

Lactoferrin Acts as an Alarmin to Promote the Recruitment and Activation of APCs and Antigen-Specific Immune Responses^{1,2}

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Lactoferrin is an 80-kDa iron-binding protein present at high concentrations in milk and in the granules of neutrophils. It possesses multiple activities, including antibacterial, antiviral, antifungal, and even antitumor effects. Most of its antimicrobial effects are due to direct interaction with pathogens, but a few reports show that it has direct interactions with cells of the immune system. In this study, we show the ability of recombinant human lactoferrin (talactoferrin alfa (TLF)) to chemoattract monocytes. What is more, addition of TLF to human peripheral blood or monocyte-derived dendritic cell cultures resulted in cell maturation, as evidenced by up-regulated expression of CD80, CD83, and CD86, production of proinflammatory cytokines, and increased capacity to stimulate the proliferation of allogeneic lymphocytes. When injected into the mouse peritoneal cavity, lactoferrin also caused a marked recruitment of neutrophils and macrophages. Immunization of mice with OVA in the presence of TLF promoted Th1-polarized Ag-specific immune responses. These results suggest that lactoferrin contributes to the activation of both the innate and adaptive immune responses by promoting the recruitment of leukocytes and activation of dendritic cells. *The Journal of Immunology*, 2008, 180: 6868–6876.

Lactoferrin is an 80-kDa protein that belongs to the transferrin superfamily, which binds Fe cations with high affinity (1, 2). It is secreted in an iron-free form from many epithelial cells into most exocrine fluids, particularly milk. In humans, its concentration varies from ~1 to 7 mg/ml in milk and in colostrum, respectively (3). Lactoferrin is a major component of the secondary granules of neutrophils, which, like many granule components, are released through degranulation upon neutrophil activation (4, 5). During inflammation, lactoferrin levels of the biologic fluids increase dramatically. This is particularly noticeable in blood, where lactoferrin concentration can be as low as 0.5–1 μ g/ml under normal conditions, but increases to 200 μ g/ml with systemic bacterial infection (6, 7). Recent reports indicate that lactoferrin expression in both neutrophils and epithelial cells can be induced (8, 9).

Lactoferrin is multifunctional and has a widely accepted antimicrobial effect against bacteria, viruses, fungi, and some parasites (10). One mechanism by which lactoferrin exerts its antimicrobial

effect depends on its iron-binding property that enables lactoferrin to sequester iron required for bacterial growth (10, 11). Lactoferrin is also capable of binding to glycosaminoglycans (in particular to heparan sulfate) of mucosal epithelial cells, resulting in the inhibition of microbial adhesion, colonization, and subsequent development of infection at mucosal surfaces (10, 12). Furthermore, lactoferrin has direct microbicidal activity that is independent of its iron-binding property (10, 12–14).

In addition to its antimicrobial effect, it has also been reported that lactoferrin has a variety of effects on the host immune system, ranging from inhibition of inflammation to promotion of both innate and adaptive immune responses (10, 15). Interestingly, recombinant human lactoferrin (talactoferrin (TLF)⁴) has recently been used as a therapeutic agent against several cancers with positive results (16, 17), including in clinical trials (18). Although the anti-inflammatory activity of lactoferrin is largely due to binding and neutralization of proinflammatory molecules such as bacterial endotoxin and soluble CD14, its capacity to promote innate immune responses is often explained by the ability of lactoferrin to promote activation of neutrophils and macrophages (10, 15, 19). We hypothesized that lactoferrin might also have a direct receptor-dependent activating effect on APCs including dendritic cells (DCs), thereby mobilizing and alerting the adaptive immune system.

In this study, we show for the first time the ability of recombinant human GMP-quality lactoferrin to recruit and activate APCs, and to enhance Ag-specific immune responses. These functional characteristics of lactoferrin are also shared by alarmins, a group of endogenous mediators of the immune system that link innate and adaptive immunity by promoting the recruitment and activation of APCs (20). Therefore, lactoferrin may act as an alarmin and rapidly mount responses to danger signals.

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⁴ Abbreviations used in this paper: TLF, talactoferrin; DC, dendritic cell; hLF, human lactoferrin; PBDC, peripheral blood DC; moDC, monocyte-derived DC; Treg, regulatory T lymphocyte; PI, propidium iodide; PTx, pertussis toxin; MCP-1, monocyte chemoattractant protein-1; SLC, secondary lymphoid tissue chemokine.

Materials and Methods

Reagents and Abs

FITC-conjugated anti-human CD4, CD18, CD80, CD83, CD86, PE-conjugated anti-human CD3, CD11b, CD14, CD19, CD29, CD56, Cy5.5-conjugated anti-human CD54, and PerCP-Cy5.5-conjugated anti-human HLA-DR were purchased from BD Pharmingen. FITC-conjugated anti-human BDCA-2 and CD1c were purchased from Miltenyi Biotec. FITC-conjugated anti-mouse CD11b, LY6G (anti-Gr-1), PE-CD11c, and PerCP-B220 were purchased from BD Pharmingen. Anti-mouse F4/80 PE-Cy5 was obtained from eBioscience.

Human recombinant lactoferrin (TLF α) was a gift from Agennix. In brief, TLF is recombinantly produced in *Aspergillus niger* var. *awamori* (nontoxigenic and nonpathogenic) (21), purified by ion-exchange chromatography, yielding a 95–99% purity, with a three-dimensional structure to be essentially identical with that of human lactoferrin (hLF) isolated from human milk (22). TLF is pharmaceutical-grade, manufactured using a GMP process, free of contaminating host-cell DNA, host-cell proteins, or mycotoxins. Human monocyte chemoattractant protein-1 (MCP-1)/CCL2, secondary lymphoid tissue chemokine (SLC)/CCL21, and Stromal Derived Factor-1 α /CXCL12 were obtained from PeproTech.

