$ versus GM-CSF. **(C)** Bovine versus human (H) LF: The average number of eosinophils adhered relative to a CFSE fluorescence standard curve is displayed $\pm$ SEM, $n = 4$, * $P < 0.04$ versus buffer control, $^{\dagger}P < 0.05$ versus GM-CSF; VCAM-1, vascular adhesion molecule-1; CFSE, carboxyfluorescein diacetate succinimidyl ester.

FIG. 3. LF-modulated CD49d and CD11b expression. Purified human blood eosinophils (2×10^6 cells/mL) were cultured in plates coated with $10 \mu\text{g/mL}$ fibronectin (FN). Cells were treated with buffer control, 100 pM GM-CSF, and/or $10 \mu\text{g/mL}$ bovine LF for 24 h. Live cells were gated (A, C) and charted (B, D) for integrin expression. CD11b and CD49d are identified via flow cytometry and reported as the gMFI. Charts represent treatment with LF alone (B) ($\pm \text{SEM}$, $n=4$, $*P=0.01$ versus CD11b buffer control) or cotreatment with LF and GM-CSF (D) ($\pm \text{SEM}$, $n=23$ $*P<0.02$ versus CD49d buffer control, $^{\dagger}P<0.0001$ versus respective buffer control, $^{\ddagger}P=0.0002$ versus CD49d GM-CSF, $^{\S}P=0.0008$ versus CD11b GM-CSF) compared to controls. The values in parenthesis indicate the ratio of CD11b gMFI/CD49d gMFI. gMFI, geometric mean fluorescent intensity.

LF-modulation of eosinophil integrin expression

Because eosinophil adhesion can be mediated by the expression of integrins (Barthel and others 2006), we examined $\alpha 4$ (CD49d) and αM (CD11b) integrin subunit expression by flow cytometry after 24 h of cell culture on fibronectin-coated wells and following treatments with vehicle control, 100 pM GM-CSF, or $10 \mu\text{g/mL}$ bovine LF. As summarized in Fig. 3B, treatment of eosinophils from 4 different donors with LF alone did not yield a detectable effect on integrin expression when compared with the control. Also, treatment with GM-CSF enhanced CD11b expression but had no discernible effect on CD49d as previously reported (Wong and others

2003). However, as shown in Fig. 3C, representative dot plots of live cells reveal enhanced CD11b expression but reduced CD49d expression upon cotreatment with GM-CSF and LF when compared with cells treated with either buffer control or GM-CSF treatment alone. In Fig. 3D, the geometric mean fluorescent intensity (gMFI) from the flow cytometric analyses of CD49d and CD11b surface expression from 23 different donors is charted. We also listed the ratio of CD11b gMFI/CD49d gMFI. These data support the general capacity of LF to upregulate CD11b expression ($P=0.0008$) but downregulate CD49d expression ($P=0.0002$) in the context of cotreatment with GM-CSF. In Fig. 4, each of the 23 donors is plotted with respect to their response to GM-CSF alone and

FIG. 4. Donor to donor variability in LF-modulated CD49d and CD11b expression. Purified human blood eosinophils from each of the 23 donors charted in Figure 3D are plotted. Identified markers represent the CD49d or CD11b gMFI of eosinophils treated with 100 pM GM-CSF $\pm 10 \mu\text{g/mL}$ bovine LF normalized to the GM-CSF treatment.

GM-CSF plus LF. Each data point is normalized to the GM-CSF treatment and the representative trends for these 23 donors and their expression of CD49d and CD11b are displayed. Using the Spearman rank correlation coefficient and comparing changes from GM-CSF to GM-CSF plus LF in these 23 donors, a significant inverse correlation was also identified ($R = -0.82$, $P < 0.0001$) between CD49d and CD11b. This response was not related to gender (13 men, 10 women), medications, or forced expiratory volume (1.0 s) percent predicted values (data not shown). However, a few donors, who did not respond to LF treatment with reduced CD49d and increased CD11b, were found to have a higher than the average fixed exhalation nitric oxide rate or a higher than average percentage of eosinophils in their white blood cell count (data not shown).

