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Statins Induce Regulatory T Cell Recruitment via a CCL1 Dependent Pathway

Emilia Mira,* Beatriz León,* Domingo F. Barber,* Sonia Jiménez-Baranda,* Iñigo Goya,* Luis Almonacid,* Gabriel Márquez,† Angel Zaballos,* Carlos Martínez-A.,* Jens V. Stein,‡ Carlos Ardavín,* and Santos Mañes2*

The statins, a group of inhibitors of the 3-hydroxy-3-methylglutaryl coenzyme A reductase, are reported to influence a variety of immune system activities through 3-hydroxy-3-methylglutaryl coenzyme A reductase-dependent and -independent mechanisms. How statin treatment regulates immune system function in vivo nonetheless remains to be fully defined. We analyzed the immunomodulatory effects of lovastatin in a Candida albicans-induced delayed-type hypersensitivity reaction in mice. In this model, lovastatin administration reduced the acute inflammatory response elicited by C. albicans challenge. This anti-inflammatory activity of lovastatin was associated with a shift from a Th1 to a Th2 immune response, as well as an increase in the percentage of regulatory T cells at the inflammation site and in the regional draining lymph node. The lovastatin-induced increase in regulatory T cells in the inflamed skin was dependent on expression of CCL1, a chemokine that is locally up-regulated by statin administration. The anti-inflammatory effect of lovastatin was abrogated in CCL1-deficient mice. These results suggest that local regulation of chemokine expression may be an important process in statin-induced modulation of the immune system. The Journal of Immunology, 2008, 181: 3524–3534.

The statins are used extensively in medical practice because of their ability to reduce cardiovascular mortality and stroke (1). Although this protective activity was initially ascribed to inhibition of cholesterol biosynthesis, it is now evident that statins are pleiotropic drugs with immunomodulatory and anti-inflammatory properties. Studies in animal models, as well as epidemiological and small-scale clinical trials, suggest that statin pleiotropism might be clinically relevant in other pathologies, including infection and sepsis (2–5), autoimmunity (6–9), and organ transplantation (10, 11).

The immunomodulatory effect of statins is thought to involve several mechanisms that act on different cell types (6, 12, 13). Statins not only prevent de novo synthesis of cholesterol, they also decrease the pool of intermediate isoprenoid metabolites, such as farnesyl pyrophosphate or geranylgeranyl pyrophosphate, thus affecting GTP-binding signaling proteins, including Ras and Rho-GTPases (2, 14, 15). Impairment of GTPase function allows statins not only prevent de novo synthesis of cholesterol, they also several mechanisms that act on different cell types (6, 12, 13).

The extent to which each of these pleiotropic activities of statins contributes to immunomodulation in vivo is not well understood. Moreover, the reported immunomodulatory activities of statins would be expected to attenuate the immune response. Paradigmatically, the capacity to mount protective immune responses to intracellular pathogens is not diminished in statin-treated patients. Indeed, regular statin use has been associated with reduced risk of bacteremia and sepsis (4, 5), as well as lower incidence and progression of specific cancer types (23).

We analyzed the in vivo effect of lovastatin in a murine model of skin delayed-type hypersensitivity (DTH)3 response to heat-inactivated Candida albicans. Skin DTH responses involve Ag uptake, processing, and transport by APC to tissue-draining lymph nodes, where Ag-specific T cells are triggered to initiate a primary immune response. Secondary exposure to the Ag results in a robust local inflammatory response. The coordinated cell and cytokine responses required to induce DTH make this experimental system ideal for in vivo analysis of the immune response.