Cell culture

Human peripheral blood enriched in mononuclear cells was obtained from healthy donors by leukapheresis (Transfusion Medicine Department, Clinical Center, National Institutes of Health, Bethesda, MD, with an approved human subjects agreement). The blood was centrifuged through Ficoll-Hypaque (Sigma-Aldrich), and PBMC collected at the interface were washed with PBS and centrifuged through an iso-osmotic Percoll (Pharmacia) gradient. The enriched monocyte populations were obtained at the very top of the gradient (top fraction). For some experiments, monocytes were further purified by magnet sorting (monocyte purification kit; Miltenyi Biotec). To obtain monocyte-derived DCs (moDCs), monocytes were cultured as described previously (23). Briefly, cells were resuspended in RPMI 1640 containing 10% FCS (Invitrogen) at 1×10^6 cells/ml in the presence of IL-4 and GM-CSF (PeproTech, both at 50 ng/ml) every 2 days. After 6 days, they were used as immature DCs. To induce maturation, immature DCs were cultured in the presence of LPS (*E. coli*; 055:B5 strain, 200 or 500 ng/ml; Sigma-Aldrich) or distinct concentrations of TLF. CD1c⁺ peripheral blood DCs (PBDCs) were purified with the CD1c⁺ purification kit (Miltenyi Biotec) following the vendor's instructions. For MLR experiments, 10^5 Percoll-enriched lymphocytes (10^5 /well) were cocultured with DCs at different ratios in 96-well round-bottom plates (Costar) for 5 days, with the addition of 1 μ Ci of [³H]TdR to each well (Amersham Pharmacia Biotech) 16 h before the end of the experiment. Cells were then harvested onto filter membranes using an Inotech harvester (Inotech Biosystems), and the amount of incorporated [³H]TdR was measured with a Wallac Microbeta counter (PerkinElmer Life and Analytical Sciences).

For regulatory T lymphocytes (Treg) studies, PBMCs were cultured at 10^6 cell/ml in RPMI 1640 10% FBS for 48 h with or without IL-2 (100 U/ml), TNF- α (50 ng/ml), or various concentrations of TLF as specified. Subsequently, cultured PBMCs were recovered, counted, and stained either with the Annexin V/PI kit to detect cell viability or with CD3, CD4, and FoxP3 (APC-anti-human FoxP3 Staining Set; eBioscience) for Treg determination.

Analysis of cell viability

Cells were recovered after the desired period of time from the plate and the wells were rinsed with cold PBS/EDTA (5 mM). Adherent cells were recovered by gently scraping the wells. After mixing the recovered volumes well, a fraction of the recovered cells was used for counting and the rest were stained with the Annexin V/PI staining set (from eBioscience) to assess early and late apoptosis by flow cytometry. Cells that did not stain for annexin V and propidium iodide (PI) were considered alive; cells that only stained for annexin V (early apoptosis) were considered "dying"; cells that were double positive or were missing after counting the recovered cells were included in the "dead" category.

Migration assays

Migration assays were performed using two different approaches to double check the effect of lactoferrin: 1) 48-well microchemotaxis chambers (NeuroProbe) and 2) 6.5-mm transwell polycarbonate inserts (Corning) as described previously. Membranes of 5- μ m pore diameter were used in both systems. Cells were washed in PBS and resuspended in RPMI 1640 (Life Technologies) supplemented with 1% BSA (Sigma-Aldrich). When pertussis toxin (PTx) from *Bordetella pertussis* (List Biological Laboratories)

was used, cells were preincubated at 37°C for 1 h in the presence of the toxin at a concentration of 100–200 ng/ml before migration experiments. Cells then were loaded in the chambers and allowed to migrate for 2 h at 37°C. In the 48-well chemotaxis assay, experiments were run in triplicates and migrated cells were fixed, stained, and counted using Bioquant Life Science (Bioquant Image Analysis) software. Transwell migration assays were performed as previously described (24) with a slight modification: experiments were run in duplicates, and migrated cells were recovered. An aliquot of the migrated cells was used for counting while the remaining cells were used to phenotype PBDCs within the PBMCs as previously described (25): HLA-DR⁺/Lin (CD3, CD11b, CD14, CD19, and CD56)^{neg} and either BDCA-2⁺ or CD1c⁺ using a FACScan cytometer (BD Biosciences). The results of migration experiments were shown as migration index, which was calculated as: (number of cells migrated in the presence of a chemotactic factor)/(number of cells migrated in the absence of a chemotactic factor).

Cytokine production

Supernatants from monocytes or DCs were collected after 48 h of treatment with or without TLF or LPS. Samples were read using a multiplex plate for IL-12p70, IL-6, TNF- α , and IL-10 (Pierce Biotech) or a 10-plex cytokine plate (Meso Scale Discovery).

Leukocyte recruitment

Female wild-type C57BL/6Ncr mice (8–10 wk old) were provided by the Animal Production Area of the National Cancer Institute (NCI; Frederick, MD). NCI-Frederick is accredited by Association for Assessment and Accreditation of Laboratory Animal Care International and follows the Public Health Service's "Policy for the Care and Use of Laboratory Animals." Animal care was provided in accordance with the procedures outlined in the "Guide for Care and Use of Laboratory Animals." Mice were injected i.p. with 1.0 ml of PBS containing various amounts of TLF or LPS. After 4 or 24 h, mice were sacrificed and cells were harvested by peritoneal lavage using 5 ml of ice-cold PBS with 5 mM EDTA. Cells were counted, phenotyped for Gr-1/F4–80 and CD11b/CD11c/B220, and analyzed using a FACScan flow cytometer (BD Biosciences).

Immunization procedure and detection of Ag-specific splenocyte proliferation and cytokine production

Eight-week-old C57BL/6 mice (three to approximately five mice per group) were injected i.p. on day 1 with 0.2 ml of PBS containing 50 μ g of OVA (Sigma-Aldrich) in the presence or absence of alum (Sigma-Aldrich) or TLF. On day 14, all mice were booster immunized by i.p. injection of 0.2 ml of PBS containing 50 μ g of OVA. On day 21, immunized mice were euthanized to remove spleens. Spleens of each group of mice were collected and pooled to make single splenocyte suspension. OVA-specific splenocyte proliferation and/or cytokine production was measured as previously described with minor modifications (26). Briefly, splenocytes (5×10^5 /well) were seeded in triplicate in wells of round-bottom 96-well plates in complete RPMI 1640 medium (0.2 ml/well) and incubated in the presence or absence of indicated concentration of OVA at 37°C in a CO₂ incubator for 60 h. The cells were pulsed with [³H]TdR (1 μ Ci/well) for the last 18 h to assess splenocyte proliferation. Alternatively, pooled splenocytes of each group were cultured in complete RPMI 1640 in 24-well plates (5×10^6 /1 ml/well) with indicated concentrations of OVA for 48 h before the culture supernatants were harvested to determine their cytokine content (Pierce SearchLight multiplex ELISA).

Statistical analysis

Data were analyzed using paired two-tailed Student's *t* test comparing untreated samples with lactoferrin-treated or LPS-treated samples, using GraphPad Prism software (GraphPad Software).