LF-modulated eosinophil integrin expression compared to NAC

The activation of eosinophil $\alpha 4\beta 1$ has been reported to occur in response to superoxide exposure (Chuang and others 2003). To test the hypothesis that GM-CSF-treated eosinophil integrin expression is altered by antioxidants, eosinophils from 9 different donors were cultured in fibronectin-coated wells and treated with vehicle control, 100 pM GM-CSF, 10 $\mu\text{g}/\text{mL}$ bovine LF, and/or 10 mM NAC for 24 h. As shown in Fig. 5, cotreatment of eosinophils with GM-CSF and LF led to enhanced CD11b expression but significantly decreased CD49d expression compared with GM-CSF treatment alone, which is similar to results presented in Figs. 3 and 4. Addition of NAC to eosinophils cultured with GM-CSF also promoted the downregulation of CD49d ($P = 0.03$) but had no effect on CD11b surface expression when compared with GM-CSF treatment alone. Incubation with LF, NAC, and GM-CSF together resulted in a synergistic downregulation of CD49 that was significantly lower than that observed with eosinophils cultured with GM-CSF plus either LF ($P = 0.03$) or NAC ($P = 0.002$) alone. The expression of CD11b in these samples was not significantly different than the controls but trended lower than eosinophils treated with GM-CSF plus LF alone.

LF-modulation of eosinophil chemokine production

Leukocyte adhesion to fibronectin via $\beta 1$ integrins can be rapidly induced and subsequently reduced by exposure to the chemokines, MIP-1 α and MCP-1 (Carr and others 1996; Weber and others 1996). To test whether LF affects the production of these chemokines, cell-free conditioned media were isolated from eosinophils cultured on fibronectin-coated surfaces and treated with vehicle control, 100 pM GM-CSF, 10 $\mu\text{g}/\text{mL}$ bovine LF, and/or 10 mM NAC for 24 h. Initial evaluation of MIP-1 α and MCP-1 via ELISA indicated that LF alone did not induce chemokine production ($n = 7$, data not shown). The additional study of 16 different donors (Fig. 6) revealed that in the presence of GM-CSF, LF, but not NAC, chemokine production was significantly induced ($P < 0.05$) compared with either vehicle or GM-CSF treatment alone. Further, in examining changes in chemokine production versus changes in integrin expression from each of the 9 donors in Fig. 5 using the Spearman rank correlation coefficient, significant correlations were revealed (Table 1). Subjects with larger decreases in CD49d from GM-CSF to GM-CSF plus LF had larger increases in both MIP-1 α ($R = -0.75$,

TABLE 1. SPEARMAN RANK CORRELATION COEFFICIENTS COMPARING CHANGES IN INTEGRIN EXPRESSION TO CHANGES IN CHEMOKINE PRODUCTION BASED ON THE TREATMENTS OF GM-CSF AND GM-CSF PLUS LF AND/OR NAC

	CD49d versus MIP-1 α	CD49d versus MCP-1	CD11b versus MIP-1 α	CD11b versus MCP-1	MIP-1 α versus MCP-1
GM-CSF-(GM-CSF + LF)	$R = -0.75$, $P = 0.03^a$	$R = -0.80$, $P = 0.01^a$	$R = +0.72$, $P = 0.04^b$	$R = +0.68$, $P = 0.05^b$	$R = +0.92$, $P = 0.001^b$
GM-CSF-(GM-CSF + NAC)	$R = +0.03$, $P = 0.95$	$R = -0.32$, $P = 0.41$	$R = +0.60$, $P = 0.10$	$R = +0.40$, $P = 0.29$	$R = +0.58$, $P = 0.11$
GM-CSF-(GM-CSF + LF + NAC)	$R = -0.33$, $P = 0.39$	$R = -0.47$, $P = 0.21$	$R = +0.97$, $P = 0.0002^b$	$R = +0.88$, $P = 0.003^b$	$R = +0.93$, $P = 0.0007^b$

Purified human blood eosinophils treated with GM-CSF, GM-CSF plus LF (GM-CSF + LF), GM-CSF plus NAC (GM-CSF + NAC), or GM-CSF plus LF and NAC (GM-CSF + LF + NAC) from each of the 9 donors in Fig. 5 were examined for statistical dependence with respect to the variables of integrin gMFI (CD49d, CD11b) and chemokine production (MIP-1 α , MCP-1). The changes in CD49d, CD11b, MIP-1 α , and MCP-1 from GM-CSF alone to (GM-CSF + LF), (GM-CSF + NAC), or (GM-CSF + LF + NAC) were expressed as ratios. The associations between changes in CD49d or CD11b with MIP-1 α and MCP-1 were evaluated using the Spearman rank correlation coefficient.

^aInverse correlation.

^bPositive correlation ($n = 9$).

GM-CSF, granulocyte-macrophage colony-stimulating factor; NAC, N-acetyl-L-cysteine; LF, lactoferrin; gMFI, geometric mean fluorescent intensity; MIP-1 α , macrophage inflammatory protein-1 α ; MCP-1, monocyte chemoattractant protein-1.

