Reversal of airway inflammation and remodeling in asthma by a bispecific antibody fragment linking CCR3 to CD300a

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Background: Mast cells (MCs) and eosinophils are critically involved in asthma-associated airway damage and remodeling. However, molecular pathways that inhibit their functions in this process have been scarcely characterized. Recently we established that cross-linking of CD300a inhibits MC and eosinophil activation.

Objective: To inhibit effector cell functions in a chronic model of experimental asthma by coaggregation of CD300a with CC chemokine receptor 3 (CCR3) using a bispecific antibody fragment (LC1).

Methods: Mast cells and eosinophils were treated with LC1 before their activation. Mediator release, survival, and intracellular signaling were assessed. Furthermore, chronic experimental asthma was induced, and starting on day 30, the mice were challenged (3 challenges/wk) for an additional 38 days. With each challenge, the mice received LC1 intranasally.

Results: LC1 inhibited MCs and eosinophil activation *in vitro* and *in vivo*. Mice that displayed airway inflammation on day 28 and were treated with LC1 completely recovered from the disease process. In the bronchoalveolar lavage fluid of these mice, cellular inflammation cytokine expression was comparable to that of saline-treated mice. Bronchoalveolar lavage fluid levels of TGF-β1 correlated significantly with reduced eosinophilia. Histologic analysis revealed significant reduction in lung inflammation, mucus production, collagen deposition, and peribronchial smooth-muscle thickening.

Conclusion: CD300a is a critical modulator of MCs and eosinophil functions in allergic settings.

Clinical implications: Specific targeting of CD300a in $CCR3^+$ cells may be a potent tool for treating airway inflammation and tissue remodeling in asthma. (J Allergy Clin Immunol 2006;118:1082-9.)

Key words: *Asthma, airway remodeling, eosinophils, CD300a, bispecific antibody*

Abbreviations used

BAL: Bronchoalveolar lavage
BALF: Bronchoalveolar lavage fluid
BsAb: Bispecific antibody
ERK: Externally regulated kinase
FACS: Fluorescence-activated cell sorting
MC: Mast cell
NK: Natural killer
OVA: Ovalbumin
PAS: Periodic acid-Schiff
PGD_2: Prostaglandin D_2
PLC: Phospholipase C

Asthma is a chronic inflammatory disease characterized by intermittent episodes of shortness of breath, coughing, wheezing, bronchial hyperresponsiveness, and airway inflammation.[1] Chronic inflammation of the asthmatic lung leads to bronchial epithelial cell damage and consequent structural changes, which in turn exacerbate the hyperresponsiveness observed in this disease.[2]

Mast cells (MCs) are the well established initiators of the allergic response. Their degranulation leads to accumulation of blood, tissue, and bronchoalveolar lavage (BAL) eosinophils that have been correlated with disease severity.[3,4] Eosinophils, by the release of their basic granule proteins, have been considered to cause the tissue damage observed in the airway epithelium of patients with asthma. In the inflamed tissue, they interact with various cell types, including MCs and T cells, aggravating and perpetuating the inflammatory response.[5,6] Furthermore, they store, synthesize, and release various cytokines and lipid metabolites, and are a rich source of TGF-β, a potent profibrotic growth factor.[7,8]

The importance of defining new pathways that suppress effector cell function in asthma has recently been bolstered by the demonstration of a critical role for eosinophils in allergic airway inflammation.[9,10] Inhibitory receptors could make up these innovative routes. They were initially characterized on cytotoxic T cells and natural killer (NK) cells and can be identified by a consensus amino acid sequence, the immunoreceptor tyrosine-based inhibitory motif, which is capable of recruiting phosphatases.[11]

We demonstrated that inhibitory receptor protein 60 (IRp60)/CD300a potently inhibits mast cell (MC) and

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eosinophil activation checkpoints—namely, recruitment, mediator release, and survival.[12,13] These observations highlighted the possibility of targeting CD300a as an approach to suppress effector cell functions in asthma. In this work, we adopted a bispecific antibody (BsAb)–based approach selectively to target CD300a on $CCR3^+$-expressing cells.

We report that selective targeting of CD300a in a murine model of chronic established asthma activates the inhibitory mechanism on MCs and eosinophils, thereby eliciting a suppressive effect on their function. In fact, a complete recovery from histopathological markers of airway inflammation and tissue remodeling was achieved. Our results emphasize the critical role of MCs, eosinophils, and CD300a in chronic allergic airway inflammation. Furthermore, they provide means for targeted inhibition of MCs and eosinophils in this disease.