Results

Lactoferrin induces migration of human peripheral blood monocytes

The chemotactic effect of recombinant human lactoferrin (TLF) was tested in vitro with PBMC in transwell migration assays. Monocytes consistently responded, but with low efficacy, whereas lymphocytes and PBDCs did not migrate at all in response to lactoferrin (data not shown). Percoll-enriched neutrophils also failed to respond to TLF (data not shown). The capacity of TLF to attract

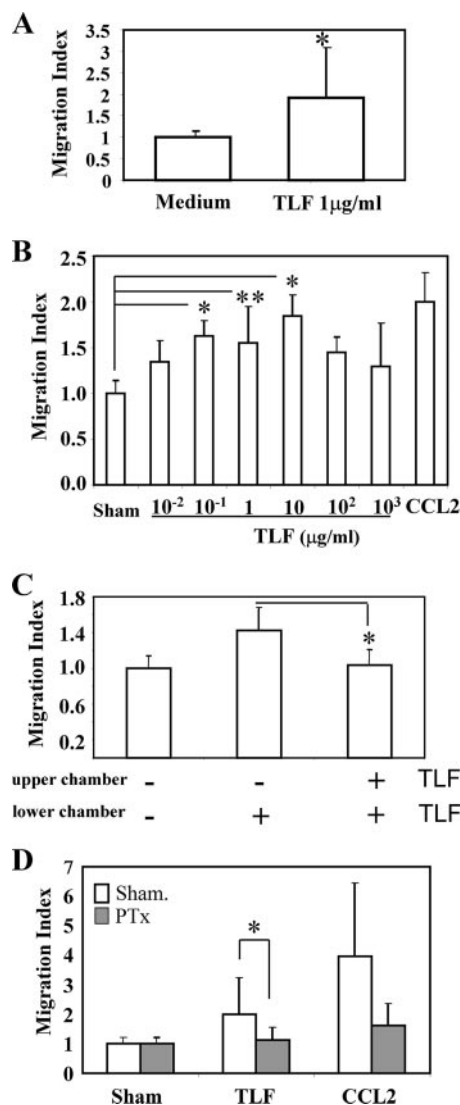


FIGURE 1. TLF induces monocyte migration. **A**, Monocyte migration was assayed using 48-well microchemotaxis chambers with the addition of monocytes ($10^6/\text{ml}$) and TLF in the upper and lower chambers, respectively. After 2 h of incubation, the membrane was scraped, fixed, and stained. Migrated monocytes were counted using Bioquant Life Science software. Results shown are the average $\pm$ SD of seven experiments. *, $p < 0.01$. **B**, Dose response of monocyte migration to TLF. One experiment of three is shown. **C**, Checkerboard analysis. Monocytes were allowed to migrate in the presence (+) of 1 $\mu\text{g}/\text{ml}$ TLF in the lower chamber or in both the upper and lower chambers. The results shown are the average (mean $\pm$ SD) of three experiments. **D**, Effect of PTx. Monocytes were pretreated with 100 ng/ml PTx for 1 h at 37°C before use in the migration assay. A total of 100 ng/ml CCL2 was used as a positive control for monocyte migration and to confirm the effect of PTx. The data show the average (mean $\pm$ SD) of four experiments. *, $p < 0.01$; **, $p < 0.05$.

monocytes was confirmed using 48-well microchemotaxis chamber assays and monocytes purified by Percoll gradient centrifugation (Fig. 1A; $p < 0.01$). When dose-response experiments were performed, TLF ranging from 100 ng/ml to 10 $\mu\text{g}/\text{ml}$ resulted in monocyte migration (Fig. 1B). Lactoferrin-induced migration was inhibited when an identical concentration of the protein was added to both the upper and lower chambers in a simple checkerboard experiment (Fig. 1C) indicating that TLF-induced monocyte migration was chemotactic rather than chemokinetic. To investigate whether lactoferrin-induced migration was mediated by a $G_i\alpha$ pro-

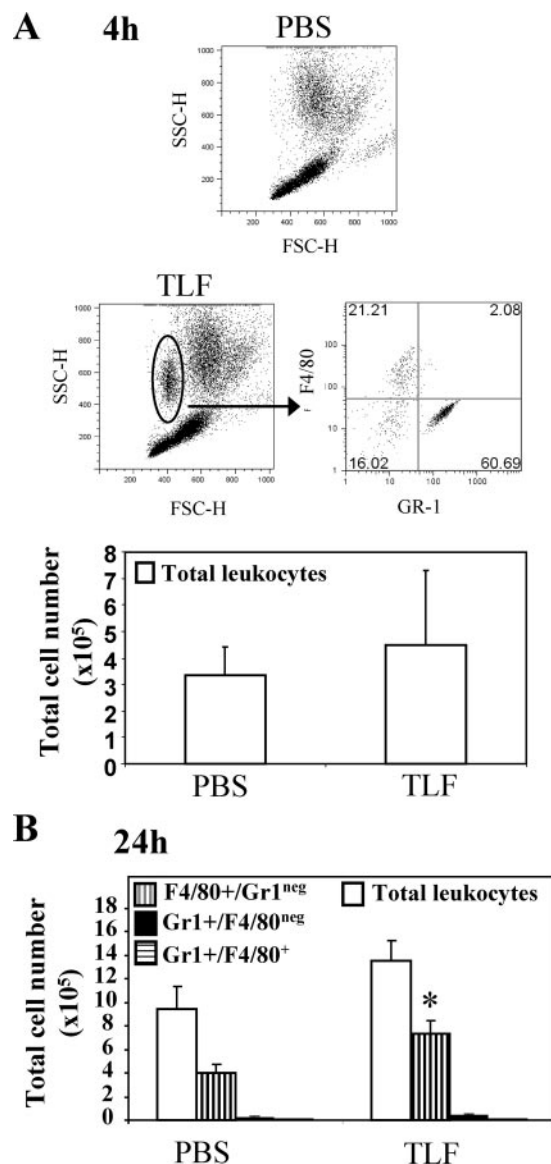


FIGURE 2. Lactoferrin recruitment of neutrophils and monocytes/macrophages in vivo. A total of 1 $\mu\text{g}/\text{ml}$ TLF or PBS was i.p. injected into C57BL/6 mice ($n = 3$). After 4 (**A**) or 24 (**B**) h, mice were sacrificed and the peritoneal cavity was rinsed with 5 ml of cold PBS containing 5 mM EDTA to recover peritoneal leukocytes. The recovered cells were counted and stained to analyze the phenotype by flow cytometry. The result of one representative experiment of three is shown. *, $p < 0.01$.

tein-coupled receptor, monocytes were pretreated with PTx. This toxin prevents $G_i\alpha$ protein association with membrane receptors, blocking the signaling cascade coming from those receptors. Pretreatment of monocytes for 1 h with PTx inhibited the migration induced by lactoferrin, indicating that the chemotactic effect of TLF was based on an interaction with $G_i\alpha$ -coupled receptor(s) (Fig. 1D). Preliminary results suggested that lactoferrin could use the CCR2 receptor. However, the chemotactic effect of TLF on monocytes could not be blocked when the cells were loaded into the chamber in the presence of MCP-1/CCL2, and conversely, the chemotaxis of monocytes toward MCP-1 could not be blocked by TLF at various concentrations, ruling out the possibility that the migration of monocytes to TLF was through CCR2 (data not shown). Similarly, Stromal Derived Factor-1 α and IL-8 failed to desensitize TLF (data not shown). Consequently, the chemotactic $G_i\alpha$ -coupled receptor used by TLF remains unknown.