FIG. 5. LF-modulated CD49d and CD11b expression compared to NAC. Purified human blood eosinophils (2×10^6 cells/mL) were cultured in plates coated with $10 \mu\text{g/mL}$ FN. Cells were treated with buffer control, 100 pM GM-CSF, 10 mM NAC, and/or $10 \mu\text{g/mL}$ bovine LF for 24 h. Live cells were gated (**A**) and charted (**B**) for integrin expression. CD11b and CD49d are identified using flow cytometry and reported as the gMFI, $\pm$ SEM, $n=9$, $^*P < 0.02$ versus respective buffer control, $^\dagger P < 0.03$ versus GM-CSF, $^\ddagger P = 0.03$ versus GM-CSF+LF, $^\S P = 0.002$ versus GM-CSF+NAC, $^\P P < 0.008$ versus GM-CSF+NAC. The values in parenthesis indicate the ratio of CD11b gMFI/CD49d gMFI. NAC, N-acetyl-L-cysteine; FN, fibronectin.

$P=0.03$) and MCP-1 ($R = -0.80$, $P=0.01$) from GM-CSF to GM-CSF plus LF. Subjects with larger increases in CD11b from GM-CSF to GM-CSF plus LF also had larger increases in both MIP-1 α ($R = +0.72$, $P=0.04$) and MCP-1 ($R = -0.68$, $P=0.05$) from GM-CSF to GM-CSF plus LF. Similar to data shown in Fig. 4, cells obtained from subjects that exhibited larger decreases in CD49d from GM-CSF alone to GM-CSF plus LF also had larger increases in CD11b ($R = -0.85$, $P=0.006$). These correlations were not significant in comparing changes between GM-CSF and GM-CSF plus NAC. Additionally, subjects with larger increases in MIP-1 α had larger increases in MCP-1 ($R = +0.92$, $P=0.001$). These correlations suggest that the combined cell signals of LF and GM-CSF enhance chemokine production that may be indirectly involved in the upregulation of CD11b and downregulation of CD49d.

Eosinophil calgranulin production

Increased expression of CD11b in neutrophils has been found to be regulated by calgranulin B (Newton and Hogg 1998; Manitz and others 2003). We have previously identified GM-CSF-induced calgranulin B (S100A9) production in eosinophils (Curran and Bertics 2011; Curran and others 2011). In Figs. 3–5, we show GM-CSF-induced CD11b ex-

pression that is enhanced by cotreatment with LF. To test whether LF enhances GM-CSF-induced S100A9 production, eosinophils from 4 different donors were cultured in fibronectin-coated wells and treated with vehicle control, 100 pM GM-CSF, or $10 \mu\text{g/mL}$ bovine LF for 24 h. Cell lysates and conditioned media were examined for calgranulin B (S100A9) by immunoblotting as detailed in Materials and Methods. As illustrated in Fig. 7, S100A9 is significantly produced in the conditioned media from eosinophils treated with GM-CSF compared with the conditioned media control ($P=0.03$). Cotreatment of LF with GM-CSF enhanced S100A9 production in the conditioned media compared with the controls of GM-CSF alone ($P < 0.05$) and control buffer ($P < 0.04$).

Discussion

Eosinophils are innate immune cells that are characteristic of both healthy and diseased tissues (Kita 2011). The mechanisms that propagate eosinophils in homeostatic and inflammatory conditions are not clearly known (Kita 2011; Woodruff and others 2011). Like the eosinophil, the protein LF is found in homeostatic (eg, mammary gland) and inflamed [eg, asthmatic lung and inflammatory bowel disease (IBD)] tissue (Kayazawa and others 2002; Monteseirin 2009;

FIG. 6. LF-modulated MIP-1 α and MCP-1 production. Purified human blood eosinophils (2×10^6 cells/mL) were cultured in plates coated with $10 \mu\text{g/mL}$ FN. Cells were treated with buffer control, 100 pM GM-CSF, 10 mM NAC, and/or $10 \mu\text{g/mL}$ bovine LF for 24 h. Cell-free conditioned media was measured in triplicate per experiment for the specified protein. Repeat experiments were averaged and are displayed as the mean concentration (pg/mL). Charted concentrations represent the chemokines MIP-1 α (A) (mean $\pm$ SEM, $n=16$, $*P < 0.003$ versus control, $^\dagger P < 0.05$ versus GM-CSF, $^\ddagger P < 0.004$ versus GM-CSF+NAC) and MCP-1 (B) (mean $\pm$ SEM, $n=16$, $*P < 0.01$ versus control, $^\dagger p = 0.001$ versus GM-CSF, $^\ddagger P < 0.004$ versus GM-CSF+NAC). MIP-1 α , macrophage inflammatory protein-1 α ; MCP-1, monocyte chemoattractant protein-1; FN, fibronectin.