Our data show that lovastatin reduced the inflammatory response to secondary Ag exposure, and that this effect was not a...
The CCL1 (also known as Scy1) gene was amplified by PCR from 129 Sv/J mouse strain genomic DNA and used to subclone 3.7-kbp NcoI-NcoI and 3.2-kbp BamHI-BamHI DNA fragments from the 5′ and 3′ regions, respectively. These DNA fragments were then subcloned at either end of a neomycin resistance gene, under the control of the phosphoglycerate kinase promoter. The herpes simplex thymidine kinase gene was also fused at the end of the cloned CCL1 sequences in the replacement targeting construct (Fig. 1A). The resulting plasmid was linearized with NcoI and electroporated into the 129 SvJ embryonic stem cell line. Gancyclovir- and G418-resistant clones were selected, and 15 μg of genomic DNA from each clone was SacI-digested, electrophoresed, and blotted onto Hybond-N membranes (Amersham Biosciences). High stringency hybridizations with CCL1-specific 32P-labeled probes were performed in RapidHyb buffer (Amersham Biosciences) (Fig. 1B). Targeted embryonic cell clones were injected individually into CD1 morulae, which were transferred to pseudopregnant CD1 females, as described (30). Chimeric males were bred to C57BL/6 females; the offspring was genotyped by Southern blot (data not shown) and PCR with specific primers (see Materials and Methods). CCL1−/− mice were backcrossed onto the C57BL/6 genetic background for 10 generations. The genotype of each animal included in the study was verified by PCR using tail DNA.

### Materials and Methods

**Generation of CCL1-deficient mice**

The CCL1 (also known as Scy1) gene was amplified by PCR from 129 Sv/J mouse strain genomic DNA and used to subclone 3.7-kbp NcoI-NcoI and 3.2-kbp BamHI-BamHI DNA fragments from the 5′ and 3′ regions, respectively. These DNA fragments were then subcloned at either end of a neomycin resistance gene, under the control of the phosphoglycerate kinase promoter. The herpes simplex thymidine kinase gene was also fused at the end of the cloned CCL1 sequences in the replacement targeting construct (Fig. 1A). The resulting plasmid was linearized with NcoI and electroporated into the 129 SvJ embryonic stem cell line. Gancyclovir- and G418-resistant clones were selected, and 15 μg of genomic DNA from each clone was SacI-digested, electrophoresed, and blotted onto Hybond-N membranes (Amersham Biosciences). High stringency hybridizations with CCL1-specific 32P-labeled probes were performed in RapidHyb buffer (Amersham Biosciences) (Fig. 1B). Targeted embryonic cell clones were injected individually into CD1 morulae, which were transferred to pseudopregnant CD1 females, as described (30). Chimeric males were bred to C57BL/6 females; the offspring was genotyped by Southern blot (data not shown) and PCR with specific primers (see Materials and Methods). CCL1−/− mice were backcrossed onto the C57BL/6 genetic background for 10 generations. The genotype of each animal included in the study was verified by PCR using tail DNA.

### Table 1. Changes in mRNA levels in the whole footpad after *C. albicans* challenge of lovastatin- and vehicle-treated mice

| Molecule | Lovastatin (2−ΔΔCt) | Vehicle (2−ΔΔCt) | Lovastatin/Vehicle Ratio
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>2.8 ± 0.2</td>
<td>4.4 ± 1.1</td>
<td>−1.6</td>
</tr>
<tr>
<td>IL-1β</td>
<td>25.7 ± 0.3</td>
<td>146 ± 37</td>
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<tr>
<td>IL-2</td>
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</tr>
<tr>
<td>IL-3</td>
<td>not detected</td>
<td>detected</td>
<td>−</td>
</tr>
<tr>
<td>IL-4</td>
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<td>0.42 ± 0.8</td>
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</tr>
<tr>
<td>IL-5</td>
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<tr>
<td>IL-6</td>
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<td>47.5 ± 10.1</td>
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<td>IL-7</td>
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<td>0.5 ± 1.0</td>
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<tr>
<td>IL-9</td>
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<td>not detected</td>
<td>−</td>
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<tr>
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<td>−</td>
</tr>
<tr>
<td>TGF-β</td>
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<td>IFN-γ</td>
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<td>1.7 ± 2.1</td>
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</tr>
<tr>
<td>CCL2</td>
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</tr>
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</tr>
<tr>
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</tr>
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<td>−1.1</td>
</tr>
<tr>
<td>CCL22</td>
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<td>2.3 ± 1.0</td>
<td>+1.5</td>
</tr>
<tr>
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<td>−8.2</td>
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<td>−1.4</td>
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<td>65.2 ± 4.9</td>
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<tr>
<td>CXCL11</td>
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<td>44.8 ± 12.4</td>
<td>+1.7</td>
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<td>CXCL12</td>
<td>2.5 ± 0.9</td>
<td>2.3 ± 0.3</td>
<td>+1.1</td>
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<tr>
<td>CXCL13</td>
<td>4.6 ± 1.8</td>
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<td>+1.0</td>
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<tr>
<td>CXCL14</td>
<td>3.9 ± 0.5</td>
<td>4.5 ± 0.7</td>
<td>+1.2</td>
</tr>
<tr>
<td>CXCL15</td>
<td>3.0 ± 1.6</td>
<td>0.4 ± 1.3</td>
<td>+7.5</td>
</tr>
<tr>
<td>CXCL16</td>
<td>3.9 ± 0.1</td>
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<td>−1.1</td>
</tr>
<tr>
<td>CCR1</td>
<td>13.4 ± 2.5</td>
<td>19.8 ± 8.1</td>
<td>−1.5</td>
</tr>
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<td>CCR7</td>
<td>5.2 ± 1.6</td>
<td>9.6 ± 2.7</td>
<td>+1.9</td>
</tr>
<tr>
<td>CCR3</td>
<td>3.5 ± 1.8</td>
<td>2.4 ± 0.7</td>
<td>+1.5</td>
</tr>
</tbody>
</table>