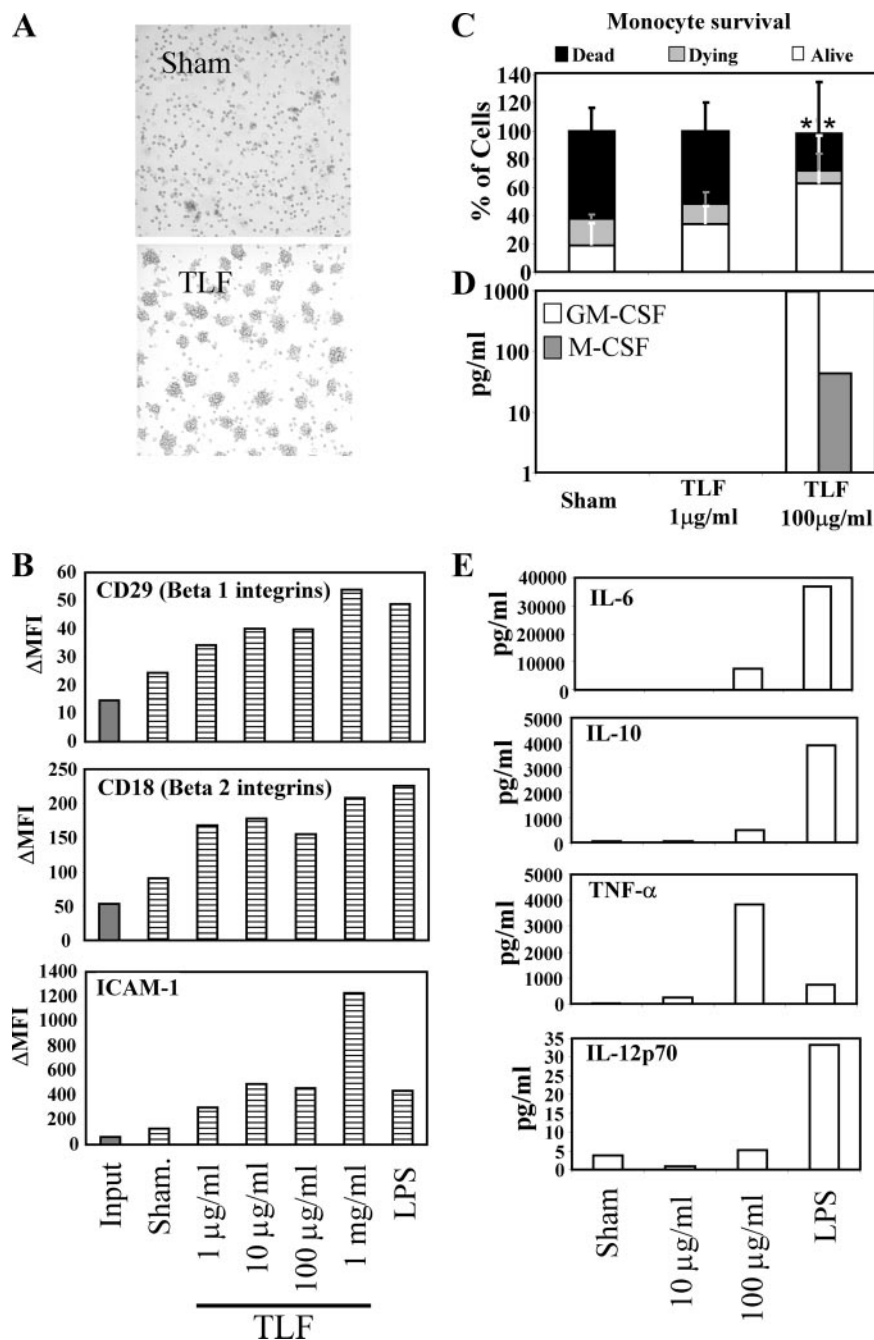


FIGURE 3. Lactoferrin activation of monocytes. Monocytes were cultured at 10^6 /ml in the presence or absence of TLF at different concentrations or LPS at 200 ng/ml for 48 h. *A*, Microscopic images ($\times 20$ magnification) showing cell distribution in monocyte cultures. TLF concentration = 10 μ g/ml. *B*, Analysis by FACS of the expression of adhesion molecules in monocytes at day 0 (Input) and after 48 h (■) culture without or with lactoferrin or LPS. Mean fluorescence intensity increment (Δ MFI) was obtained by subtracting the MFI of the isotype control from the experimental MFI. The results of one representative experiment of two are shown. *C*, Monocytes were treated for 24 h under conditions specified and counted by flow cytometry and stained with FITC-conjugated annexin V and PI. Double-negative (DN) and annexin V⁺ events are plotted. Data are presented as the percentage of cells relative to the initial population plated (100%). The results are the average (mean $\pm$ SD) of four individual experiments. DN populations were compared to analyze statistical significance. **, $p < 0.05$. *D* and *E*, Supernatants of 48-h cultured monocytes were measured for cytokine concentrations. The results of one experiment representative of three and one of two, respectively, are shown.

Lactoferrin causes recruitment of neutrophils and monocytes/macrophages in vivo

Next, we examined the *in vivo* chemotactic effects of lactoferrin. TLF was injected into the peritoneal cavity of C57BL/6Ncr mice to determine whether it could induce *in vivo* infiltration of inflammatory cells at two different time points: after 4 h, there was a clear recruitment of a subpopulation consisting mainly in Gr-1⁺/F4/80^{neg} neutrophils (Fig. 2*A*). After 24 h, that particular cell type had disappeared, but the numbers of infiltrating F4/80⁺/Gr-1^{neg} monocytes/macrophages were increased ($p < 0.01$) (Fig. 2*B*).