METHODS

Antibodies and reagents

The following reagents were used: media, reagents, and buffers (Biological Industries, Beit Haemek, Israel); rat anti-mouse CCR3, fluorescein isothiocyanate–conjugated rat anti-mouse CCR3, and anti-mouse CD300a clones (R&D Systems, Minneapolis, Minn); anti-rat IgG_1, allophycocyanin (APC)-conjugated anti-mouse CD3, goat anti-rat fluorescein isothiocyanate, and goat anti-rat phycoerythrin (e-Bioscience, San Diego, Calif); APC-conjugated anti-mouse B220 (Miltenyi Biotech, Auburn, Calif); Cy5-conjugated anti-rabbit antibodies (Jackson Immunoresearch, West Grove, Pa); and phospho-specific antibodies (Cell Signaling, Danvers, Mass).

BsAb design and construction

Bispecific antibody fragments were generated as previously described.[14] Additional controls (ie, isotype match and anti-CCR3 $F[ab']_2$ fragments) were generated similarly and are termed *control antibodies* throughout the study (see this article's Fig E1 in Online Repository at www.jacionline.org).

Animal models

BALB/c mice (females, 6-8 weeks, Harlan Laboratories, Jerusalem, Israel) were subjected to experimental protocols that were approved by the Institutional Ethical Committee for Animal Experimentation.

Chronic allergic eosinophilic airway inflammation was performed as follows (Fig 1, *A*): mice (4-6 mice/group/time point; n = 3) were sensitized with ovalbumin (OVA; 100 μg) and alum (1 mg) intraperitoneally (in 200 μL saline/mouse) on days 0 and 14. On days 24 and 27, mice were challenged intranasally (50 μg/50 μL OVA/mouse), and on day 28, a group of mice was killed for verification of airway inflammation. Another group of mice received intranasal saline, OVA, OVA + LC1, or OVA + control antibody treatment. After 24 and 48 hours, a group of mice was killed to dissect the acute effects of LC1 on airway inflammation. The rest of the mice received treatment on day 30 and afterward 3 per week until day 66. A group of mice was killed 1 per week for monitoring the long-term effects of LC1. BAL was performed and assessed for differential cell counts and cytokines.[15]

Histopathology

Lungs were fixed in 2.2% formaldehyde, sectioned, and stained by Hematoxylin and Eosin (H&E), periodic acid-Schiff (PAS), or Masson Trichrome to evaluate inflammatory score, mucus production, and extracellular matrix (ECM) deposition. Tissue sections were examined by 2 blind observers, and total inflammatory score was graded. For quantification of airway mucus expression, PAS-positive epithelial cells in individual bronchioles were counted and calculated as described.[16] Trichrome-stained areas (total pixels) were calculated from at least 3 medium-sized bronchioles (Adobe Photoshop, San Jose, Calif) using the following mathematical algorithm:

$$S_{trichrome} = \frac{\sum \alpha_n}{2\pi\left(2\Delta_n r_n - r_n^2\right)}$$

α, angle (radians); r, distance to stained area from bronchiole center; Δ, width of stained area within selected slice; n, number of calculated slices. Peribronchial smooth muscle thickness (mean pixels) was calculated from at least 10 different positions per bronchiole (Adobe Photoshop).

In vivo LC1 binding

LC1 (5 μg/mouse) was administered intranasally to OVA-challenged mice on day 28 (Fig 1, *A*). One hour after LC1 administration, mice were killed, and BAL was performed and cells from the BAL incubated with secondary antibodies. Differential cell populations were electronically gated (see this article's Methods in the Online Repository at www.jacionline.org for gating criteria) and assessed for LC1 binding by fluorescence-activated cell sorting (FACS).

In vitro cell activation

Ovalbumin-challenged mice were killed on day 28 (Fig 1, *A*). Bronchoalveolar lavage fluid (BALF) cells (3.5×10^5/200 μL) were incubated in 96-well plates (Nunc, Rochester, NY) in supplemented RPMI-1640 with LC1 or control antibodies (1-5 μg/mL; 30 minutes; 37°C). Cells were washed, incubated with IL-5 (20 ng/mL, Peprotech, Rocky Hill, NJ), and assessed for survival and syk phosphorylation. In these assays, total eosinophil numbers reached $4.2 \times 10^5 \pm 3.5 \times 10^4$ (purity, 68.7% ± 12.4%).

For survival, annexin V–propidium iodide protocol was used (R&D Systems) according to the manufacturer's instructions and analyzed by FACS. Syk phosphorylation was assessed by intracellular FACS as described.[12]

In vivo signal transduction assays

Bronchoalveolar lavage fluids of variably treated mice (day 28) was obtained 6 hours after OVA and/or antibody treatment (Fig 1, *A*). BALF cells were fixed, permeabilized, blocked, and stained with anti-phospho–externally regulated kinase (ERK) 1/2, phospholipase C (PLC)–γ1/2, followed by secondary antibodies. Phosphorylation was assessed by FACS.[12]

MC culture and activation and mediator analysis

See this article's Methods in the Online Repository at www.jacionline.org.