a  ΔΔCt coefficient was calculated using ΔCt values for the PBS-injected footpad as reference, and the relative expression value was calculated as 2−ΔΔCt. Values >1 indicate increase in mRNA expression for the corresponding gene in the C. albicans-injected footpad compared with the PBS-injected control footpad. Values <1 indicate down-modulation of the mRNA. Data are mean ± SEM (n = 6).

b Ratio between lovastatin and vehicle 2−ΔΔCt values; +, indicates lovastatin/vehicle ratio; −, indicates vehicle/lovastatin ratio.

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**FIGURE 1.** Generation of the CCL1 knockout mouse. A. The WT allele of the CCL1 gene, a fragment of the targeting construct, and the mutant allele are shown. The 5′ and 3′ probes used for Southern hybridization are indicated. B, Southern blot analysis of SacI- or SphI-digested genomic DNA prepared from targeted embryonic stem cell clones indicates the presence of mutant alleles (arrow). Blots of SacI- and SphI-digested DNA were probed with 32P-labeled probes A (NcoI-NcoI) and B (BamHI-SacI), respectively. C, PCR analysis of genomic DNA isolated from CCL1+/+, CCL1−/−, and CCL1−/− mice tails was amplified by two separate PCR using specific primers (see Materials and Methods). Polynucleotides of 604 and 421 bp were amplified for the WT and targeted alleles, respectively.

A consequence of a defective primary response, vascular permeability, or diapedesis of immune cells. Lovastatin changed the expression of specific cytokines, promoting a shift from a Th1 to a Th2 response. In the Ag-challenged footpad, lovastatin also increased expression of CCL1, a chemokine that triggers chemotaxis of Th2 and a subset of T regulatory (Treg) cells (24–29). Lovastatin treatment increased the percentage of Treg cells at inflammatory sites and in regional tissue-draining lymph nodes, compared with vehicle-treated animals. In contrast, the percentage of Treg cells was similar in the inflamed footpads of vehicle- and lovastatin-treated CCL1-deficient mice, suggesting that statin-induced CCL1 up-regulation affects Treg cell recruitment to the inflammation site. Our data highlight control of chemokine expression as a major mechanism for the immunomodulation induced by the statins in vivo.
Induction of DTH response to C. albicans