Lactoferrin promotes activation and survival of monocytes

In the course of treating monocytes with TLF, it was observed that the cells started to form clusters within 24 h (Fig. 3*A*). This process was presumed to be based on the activation of adhesion molecules (27). To confirm this, the surface expression level of β_1 , β_2 inte-

grins and ICAM-1 in these monocytes was analyzed by flow cytometry. Concentrations of 1 μ g/ml or higher of lactoferrin were able to induce a clear increase in the expression of both integrin types and ICAM-1 molecules (Fig. 3*B*) in the whole monocyte population. We also observed that the number of monocytes present in TLF-treated cultures was higher compared with the untreated samples. To determine whether cell survival was affected after 48 h, cells were recovered, counted, compared with the number of cells plated at day 0, and analyzed for annexin V and PI staining. TLF treatment dose-dependently rescued cultured monocytes from spontaneous apoptosis (Fig. 3*C*). Although monocytes cultured in medium alone had a percentage of survival of 18.9 ± 15.9 , monocytes cultured in the presence of 100 μ g/ml TLF increased the survival to 63.0 ± 33.6 ($p < 0.01$). Analysis of GM-CSF and M-CSF levels in these supernatants showed that 100 μ g/ml TLF induced considerable production of these cytokines,

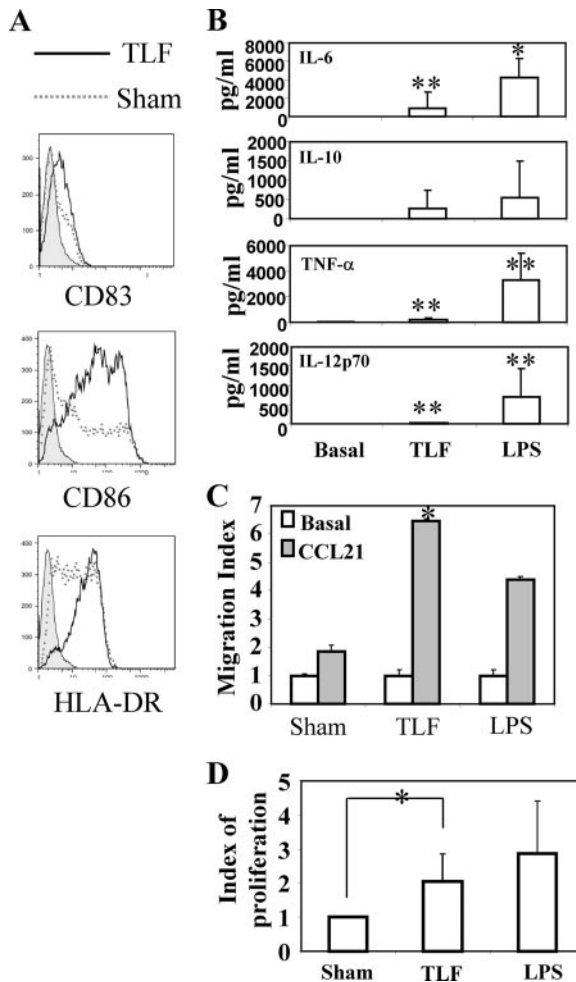


FIGURE 4. Lactoferrin induction of moDC activation. **A**, moDCs were cultured in the absence (sham) or presence of TLF at 10 $\mu\text{g/ml}$. After 48 h, the expression of CD83, CD86, and HLA-DR was analyzed by flow cytometry. Shown is the overlay histograms of isotope-control (filled), sham (dashed lines), and TLF treatment (bold lines) of one experiment representative of seven. **B**, Supernatants of moDCs cultured for 48 h in the absence (sham) or presence of TLF (10 $\mu\text{g/ml}$) or LPS (200 ng/ml) were measured for cytokines by ELISA. The results shown are the average (mean + SD) of five separate experiments. **C**, The migration of moDCs incubated in the absence (sham) or presence of TLF (10 $\mu\text{g/ml}$) or LPS (500 ng/ml) for 48 h in response to SLC/CCL21 were measured by 48-well microchemotaxis chamber assay. The results of one experiment representative of two are shown. **D**, moDCs cultured without or with TLF (10 $\mu\text{g/ml}$) or LPS (1 $\mu\text{g/ml}$) for 48 h were mixed with allogeneic PBLs ($10^5/\text{well}$) at a ratio of 1:100 and cultured (in triplicate) for 4–5 days. The cultures were pulsed with [^3H]TdR (1 $\mu\text{Ci/well}$) for the last 16 h before harvested for the measurement of [^3H]TdR incorporation. Lymphocyte proliferation was shown as proliferation index, which was calculated as: (cpm of the well with TLF- or LPS-treated DCs)/(cpm of the well with sham-treated DCs). Data represent the average (mean + SD) of seven separate experiments; *, $p < 0.01$; **, $p < 0.05$.

which may explain the increase in cell survival of monocytes when in the presence of high concentrations of lactoferrin (Fig. 3D). We also determined whether TLF could induce other cytokine production by human monocytes. As shown by Fig. 3E, TLF at 100 $\mu\text{g/ml}$ stimulated production of considerable IL-6 and TNF- α (>1000-fold increase compared with sham-treated cells). IL-10 levels were only doubled in response to TLF, whereas LPS induced a 1000-fold increase. There was no effect on IL-12p70 production.