Data analysis

For FACS assays, at least 10,000 events were acquired, and electronically gated eosinophils were analyzed (Becton-Dickinson FacsCalibur, CellQuest software, Franklin Lakes, NJ).

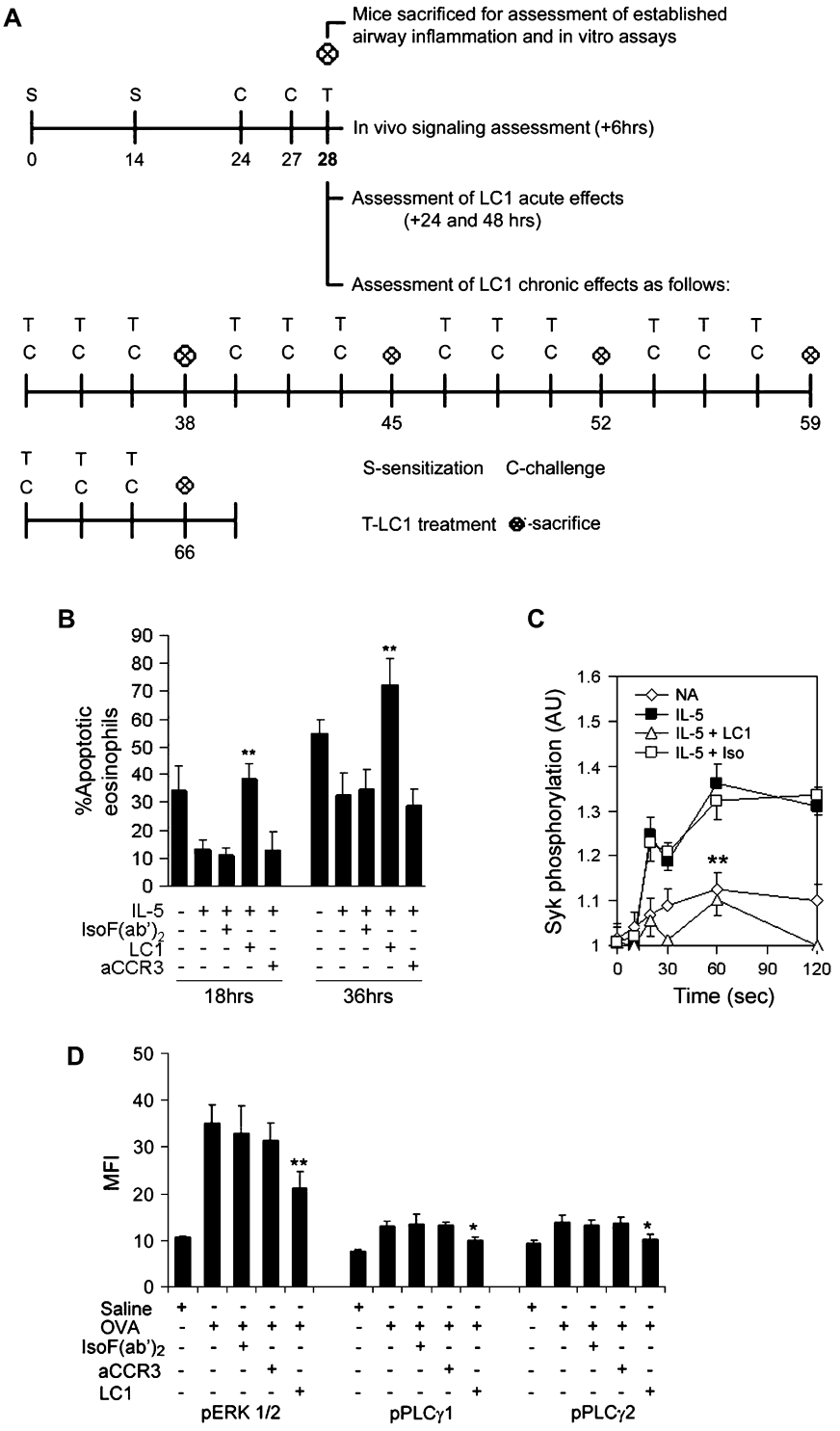

FIG 1. LC1 inhibits eosinophil survival and signaling. **A**, The study design. **B**, Effect of LC1 on eosinophil survival was assessed by FACS (annexin V–propidium iodide). **C**, Effect of LC1 on Syk phosphorylation *in vitro* was assessed by intracellular FACS. **D**, Effect of LC1 on ERK1/2 and PLCγ phosphorylation *in vivo* was assessed by intracellular FACS. Data are means ± SDs; n = 3. $*P < .05$; $**P < .01$.