All experiments involving the use of animals were supervised by the Centro Nacional de Biotecnología Ethics Committee, and performed according to European Union guidelines. Twelve-week-old C57BL/6 female mice (The Jackson Laboratory) were rested for 1 wk in an air-filtered enclosure before use; C. albicans mice were bred in our facility. Mice were treated daily with lovastatin (Sigma-Aldrich; 200 µg/mouse in absolute ethanol; 10 mg/kg or 1 mg/kg, i.p.) or vehicle during the experimental period. One week after the start of the treatment, mice were sensitized with heat-inactivated C. albicans (strain 3153, serotype A; National Collection Pathogenic Fungi, Bristol, U.K.; 10 mg/kg, i.p.) or vehicle during the experimental period. One week after the sensitization, mice were injected with FITC-BSA in PBS (2 mg/ml, 500 µl, s.c.); control right footpads of each mouse received 50 µl of PBS, i.p.). Mice treated with lovastatin were challenged on the following day (10 mg/kg, i.p.). Mice treated with vehicle were injected with an equal volume of PBS. The guinea pig footpad model of DTH was performed as described previously (8), in which footpad swelling was measured and FITC-BSA accumulation in footpad extracts was quantified. Mice sensitized and challenged as above were injected with FITC-BSA accumulation in footpad extracts was quantified. Mice sensitized and challenged as above were injected with FITC-BSA accumulation in footpad extracts was quantified.
injected into the tail vein of age- and sex-matched untreated or lovastatin-treated mice. After 2 h, mice were sacrificed, and percentages of red- and green-labeled adoptively transferred cells in peripheral and mesenteric lymph nodes, Peyer’s patches, blood, and spleen were calculated by FACS. The ratio of the input population was also determined and used as a correction factor. A homing ratio was calculated for each organ as follows: (percentage of lovastatin-treated adoptively transferred cells/percentage of vehicle-treated adoptively transferred cells) × (correction factor input). Switching dyes did not influence homing behavior.

Casein-induced peritonitis model

Peritoneal exudates were induced by casein injection, basically as reported (32). BALB/c mice were injected in a casein sodium solution (Sigma-Aldrich; 0.2% w/v in PBS, 2 ml, i.p.). After 3 h, cells from peritoneal exudates were recovered by washing with 5 ml of PBS and analyzed by FACS.

Adoptive transfer of sensitized T cells

Lovastatin- and vehicle-treated mice were sensitized with C. albicans as for DTH responses; 1 wk after sensitization, T cells were isolated from splenocytes of these donor mice by negative selection and injected into naive recipient mice (15 × 10⁶ cells, 200 μl of RPMI 1640 plus 1% FCS, i.v.). After 24 h, the mice were challenged with the same Ag in the left footpad, and the swelling increase was calculated. Unsensitized control donor mice were included in each experiment.

FIGURE 4. Effect of lovastatin on draining PO-LN infiltration by specific cell types. A and B, PO-LN at days 2 and 3 after C. albicans challenge were disaggregated, and cells were analyzed by flow cytometry after appropriate staining. The increase in absolute cell number was calculated as above. Results are mean ± SEM of four mice per group in a representative experiment (n = 3).

FIGURE 5. Effect of lovastatin on leukocyte migration in vitro and in vivo. A, T cells isolated from lymph nodes preincubated with vehicle, lovastatin, mevalonate, or lovastatin plus mevalonate were assayed for chemotaxis toward basal medium or medium containing CCL21 in Transwell assays. Migrating cells were calculated by FACS analysis; data are mean ± SD of duplicates in one representative experiment of two (+, p < 0.001, two-tailed t test). B and C, Lovastatin does not affect homostatic T cell homing. T cells isolated from vehicle- or lovastatin-treated mice were differentially labeled, mixed at a 1:1 ratio, and reinjected into age- and sex-matched vehicle-treated (B) or lovastatin-treated (C) mice. Red- and green-labeled adoptively transferred cells in the indicate organs were counted by FACS, and the homing ratio was calculated (see Materials and Methods). Data are representative of one experiment of three (n = 4). D, Lovastatin does not affect leukocyte migration to the peritoneum. Peritoneal exudates were induced by casein injection; cells in exudates were analyzed by FACS with the indicated markers. The graph shows the difference in the number of cells recovered in exudates of casein- and PBS-injected mice; data are mean ± SEM (n = 5) in one experiment of three.

TagMan low-density arrays and quantitative RT-PCR

Total RNA from C. albicans- and PBS-injected footpads was purified (TRI-Reagent; Sigma-Aldrich) and cDNA synthesized (high-capacity cDNA archive kit; Applied Biosystems). Quantitative real-time PCR was performed with TaqMan low-density arrays in a 7900HT sequence detection system (Applied Biosystems). The complete list of the genes analyzed is given in Table I; each gene was assayed in duplicate in the same card. Differences in cycle threshold (ΔCt) for each gene in C. albicans- and PBS-injected footpads were calculated using 18S rRNA as an internal control. The ΔΔCt coefficient was then calculated using ΔCt values of the PBS-injected footpad as reference, and increase/decrease in mRNA expression was calculated as 2−ΔΔCt. Variations in mRNA expression from at least six mice per group were averaged, and the lovastatin/vehicle or vehicle/lovastatin quotient was calculated.