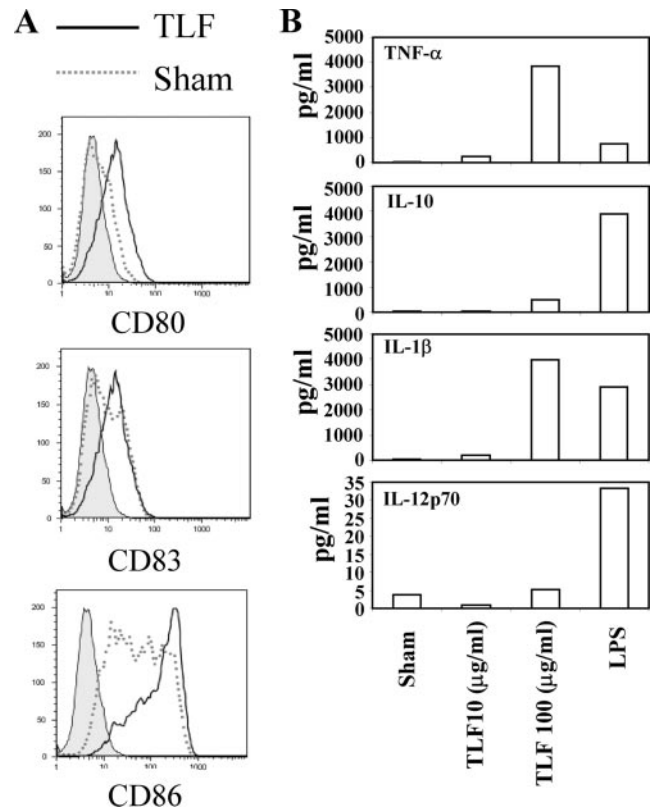


FIGURE 5. Lactoferrin enhancement of the maturation of PBDCs. **A**, PBDCs were cultured for 40 h in RPMI 1640 containing 5% FCS with or without TLF (100 $\mu\text{g/ml}$) or LPS (200 ng/ml). The cells were subsequently stained to analyze by flow cytometry CD80, CD83, and CD86 expression by gating on CD1c^+ PBDCs subset. Data shown are the results of one experiment representative of four. **B**, Purified CD1c^+ PBDCs were cultured ($10^5/\text{well}$) in RPMI 1640-5% FCS for 48 h in the presence of TLF (10 $\mu\text{g/ml}$) or LPS (500 ng/ml). The supernatants were collected for the measurement of cytokine production. The results of one experiment representative of two are shown.

Lactoferrin induces DC maturation

DCs play an important role in inducing T cell responses, being the most potent APC for initiating primary immune responses. The capacity of DCs to present Ags is dependent on their activation and maturation status (28). When TLF was added into human moDC cultures, up-regulation of costimulatory molecules such as CD80, CD83, and HLA-DR was observed (Fig. 4A). The increase in phenotypic maturation markers on moDCs was accompanied by an increase in the production of IL-6, TNF- α , and IL-12p70 (Fig. 4B; $p < 0.05$). In addition, upon treatment with TLF moDCs acquired the capacity to migrate to SLC/CCL21, suggesting induction of a functional CCR7 (Fig. 4C). To determine whether TLF-treated DCs were functionally mature, the capacity of TLF-treated DCs to induce allogeneic T cell proliferation in a MLR was assayed. DCs treated with TLF stimulated greater proliferation of allogeneic lymphocytes than sham-treated DCs, indicating that DCs treated with TLF were functionally mature (Fig. 4D). These results indicate that lactoferrin has the capacity to induce both phenotypic and functional maturation of moDCs.

We also studied the effect of lactoferrin on freshly isolated CD1c^+ PBDCs. Although PBDCs undergo spontaneous maturation during in vitro cultures as reported previously (29), addition of TLF further enhanced the expression of CD80, CD83, and CD86 (Fig. 5A). In addition, PBDCs treated with TLF produced high amounts of proinflammatory cytokines such as IL-1 β and TNF- α

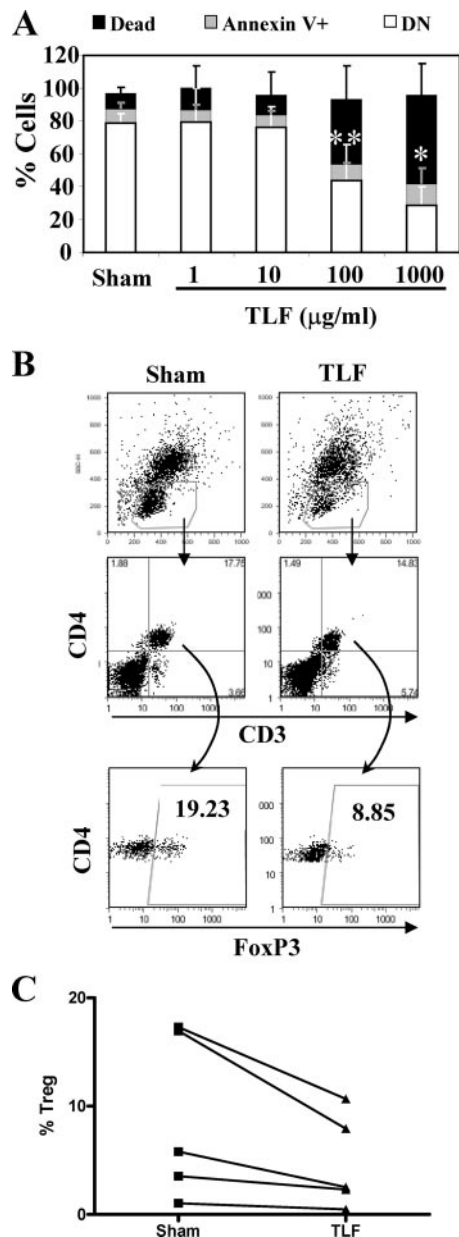


FIGURE 6. Lactoferrin reduction of lymphocyte and Treg numbers in vitro-cultured PBMCs. *A*, PBMCs cultured at 1×10^6 /ml in the absence (sham) or presence of TLF (1–1000 µg/ml) for 48 h were stained with FITC-conjugated annexin V and PI and analyzed by flow cytometry. Lymphocytes were gated based on forward scatter and side scatter characteristics. Data are presented as the percentage of cells with respect to the initial population plated (100%); DN populations were compared with analyze statistical significance. *B* and *C*, PBMCs were cultured in RPMI 1640 containing 10% FBS, 100 U/ml IL-2, and 50 ng/ml TNF- α in the absence (sham) or presence of 100 µg/ml TLF for 48 h. *B*, Subsequently, the cultured cells were stained for CD3, CD4, and FoxP3, and analyzed by flow cytometry. Dot plots show the phenotype of one representative experiment of five and the numbers within each gate correspond to the percentage of gated events. *C*, The percentage of Treg (CD3⁺/CD4⁺/FoxP3⁺) within total CD3⁺ T lymphocyte population of five individual experiments are plotted. *, $p < 0.01$; **, $p < 0.05$.

(Fig. 5*B*). Therefore, TLF can induce the maturation of not only moDCs, but also PBDCs.