Data are means ± SDs of 3 independent experiments as analyzed by ANOVA followed by paired Student *t* test. Significant values were considered at $P < .05$.

RESULTS

Cellular expression of leukocyte mono-Ig loke receptor 1 (LMIR-1)/CD300a

The murine homologue of IRp60[12] is LMIR-1[17] (NM_170758.2, NP_739564), collectively termed CD300a. CD300a expression was found to be restricted to neutrophils, monocytes, eosinophils, and basophils, but not lymphocytes (see this article's Fig E2 in the Online Repository at www.jacionline.org). Because antibody clone 712224.111 yielded the highest recognition level, all further experiments were conducted using this antibody.

Design and construction of a BsAb recognizing CCR3 and LMIR-1/CD300a (LC1)

The BsAb was designed to recognize CCR3 and CD300a. Because murine MCs and basophils express CCR3,[18,19] we determined whether LC1 binds these cells. LC1 binds to MCs and basophils (mean fluorescence intensity, 4.3 ± 5.6 and 9.7 ± 1.3, respectively). However, it preferentially recognizes eosinophils (mean fluorescence intensity, 37.4 ± 4.2; $P < .001$). Importantly, LC1 does not recognize lymphocytes (see this article's Fig E3 in the Online Repository at www.jacionline.org).

Next, LC1 was administered intranasally to OVA-challenged mice. One hour afterward, mice were killed, and LC1 binding was assessed on the various BALF cell populations (see this article's Fig E4 at www.jacionline.org). Although neutrophils and monocytes were recognized by LC1, the main population stained consisted of eosinophils. Lymphocytes displayed comparable staining intensities to isotype-matched control antibody staining (data not shown).

LC1 inhibits MC and eosinophil activation *in vitro*

Prolonged eosinophil tissue survival is a hallmark of allergic inflammation. Thus, we examined the ability of LC1 to inhibit eosinophil survival. For this, BALF eosinophils were obtained, recovered, pretreated with LC1 or control antibodies, and cultured with IL-5 (Fig 1, *A*). Cells treated with IL-5 displayed low levels of apoptosis compared with untreated cells. Eosinophils incubated with IL-5 and LC1 demonstrated increased apoptosis (38.4% ± 5.2% and 72.4% ± 9.3%, 18 and 36 hours, respectively; $P < .01$). Notably, eosinophils preincubated with control antibodies displayed apoptotic levels similar to IL-5–treated cells (Fig 1, *B*).

The ability of LC1 to inhibit eosinophil survival suggests that LC1 is capable of inhibiting eosinophil signaling. To examine this hypothesis, we assessed the effects of LC1 on syk phosphorylation.[20] For this, BALF eosinophils were obtained and incubated with IL-5 and LC1 or control antibodies. Whereas IL-5–treated cells displayed enhanced syk phosphorylation, LC1-treated cells displayed syk phosphorylation comparable to nonactivated cells (Fig 1, *C*). This effect was specific to LC1, because isotype-treated cells exhibited the same phosphorylation pattern as IL-5–treated cells.

Because LC1 recognized MCs as well, we assessed whether it is capable of inhibiting MC mediator release *in vitro*. Interestingly, LC1 inhibited IgE-dependent tryptase release from bone marrow-derived MC (BMMC) in a dose-dependent fashion (see this article's Fig E5 in the Online Repository at www.jacionline.org).

LC1 inhibits eosinophil signaling *in vivo*

Next, we aimed to verify whether treatment with LC1 will inhibit eosinophil signaling *in vivo*. LC1 was administered to OVA-challenged mice, and 6 hours later, the mice were killed (Fig 1, *A*). Thereafter, BALF cells were obtained, and signaling molecules were assessed on gated eosinophils. LC1-treated mice displayed reduction in ERK1/2 phosphorylation and complete abrogation of PLCγ signaling, whereas mice treated with control antibody displayed augmented phosphorylation (Fig 1, *D*).