T cell stimulation assays

CD4⁺ T cells were isolated from cell suspensions of PO-LN from vehicle- and lovastatin-treated mice by magnetic cell sorting (MACS; Miltenyi Biotech) after incubation with biotin anti-CD4 mAb and streptavidin-conjugated MACS microbeads. Purity of CD4⁺ T cell preparations was >95%. CD4⁺ T cells were stimulated for 48 h with anti-CD3e (145-2C11) and anti-CD28 (37.51; BD Pharmingen) Abs, and IFN-γ and IL-4 were measured by ELISA (BD Pharmingen).

Immunohistochemistry

Footpads were snap frozen in tissue-freezing medium (Jung), and 4-μm crossections were acetone fixed and stained with anti-CCL1 (R&D Systems), anti-CD31 (MEC13.3; BD Pharmingen), biotinylated anti-CD3e
Lovastatin does not affect T cell activation in the sensitization phase. A, MHCII staining of spleen B220⁺ and CD11c⁺ cells 1 wk after priming with C. albicans. MHCII staining of cells from lovastatin-treated (dashed line) and vehicle-treated (solid line) mice is shown. B, Activation and memory markers in CD4⁺ and CD8⁺ T cells isolated from the spleen of vehicle- and lovastatin-treated mice 1 wk after C. albicans priming. Data are mean ± SEM of the percentage of double-positive cells for the indicated markers (n = 5 mice/group in a representative experiment of three). C, Sensitized T cells from vehicle- or lovastatin-treated mice were adoptively transferred to untreated, naive recipient mice, which were challenged with the same Ag in the footpad, and swelling was recorded. Data are mean ± SEM (n = 4 mice/group) in one representative experiment of three.

FIGURE 6. Lovastatin does not affect T cell activation in the sensitization phase. A, MHCII staining of spleen B220⁺ and CD11c⁺ cells 1 wk after priming with C. albicans. MHCII staining of cells from lovastatin-treated (dashed line) and vehicle-treated (solid line) mice is shown. B, Activation and memory markers in CD4⁺ and CD8⁺ T cells isolated from the spleen of vehicle- and lovastatin-treated mice 1 wk after C. albicans priming. Data are mean ± SEM of the percentage of double-positive cells for the indicated markers (n = 5 mice/group in a representative experiment of three). C, Sensitized T cells from vehicle- or lovastatin-treated mice were adoptively transferred to untreated, naive recipient mice, which were challenged with the same Ag in the footpad, and swelling was recorded. Data are mean ± SEM (n = 4 mice/group) in one representative experiment of three.

Results
Effects ofLovastatin on inflammation in the DTH reaction to C. albicans Ags
To study the immunomodulatory role ofLovastatin, we established a DTH response to heat-inactivated C. albicans (Fig. 2A). Lovastatin- or vehicle-treated mice were primed i.p. with Ag, and challenged (s.c.) 1 wk later in the left hind footpad; as an internal control, the right hind footpad received the same volume of PBS. Swelling was measured daily in both footpads for 4 days (Fig. 2B). Maximum swelling occurred on days 1 and 2 postchallenge; as predicted, lovastatin consistently reduced, but did not prevent Ag-induced inflammation (Fig. 2B). TheLovastatin dosage used in these experiments (10 mg/kg) yields plasma levels in mice of the active metaboliteLovastatin hydroxyacid comparable to those found in humans treated with 40 mg ofLovastatin (34). Nonetheless, a slight reduction in footpad inflammation was also observed in mice treated with Lovastatin at 1 mg/kg (data not shown).

The effect of statins on vascular permeability is debated (17, 35, 36). To address a possible effect on the vasculature, we injected vehicle- andLovastatin-treated mice with FITC-BSA and analyzed vascular leakage. Vascular permeability increased moderately and transiently at day 2 postchallenge inLovastatin-treated compared with vehicle-treated mice; permeability was subsequently recovered (data not shown). We next determined cell infiltration in swollen footpads after collagenase digestion of minced dermis. To estimate the Ag-induced increase in cell number, we subtracted the number of cells recovered from PBS-injected controls from that of C. albicans-challenged footpads. In vehicle-treated mice, cell numbers increased linearly and peaked at day 3 postchallenge; Lovastatin reduced the number of infiltrating cells in the footpad from day 2 postchallenge (Fig. 2C). Positive correlation of swelling and cell number increase was significant for days 2 and 3 after challenge.
both mouse groups (Fig. 2D; p = 0.56 and p = 0.78 for vehicle and lovastatin treatment, respectively; p < 0.001). Because lovastatin did not affect vascular leakage in our system, these results suggest that the anti-inflammatory effects of lovastatin are largely mediated by reducing cell recruitment.