Lactoferrin reduces Treg in cultured PBMCs

Treg consist of a subset of CD4⁺ T cells that express the transcriptional factor FoxP3 and play a critical role in down-regulating

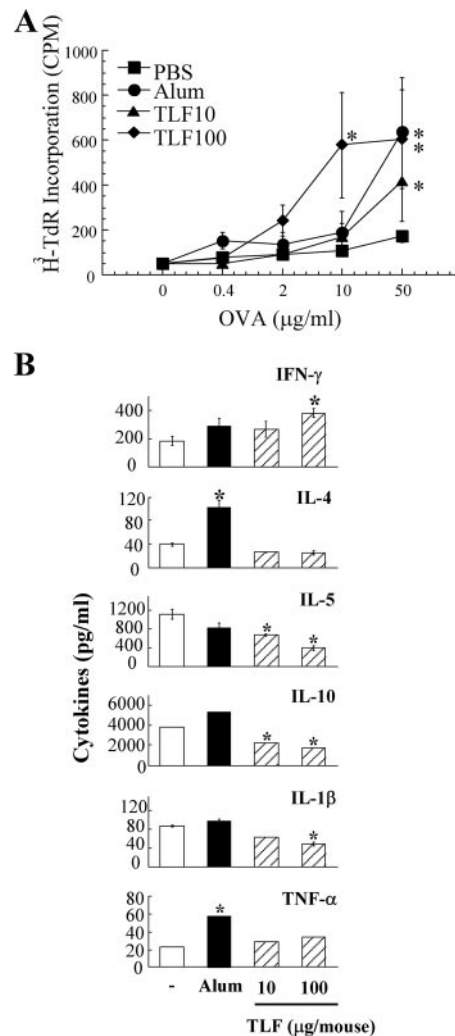


FIGURE 7. Lactoferrin enhancement of Ag-specific immune response. C56BL/6 mice ($n = 4$) were i.p. immunized with OVA (50 µg/mouse) in the absence or presence of TLF or alum (as a positive control) on day 1, boosted i.p. with OVA alone on day 14, and euthanized on day 21 for the removal of spleens and preparation of single splenocyte suspension. *A*, OVA-specific proliferation of splenocytes. Splenocytes were cultured in triplicate in a 96-well plate at 5×10^5 /well in the presence of various concentrations of OVA for 60 h and pulsed with 0.5 µCi/well of [³H]TdR for the last 18 h. The proliferation of splenocytes was measured as [³H]TdR incorporation and shown as the average (mean $\pm$ SD) of four mice. *B*, Pooled splenocytes of each group were cultured in duplicate in a 24-well plate at 5×10^6 /ml in RPMI 1640 containing 10% FBS and 50 µg/ml OVA for 48 h before harvesting the supernatants for cytokine measurement. Shown are the average (mean $\pm$ SD) cytokine concentration of duplicate wells. Similar results were obtained in two independent experiments; *, $p < 0.01$ compared with the group immunized with OVA alone.

immune activation (30). Because bovine lactoferrin has been shown to inhibit the proliferation of T cells (31), we determined whether TLF could also affect Tregs by incubating PBMCs in the absence or presence of TLF followed by analysis of Treg content. In contrast to promoting monocyte survival (Fig. 3*C*), TLF at high concentrations reduced the number of viable lymphocytes after 48 h of culture (Fig. 6*A*). To look at the effect on Treg, PBMCs were cultured in medium containing low concentrations of IL-2 (100 U/ml) and TNF- α (50 ng/ml) to prevent Treg from spontaneous apoptosis as reported previously (32). Interestingly, addition of TLF at 100 µg/ml in this culture system decreased the proportion of Treg (defined as CD3⁺/CD4⁺/FoxP3⁺) in comparison with

sham-treated cultures (Fig. 6, *B* and *C*), indicating that Tregs are more sensitive to TLF than other lymphocytes. TLF at 1 $\mu\text{g/ml}$ did not affect the Treg numbers (data not shown).

Lactoferrin enhances Ag-specific immune response

The capacity of TLF to activate APCs including monocytes and DCs suggested that it might act as an adjuvant to promote Ag-specific immune response. To validate this possibility, C57BL/6 mice were immunized with OVA in the absence or presence of TLF or alum (as a positive control) on day 0, boosted with OVA alone on day 14, and their spleens were removed on day 21 for the determination of OVA-specific proliferation and cytokine production. As expected, splenocytes from mice immunized with OVA in the presence of alum incorporated significantly more [^3H]TdR than cells of mice immunized with OVA alone, particularly when the concentration of OVA used for in vitro stimulation reached 50 $\mu\text{g/ml}$ (Fig. 7*A*). Importantly, splenocytes of mice immunized with OVA in the presence of TLF also showed enhanced proliferation upon in vitro stimulation with OVA (Fig. 7*A*), suggesting that TLF enhanced mouse anti-OVA immune response. Compared with splenocytes of negative control mice, splenocytes of mice immunized with Ag plus TLF, upon in vitro OVA stimulation, produced predominantly IFN- γ with a simultaneous reduction in IL-4, IL-5, IL-10, and IL-1 β , demonstrating the capacity of TLF to promote a Th1-type T cell response (Fig. 7*B*). Alum, as expected, enhanced predominantly Th2 responses as evidenced by up-regulation of IL-4, rather than IFN- γ (Fig. 7*B*).

Discussion

Ever since its discovery almost 50 years ago, lactoferrin has been widely studied as an antimicrobial as well as a modulator of inflammation and immune defense (2, 10, 11, 15). More recently, TLF, a recombinant hLF has been shown to suppress the growth of implanted tumors in mouse models (16, 33) and to exhibit antitumor activity in phase I clinical trials (18). In this study, we have shown that TLF is able to: 1) chemoattract human monocytes in vitro and induce the recruitment of mouse phagocytes including neutrophils and monocytes/macrophages in vivo, 2) activate human APCs including monocytes/macrophages and DCs, 3) reduce human Treg content in cultured PBMCs, and 4) enhance Ag-specific mouse Th1 immune responses upon coadministration with the Ag. By using a Food and Drug Administration-approved, GMP level recombinant hLF generated in eukaryotic cells, this study avoids the potential endotoxin contamination problem associated with the use of lactoferrins purified from bovine milk or generated in *Escherichia coli*. Furthermore, hLF shares only 68% sequence with its bovine counterpart (21, 34–36). Therefore, our results may have more relevance for understanding the effect(s) of lactoferrin on human immune cells and immunity than those studies in which the bovine lactoferrin was used (37–40).

Based on the inhibition of TLF-induced monocyte migration by PTx, it is clear that the capacity of TLF to chemoattract monocytes is direct and mediated by a G α_i protein-coupled receptor(s), although the precise identity of the receptor(s) remains to be determined. In contrast to a previous report showing neutrophil mobility enhanced by lactoferrin (41), we did not detect any effect of TLF on the migration of human neutrophils nor on mouse neutrophils or monocytes in vitro (data not shown) within a wide range of doses (0.1–1000 $\mu\text{g/ml}$). However, i.p. injection of TLF in mice did cause the recruitment of neutrophils to the peritoneal cavity within 4 h, illustrating an in vivo neutrophil-mobilizing capacity for TLF. This may be due to an indirect effect of lactoferrin inducing production of chemoattractants by cells present in the peritoneal cavity (macrophages or epithelial cells). Because monocytes may dif-

ferentiate into DCs upon recruitment into tissues (42), lactoferrin's capacity to induce the recruitment of monocytes/macrophages potentially promotes the accumulation of DCs at inflammatory sites where neutrophil infiltration and degranulation often occur. Lactoferrin can thus potentially enhance both innate and adaptive immunity.