LC1 inhibits eosinophil and MC mediator release *in vivo*

Our findings suggest that LC1 will inhibit eosinophil effector functions *in vivo* as well. Hence, a murine model of chronic established asthma in which BALF and lung eosinophilia were present was employed (Fig 1, *A*). Assessment of eosinophil peroxidase content in the BALF revealed a significant reduction after LC1 treatment (see this article's Fig E6, *A*, in the Online Repository at www.jacionline.org). Because LC1 preferentially binds to eosinophils *in vivo*, we determined whether LC1 will affect MC activation *in vivo* as well. Interestingly, MC-derived tryptase but not PGD_2 was significantly reduced after LC1 treatment (see this article's Fig E6, *B* and *C*, in the Online Repository at www.jacionline.org). In addition, LC1 was capable of inhibiting MC activation and consequent blue dye exudation in murine passive cutaneous anaphylaxis (data not shown).

LC1 inhibits BALF inflammation in a murine model of chronic established asthma

After LC1 treatment, eosinophil counts in the BALF drastically decreased, reaching statistical significance after 48 hours. Eosinophil counts steadily decreased and on day 59 were not different from those of saline-treated mice (Fig 2, *A*). This was not a result of inhibition of local chemokine expression, because eotaxin-2 was still present at these time points (data not shown). Lymphocyte accumulation in the BALF was monitored as well. Following a single treatment, lymphocyte accumulation was not impaired at 24 and 48 hours. However, at day 38, lymphocyte counts significantly decreased, and were comparable to those of saline-treated mice at day 59 (Fig 2, *B*). Notably, both eosinophil and lymphocyte counts were not affected by control antibody treatment.

The T_H2 cytokine profile in the BALF paralleled lymphocyte clearance. In fact, the high cytokine levels