Legrand and Mazurier 2010; Kita 2011). LF is secreted by activated neutrophils, a cell type also recruited in tandem with eosinophils in certain cases of asthma and IBD (Sampson 2000; Jeziorska and others 2001; Legrand and Mazurier 2010). Of interest, treatment of eosinophils with LF reportedly reduces eotaxin-stimulated eosinophil migration (Bournazou and others 2010), suggesting that LF may differentially affect cell signals associated with the expression of eosinophil adhesion molecules. To understand the nature of LF-induced eosinophil responses, we tested the hypothesis that LF can significantly alter the function, expression, and production of proteins involved in migration by examining eosinophil adhesion, integrin detection, and the production of chemokines and calgranulins.

Eosinophil adhesion to fibronectin and VCAM-1 can occur via binding interactions involving the integrins $\alpha 4$, αM , $\beta 1$, $\beta 2$, and $\beta 7$ (Anwar and others 1994; Walsh and others 1996; Matsumoto and others 1997; Barthel and others 2006). The

cytokine GM-CSF is known to induce eosinophil adherence to fibronectin (Anwar and others 1993; Bates and others 2010), whereas, LF has been indicated to inhibit the adhesion of various cell lines to fibronectin (Sakamoto and others 2006). In response to 1 h culture, we show that increasing concentrations of LF inhibit GM-CSF-induced adhesion in response to fibronectin but not a characterized $\beta 2$ ligand, BSA (Zhu and others 2000). We also identified that LF inhibits GM-CSF-induced adhesion to VCAM-1 via EPO assay (Fig. 2A) and a CFSE labeling adhesion assay (Fig. 2B). Interestingly, GM-CSF treatment enhanced adhesion via CFSE labeling (Fig. 2B) but not via EPO assay (Fig. 2C) in response to the VCAM-1 substrate, suggesting that donor to donor responses vary or that possibly during cell culture, EPO may be nonspecifically released from cells and thereby adhere to wells as has been previously described (Adamko and others 2004). In Fig. 2C, we assessed a comparison of bovine LF, derived from milk and used throughout this article, with human LF obtained from neutrophils. As shown, both variants of LF inhibited adhesion to fibronectin and VCAM-1 in the presence of GM-CSF. Differences in efficacy with respect to VCAM-1 could be due to differences in charge between bovine (+18) and human (+11) LF (Harmsen and others 1995), particularly if LF binds eosinophils via charge interactions. Alternatively, the structure of LF and downstream cell signals of the molecule have been reported to differ depending upon the iron bound to LF (Jiang and others 2011; Baker and Baker 2012; Jiang and Lonnerdal 2012). Bovine LF used in this study was 10% to 20% iron-saturated, whereas, the amount of iron bound to the human LF was not available.

After 24 h culture, we found that GM-CSF induced CD11b but not CD49d expression, whereas, LF treatment alone had no effect on the immunodetection of these integrins (Fig. 3B) or cell viability (data not shown). We also noted that co-treatment with GM-CSF and LF enhanced CD11b but downregulated CD49d expression (Figs. 3D and 4) and did not affect cell viability (data not shown). One potential mechanism for these data comes from the observation that LF may function, at least in part, as a superoxide dismutase and thus converts superoxide (O_2^-) to hydrogen peroxide (H_2O_2 ; Comhair and Erzurum 2010). Because CD49d and CD11b are respectively induced by O_2^- and H_2O_2 (Nagata and others 2000; Chuang and others 2003), LF-mediated functions as an antioxidant may reduce CD49d but enhance CD11b expression.

Because, as mentioned above, the regulation of CD49d and CD11b has been shown to be redox sensitive (Nagata and others 2000; Puig-Kroger and others 2000; Chuang and others 2003), we compared LF-induced responses in the presence of GM-CSF to similar treatments with NAC. Results demonstrated that NAC did induce CD49d downregulation but not CD11b upregulation (Fig. 5B). Interestingly, the NAC-modulated cell scatter was distinctly different from LF in that the NAC population was uniquely more uniform than that of LF (Fig. 5A), suggesting that the effects of LF may be directed to a distinct population subset. Including both LF and NAC in GM-CSF cultures enhanced CD49d downregulation compared with LF or NAC treatments alone (Fig. 5B). Interestingly, inclusion of LF in GM-CSF plus NAC treatments enhanced CD11b expression compared with NAC plus GM-CSF alone (Fig. 5B). These data support the idea that LF can operate