Another critical finding of this study is the capacity of lactoferrin to activate APCs including monocytes/macrophages and DCs. TLF induced monocyte clustering due to an increase in the expression of adhesion molecules. TLF also rescued monocytes from the spontaneous in vitro apoptosis (43, 44). This is likely to be due to an induction in GM-CSF and M-CSF production by monocytes, which may help these precursor APCs to exert their surveillance function in the tissues for a longer period. TLF stimulated production of proinflammatory cytokines such as IL-6 and TNF- α by monocytes at similar concentrations as reported for the promotion of monocyte cytotoxicity by purified bovine lactoferrin (45). Although certain aspects of lactoferrin's monocyte-activating effect have previously been sporadically reported (41, 45), the effect of lactoferrin on DC maturation remains to be determined. Here, we have demonstrated for the first time the capacity of lactoferrin to induce the maturation of both moDCs and CD1c $^+$ PBDCs. Co-culture of moDCs with TLF resulted in up-regulation of expression costimulatory and MHC molecules, induction of DC proinflammatory cytokines including IL-12p70, and DC acquisition of the capacity to migrate to lymphoid-homing chemokine SLC/CCL21 (Fig. 4). TLF also induced the maturation of CD1c $^+$ PBDCs as evidenced by up-regulation of costimulatory molecules (CD80, CD83, and CD86) and induction of proinflammatory cytokines such as TNF- α and IL-1 β (Fig. 5). Furthermore, DCs treated with TLF induced a greater lymphocyte proliferation compared with untreated cells in allogeneic cell cultures (Fig. 4*D*). Similar results were obtained by M. Spadaro, C. Caorsi, P. Ceruti, A. Varadhachary, G. Forni, F. Pericle, and M. Giovarelli, when TLF was used to induce maturation of moDCs (unpublished observation). These activation effects indicate that lactoferrin can enable DCs to mature and develop the capacity to induce adaptive immune responses. Thus, only in danger conditions, when neutrophils are induced to release the content of their secondary granules, does lactoferrin become available to the recruited APCs at concentrations that favor their activation and maturation. We consider that doses around 100 $\mu\text{g/ml}$ might have relevant clinical importance, because of their critical effect on the survival and activation of APCs, and this should be taken into consideration for future vaccines or treatments. As lactoferrin levels in milk, and especially in colostrum, are also very high, it is possible that lactoferrin also promotes development of the newborn immune system by activating APCs positioned along the gastrointestinal tract, in addition to its antimicrobial capacities and the effect exerted on the intestinal epithelium (46).

It has been previously reported that lactoferrin can inhibit lymphocyte proliferation and cytokine production (31, 47). Our data showing the induction of lymphocyte death in cultured PBMCs by high doses (>100 $\mu\text{g/ml}$) of TLF may account for the reported inhibitory effect. Of substantial interest is our data showing that TLF caused a greater reduction of Treg (CD4 $^+$ /FoxP3 $^+$) in cultured PBMCs (Fig. 6). Although how TLF suppresses Treg more than other lymphocytes in this culture system is unclear, this preferential reduction of Treg by TLF would definitively favor induction of immune responses.

Given the critical roles of APCs in the induction of immunity, the effects of TLF on APC migration, recruitment, activation/maturation, and Treg reduction suggest that lactoferrin may enhance the Ag-specific immune response. Indeed, coadministration of

TLF with an Ag (OVA) enhanced specific immune response in mice (Fig. 7). Many endogenous antimicrobial peptides and proteins have been documented to exhibit adjuvant effects, such as α -defensins, β -defensins, and cathelicidins (26, 48, 49). However, α -defensins and cathelicidin promote both Th1 and Th2 immune responses (26, 48) whereas β -defensin 2 preferentially enhances Th1 response (49). The TLF-enhanced anti-OVA immune response is predominantly of a Th1 type as evidenced by the up-regulation of Ag-specific IFN- γ production and a concomitant down-regulation of IL-4 and IL-10 (Fig. 7). The capacity of bovine lactoferrin to enhance delayed-type hypersensitivity reaction (39) can easily be explained by lactoferrin's Th1-polarizing effect demonstrated in our study. Therefore, lactoferrin is a promising adjuvant for use in cancer vaccines, because Th1 immune response is required for tumor rejection. In this regard, TLF has been shown to increase IFN- γ production and cytotoxic capacity of T lymphocytes in tumor-implanted mice (33).

Lactoferrin concentrations in normal human secretions are reported to be 35 μ g/ml in bronchial mucus, 46 μ g/ml in synovial fluid, up to 2.2 mg/ml in lacrimal fluid, and 5–7 mg/ml in colostrums (3, 15). During inflammation, lactoferrin levels in biologic fluids can increase >200-fold (6, 7). The effective concentrations of lactoferrin on monocyte migration, leukocyte recruitment, APC activation, and Treg reduction range from 1 to 1000 μ g/ml, which is apparently achievable in vivo. Although TLF-induced APC migration and recruitment occurs at low μ g/ml levels, its effect on APC activation and Treg reduction become more obvious at ≥ 100 μ g/ml. This may be relevant in the context of inflammatory and immune reactions: In the initial phase of inflammation, low concentration of lactoferrin may facilitate APC recruitment, while with the progression of inflammation, lactoferrin accumulates, due to additional neutrophil degranulation and induced production, to reach higher concentrations, which would promote DC maturation and reduce Treg, thereby promoting the induction of adaptive immune responses. As neutrophil infiltration is transient (50), and lactoferrin can be rapidly cleared by the liver (51), by the time Ag-specific effector lymphocytes traffic into sites of inflammation, it is likely that lowered lactoferrin levels would no longer be lymphotoxic.

Alarmins are defined as endogenous mediators rapidly released by cells of the host innate immune system in response to infection and/or tissue injury, which possess the dual activities of recruiting and activating APCs, and consequently are capable of enhancing Ag-specific adaptive immune responses (20, 52). Lactoferrin is an endogenous mediator rapidly released from neutrophils and many epithelial cells. The results of the present study demonstrate that lactoferrin is capable of inducing the recruitment and activation of APCs, thus enhancing Ag-specific immune response in vivo. Therefore, lactoferrin can be considered a bona fide alarmin that favors a Th1-type immune response.

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Disclosures

Dr. A. Varadhachary is an employee of Agennix, developer of the talactoferrin alfa.

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