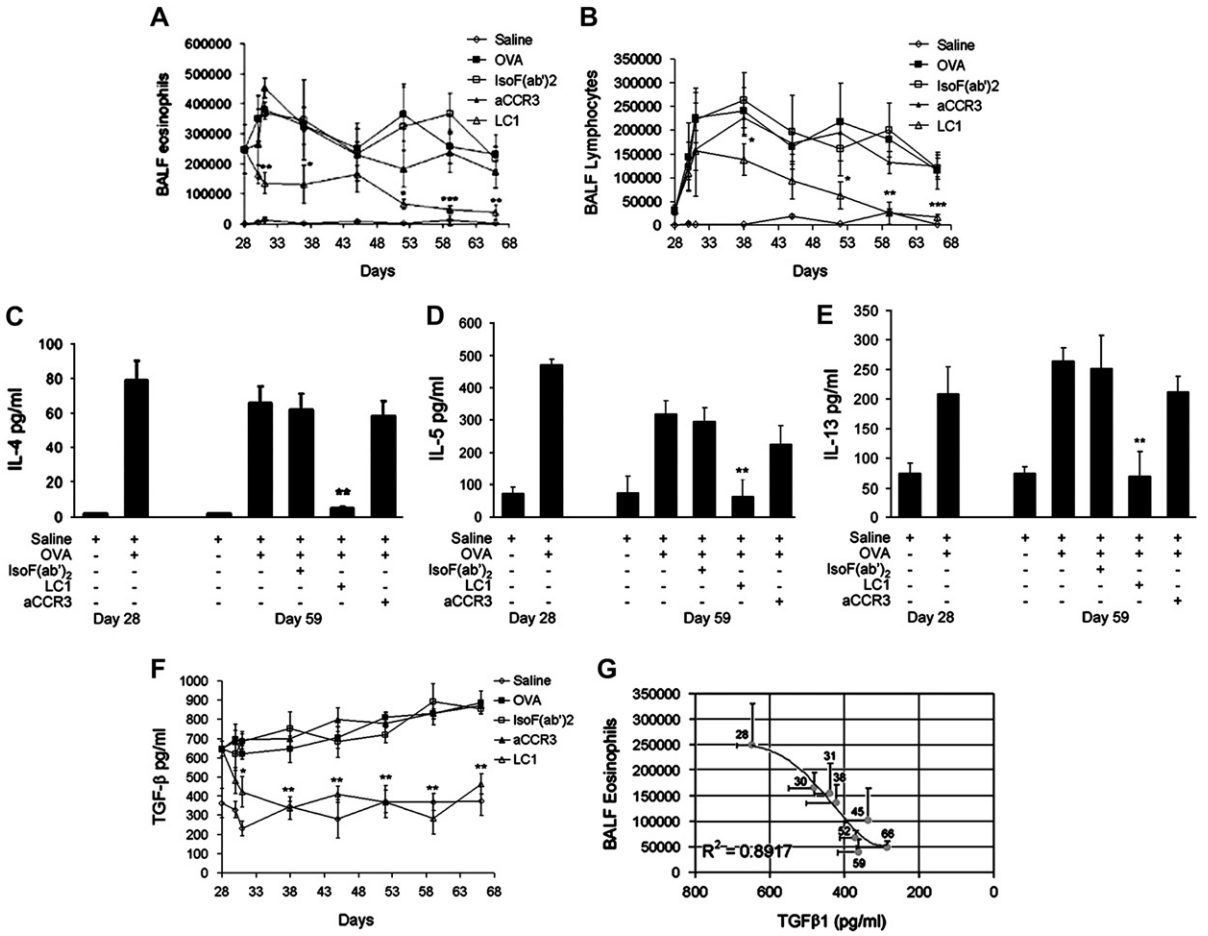

FIG 2. LC1 inhibits BALF inflammation. Effects of LC1 on BALF eosinophil **(A)** and lymphocyte **(B)** counts (FACS analysis). Effects of LC1 on the BALF expression of IL-4 **(C)**, IL-5 **(D)**, and IL-13 **(E**; ELISA). Effects of LC1 on BALF TGF-β1 expression (**F**; ELISA). Computed correlation between TGF-β1 and eosinophil counts (**G**; Excel, Microsoft, Redmond, Wash). *aCCR3,* Anti-CCR3; *IsoF(ab')2,* isotype control. Data are means ± SDs; n = 3. **P* < .05; ***P* < .01; ****P* < .001.

observed at day 28 were completely abrogated at day 59 after LC1 treatment (Fig 2, *C-E*). Significantly, mice treated with control antibodies demonstrated levels similar to those of OVA-treated mice.

LC1 inhibits eosinophil-derived TGF-β1 in the BALF

Eosinophils could be a prominent source of TGF-β1 in the asthmatic lung. Thus, we aimed to determine whether LC1 was able to inhibit TGF-β1 expression in the BALF. Although the levels of TGF-β1 gradually increased during induction of the chronic protocol in OVA and control antibody-treated mice, LC1-treated mice displayed a dramatic inhibition of TGF-β1. Furthermore, the levels of TGF-β1 reached levels comparable to those of saline-treated mice after 1 week of treatment (day 38; $P < .01$; Fig 2, *F*). Interestingly, reduction in eosinophil numbers in the BALF highly correlated to TGF-β1 expression in LC1-treated mice ($R^2 = 0.8917$; Fig 2, *G*). However, the correlation between BALF IL-5 and eosinophil numbers was significantly lower ($R^2 = 0.4325$) and correlated with the reduction in lymphocyte counts ($R^2 = 0.7265$; data not shown).

LC1 reverses lung inflammation

To assess the effects of LC1 on tissue inflammation, the lungs of saline-treated, OVA-treated, control antibody-treated, and LC1-treated mice were examined for peribronchial, perivascular, and alveolar space inflammation. OVA-challenged mice killed on day 28 manifested, as expected, significant lung inflammation (data not shown). Throughout the chronic protocol, OVA-treated mice displayed evident peribronchial, perivascular, and alveolar space inflammation (Fig 3, *A-D*). Furthermore, the bronchial epithelium was injured, and smooth muscle layer was significantly thicker than that of the saline-treated mice. In contrast, LC1-treated mice displayed gradual reversion of the inflammatory state, reaching comparable