FIG. 7. Immunoblot detection of intracellular and extracellular eosinophil S100A9 in response to LF. Purified human blood eosinophils (5×10^6 /mL) were cultured in plates coated with $10 \mu\text{g}/\text{mL}$ FN. Cells were suspended in 0.1% human serum albumin RPMI and treated with buffer control, 100 pM GM-CSF or $10 \mu\text{g}/\text{mL}$ bovine LF for 24 h. Total eosinophil cell lysates from 5×10^5 cells and the total corresponding conditioned media were loaded onto a 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel. A representative immunoblot is displayed in (A). Data were also quantified with ImageJ software, expressed as S100A9 mean band densitometry relative to actin controls and normalized to the respective media control, $n=4$, $*P<0.04$ versus conditioned media buffer control, $^\dagger P<0.05$ versus conditioned media GM-CSF control.

synergistically with NAC via CD49d but not via CD11b expression. Thus, LF may have additional functions other than serving as an antioxidant.

To further investigate the LF-induced CD49d and CD11b modulation, we examined the production/release of chemokines (MIP-1 α and MCP-1) and calgranulin B (S100A9), which are respectively known to regulate CD49d and CD11b expression (Carr and others 1996; Weber and others 1996; Newton and Hogg 1998; Manitz and others 2003). In Fig. 6, GM-CSF treatments with LF alone or in combination with NAC upregulated chemokine production compared with controls, whereas, NAC did not exhibit a response. The increased production of chemokines in GM-CSF plus LF-treated samples was also revealed to be correlative with reduced CD49d and increased CD11b immunodetection (Table 1). These associations were not revealed in GM-CSF plus NAC treatments, suggesting that LF and NAC do not operate along similar cell signaling pathways. We also assessed eosinophil intracellular and extracellular production of S100A9. The levels of extracellular S100A9 increased in response to GM-CSF treatment, as we have previously described (Curran and Bertics 2011), and the addition of LF enhanced the effect. Combined, these data suggest that in the presence of GM-CSF, LF induces the production of chemokines (MIP-1 α , MCP-1, and S100A9) that may then alter eosinophil integrin expression and function. Because eosinophil priming with GM-CSF enhances cell signaling (Bates and others 2010) and the sensitivity of integrins to bind ligands (Lampinen and others 2004), the altered responses (chemokine production and integrin expression) to LF cotreatment may be an effect of the increased binding affinity of the LF receptor for its ligand and/or increased cell signals associated with GM-CSF priming.

Previous research has indicated that cationic-charged surfaces coupled with GM-CSF treatment induce increased

eosinophil mediator release associated with the increased density of CD18 expression (Adamko and others 2008). The N1-terminal domain of bovine and human LF has a net positive charge and contains a heparin-binding peptide fragment termed lactoferricin that is known to survive digestion (van Berkel and others 1997; Kuwata and others 1998; Shimazaki and others 1998; Vogel and others 2002). Studies involving nasal polyp fibroblasts and the Caco-2 colon cancer cell line indicate that LF-binding interactions with these cells is inhibited by heparin (Ashida and others 2004; Nadolska and others 2010). Heparin and anti-thrombin III have been shown to modulate eosinophil chemokine production and migration via HSPGs (Culley and others 2003; Feistritzer and others 2004), indicating that the LF-induced eosinophil chemokine production (Fig. 6), S100A9 release (Fig. 7), CD49d downregulation, and CD11b upregulation (Figs. 3–5) may partially arise by virtue of the heparin-binding activity that has been ascribed to LF (Wu and others 1995).

In the pathobiology of atopic disease, eosinophils are known to accumulate in the skin, lung, and gastrointestinal tract (Simon and others 2010). Eosinophils are also identified in various tumors and in some clinical cases of multiple sclerosis where patients were treated with an anti-CD49d antibody called natalizumab (Samoszuk 1997; Abbas and others 2011). LF is an identified immunoregulator in diseases such as asthma (Kruzel and others 2006), cancer (Ward and others 2005), and multiple sclerosis (Zimecki and others 2007) where the integrin $\alpha 4 \beta 1$ has been found to be pivotal to the progression of these diseases (Singh and others 2004; Garmy-Susini and others 2010; Skarica and others 2011). The conditions by which LF may regulate the function of integrins on eosinophils or additional cell types may be integral in understanding disease development and immunotherapeutic treatment.

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Author Disclosure Statement

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