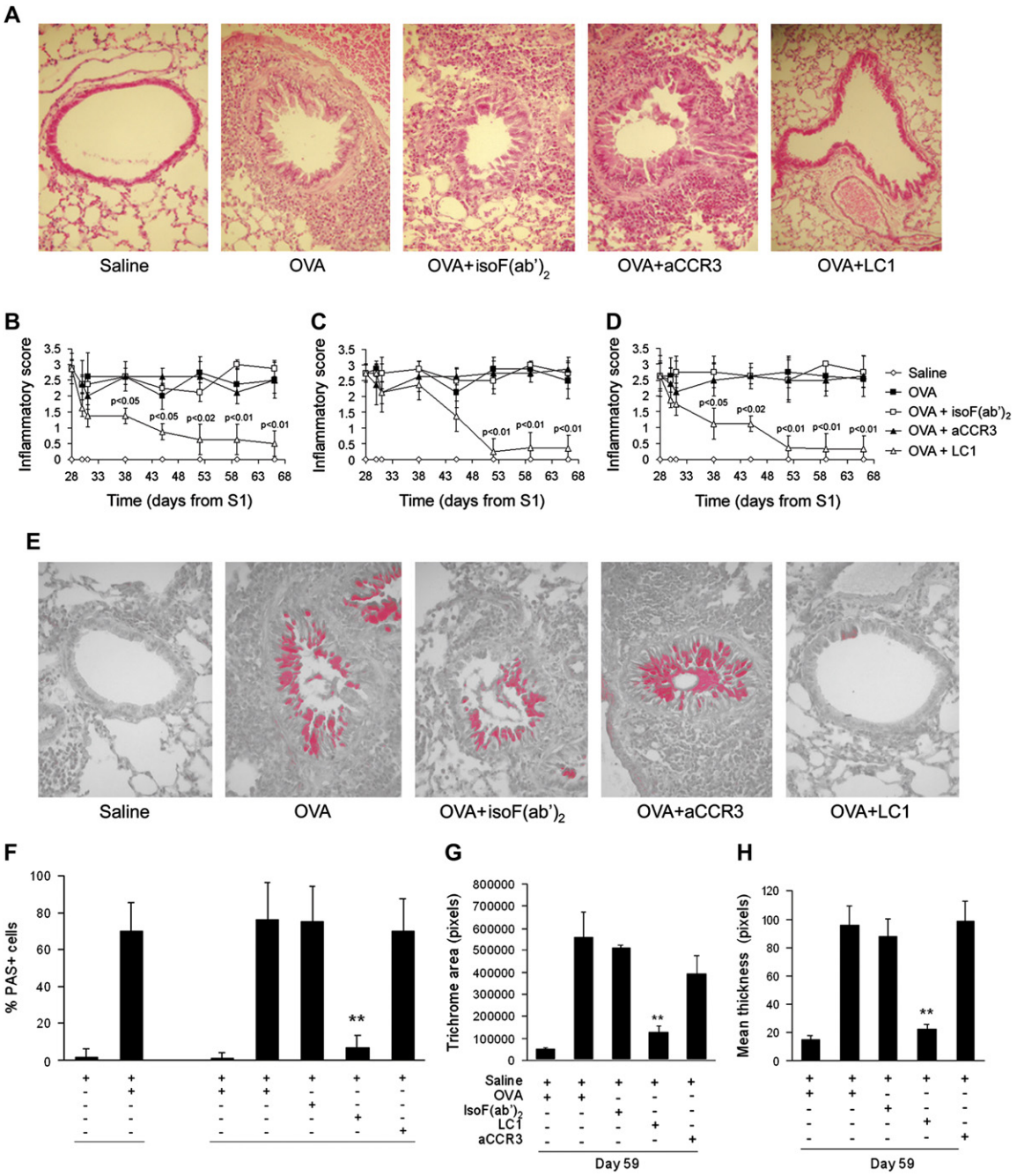

FIG 3. LC1 reverses lung inflammation and inhibits tissue remodeling. Effects of LC1 on lung inflammation (H&E, representative photomicrographs; **A**) and quantified compartmented parameters: peribronchial **(B)**, perivascular **(C)**, and alveolar **(D)**. Effects of LC1 on mucus expression (PAS, representative photomicrographs and quantification; **E and F**). Effects of LC1 on ECM deposition **(G)** and on smooth muscle thickness **(H)**. Data are means ± SDs; n = 3. **P* < .05; ***P* < .01; ****P* < .001.

characteristics to those of saline-treated mice at day 53. Remarkably, control antibody-treated mice showed a similar inflammatory phenotype to OVA-treated mice.

LC1 inhibits lung remodeling

Hallmark characteristics of tissue remodeling in asthma are increased mucus production, goblet cell hyperplasia, and deposition of extracellular matrix components.[1,2] Thus, we evaluated the effects of LC1 treatment on these parameters. OVA-treated mice displayed significant PAS staining throughout the epithelium and inside the airways. As opposed to control antibody-treated mice that showed similar PAS staining to OVA-treated mice, LC1-treated mice displayed negligible staining (day 59; Fig 3, *E* and *F*).

Moreover, LC1-treated mice exhibited reduced ECM deposition in comparison with OVA-treated and control antibody-treated animals (Fig 3, *G*). Assessment of peribronchial smooth muscle thickness in LC1-treated mice revealed a significant decrease in mean thickness in comparison with OVA-treated and control antibody-treated mice (Fig 3, *H*).

DISCUSSION

We report that selective activation of the inhibitory receptor CD300a on $CCR3^+$ cells reverses airway inflammation and inhibits remodeling in a murine model of chronic established asthma. A central issue in controlling allergic diseases is the identification of new inhibitory pathways that will counteract the functions of participating effector cells. Therefore, the development of anti-MC and eosinophil-specific therapies is currently encouraged. We have recently demonstrated that CD300a is a potent inhibitor of key MC and eosinophil checkpoints.[12] Hence, it is a natural candidate for suppressing their functions *in vivo*.

In this study, we generated a BsAb recognizing CD300a and CCR3, termed LC1. LC1 preferentially recognizes eosinophils and, to a much lesser extent, MCs and basophils.[19] In humans, the expression of CCR3 is not confined to eosinophils and other cells; MCs, basophils, dendritic cells, lymphocyte subsets, and structural cells express it as well.[21,22] Thus, using BsAb for specific targeting of human cells may need different cell-specific markers.

In addition, although the expression of CD300a is not restricted to eosinophils, our preliminary studies demonstrate that only coexpression of CD300a and CCR3 on the surface of a cell will elicit an inhibitory response via CD300a (data not shown). Furthermore, our results demonstrate that LC1 displays the highest reactivity toward eosinophils. Importantly, in mice, CD300a is not expressed by lymphocytes. Therefore, the inhibitory response is not a result of inhibition of lymphocyte activation.

We report that LC1 inhibits eosinophil survival and signaling. Eosinophil activation involves several signaling cascades, including Janus-activated, Src-activated, and mitogen-activated kinases and calcium influx.[20,23-25] Interestingly, LC1 was unable to abrogate ERK1/2 signaling and decreased ERK1/2 phosphorylation by only ~50%. This limited inhibition of ERK1/2 phosphorylation may be a result of intracellular trafficking of ERK to a cellular compartment that is protected from phosphatase activity.[26]

In vitro experiments suggest that LC1 acts mainly on eosinophil and MC survival and activation while inhibiting chemotaxis to a lesser extent. Indeed, the numbers of peripheral blood eosinophils were unaltered in LC1-treated and OVA-treated mice (data not shown).

Strikingly, intranasal administration of LC1 was capable of causing a complete inhibition of lung and BALF cellular inflammation. These findings differ from studies using anti-CCR3 antibodies or studies using eosinophil-deficient mice.[9,10,27] Our data demonstrate an inhibitory effect of LC1 on both MCs and eosinophils. This may alter the responsiveness of MCs to allergen challenge and stop the positive feedback mechanism caused by MC-eosinophil interactions. Indeed, eosinophils can activate MCs via major basic protein (MBP), stem cell factor (SCF), and nerve growth factor (NGF). On the other hand, eosinophils can be activated by MC mediators such as histamine, tryptase, and GM-CSF. Furthermore, because both cells can interact with T cells, LC1 could inhibit this cross-talk as well.[7,28] Interestingly, LC1 was not capable of inhibiting the production of PGD_2, a hallmark MC prostaglandin, indicating only a partial inhibitory effect on MC activation. Nevertheless, CD300a is capable of inhibiting MC-mediated PGD_2 release because coaggregation of FcεRI-bound IgE to CD300a inhibits PGD_2 release *in vivo* and *in vitro* (Bachelet I et al, Unpublished data, 2005).

One of our most striking observations is that TGF-β1 expression was highly correlated to eosinophil clearance. The data fitted best into a sigmoidal correlation, suggesting that eosinophils are not the only source for TGF-β1. Airway epithelial cell–derived TGF-β1 has been described in the development of airway wall remodeling in asthma.[29] Therefore, possibly, eosinophil clearance leads to reduced epithelial damage and activation, which in turn results in decreased TGF-β1 expression. In addition, other immunoregulatory cells such as T regulatory cells (Tregs) and natural killer (NK) cells constitutively produce TGF-β1. Interestingly, CD2 antibody stimulation of NK cells is a potent activator for TGF-β1 production.[30,31] We have recently shown that CD48, a ligand for CD2, is upregulated on eosinophils after allergen challenge.[32] Hence, eosinophils not only are a source for TGF-β1 but also are capable of promoting other cells to secrete this factor, stressing their role as potent profibrogenic cells.

We found that inhibition of MC and eosinophil functions reverses mucus production, suggesting that these cells are regulators of mucus production in the lung as well, extending the observations of Yu et al,[33] Lee et al,[9] and Humbles et al[10] and all showing significant decreases in mucus production in MC- or eosinophil-deficient mice. Certainly, in LC1-treated mice, expression of IL-13 and IL-4, 2 main contributors to mucus production, was abrogated.

Subepithelial fibrosis airway smooth muscle thickening and excessive mucus production from hyperplasic goblet cells are hallmark features of asthma-related structural changes.[1,2,34] In addition to TGF-β1, MCs and eosinophils are a source for other agents that can modulate airway remodeling such as b fibroblast growth factor (b-FGF), NGF, vascular endothelial growth factor (VEGF), and MMPs.[4,7,35] Hence, their inhibition would reduce this feature of asthma.

Taken together, our results demonstrate that targeting CD300a provides a new approach to suppress critical effector functions *in vivo*. Furthermore, our study emphasizes a fundamental role for MCs and eosinophils in chronic allergic airway inflammation. Finally, it is likely to assume that targeting inhibitory receptors on these cells

will form the backbone concepts of immunotherapy in the near future.

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