Lovastatin decreases footpad infiltration of specific leukocyte types

We characterized the leukocyte infiltrate in the footpad by flow cytometry after staining cells with the appropriate Abs. The most abundant dermis-infiltrating cells were myeloid cells (CD11b+), specifically neutrophils and monocytes (CD11b+Gr1−) (Fig. 3). CD11b+Gr1− cell number increased with time, whereas macrophages (CD11b+F4/80+) infiltrated the footpad only transiently at day 3 postchallenge. Most of the CD3+ TCR+ T cells infiltrating the footpad were CD4+CD8−, and few CD4+ and CD8+ T cells were found; in all cases, T cell infiltration was delayed compared with neutrophils. A similar pattern was observed for NKT cells (CD3+NK1.1+); in contrast, NK cell (CD3+NK1.1+) infiltration showed a bell-shaped curve with a maximum at day 2 postchallenge. Finally, B cells were comparable to macrophages in number and infiltration time course. Lovastatin treatment did not alter the relative percentage of any cell type in the inflamed footpad (data not shown), but drastically reduced absolute numbers of infiltrating neutrophils, macrophages, CD3+CD4+CD8− T cells, NKT cells, and NK cells (Fig. 3); CD4+, CD8+, and B cells were only minimally affected or unaffected by lovastatin treatment.

Lovastatin reduces leukocyte numbers in the regional draining lymph node

We analyzed the number and cell types infiltrating the draining PO-LN of Ag-injected footpads from vehicle- and lovastatin-treated mice. Analyses were performed at days 2 and 3 postchallenge, because maximum swelling and the most acute anti-inflammatory effects of lovastatin were observed at this time. As for the inflamed dermis, lovastatin decreased total cell numbers in PO-LN (Fig. 4A), but did not alter the relative percentage of each cell type (data not shown). Lovastatin reduced the numbers of B and T cells (CD4+ and CD8+) on days 2 and 3, whereas a clear reduction was observed in macrophages and monocytes on day 3 postchallenge. Lovastatin also affected the dendritic cell (DC) subtypes found in the PO-LN (37); the numbers of plasmacytoid DC, inflammatory, and CD8− DC were reduced only at day 2, whereas dermal and CD8+ DC numbers were diminished only at day 3 postchallenge (Fig. 4B).

Lovastatin effects on leukocyte mobilization and T cell activation

Because statins could influence the immune system at different levels, including leukocyte trafficking, Ag presentation, and T cell activation, we analyzed the effect of lovastatin treatment on these processes during the DTH reaction. Subtoxic lovastatin doses inhibited T cell chemotaxis toward the chemokine CCL21 in Transwell assays in an 3-hydroxy-3-methylglutaryl coenzyme A reduce-tase-dependent manner (Fig. 5A). We nonetheless found that T cells from lovastatin- or vehicle-treated mice showed similar in
vivo homing to secondary lymphoid organs (SLO) when adoptively transferred into vehicle-treated (Fig. 5B) or lovastatin-treated (Fig. 5C) hosts. Moreover, there was no difference in the number of cells recovered from SLO of untreated or lovastatin-treated hosts (88 ± 13 × 10^6 vs 91 ± 6 × 10^6 cells, respectively). In a case-injected peritonitis assay, we also found that lovastatin treatment did not affect mobilization of myeloid cells to the peritoneal cavity compared with vehicle-treated mice (Fig. 5D). These results suggest that, at this dose, lovastatin does not affect in vivo mobilization of myeloid or T cells in physiological conditions.

We analyzed the effects of lovastatin on the APC and/or T cell compartments. Concurring with previous data (38), lovastatin reduced MHC class II expression in spleen CD11c^+ cells 1 wk after priming with *C. albicans* (Fig. 6A), although the reduction was slight at the dose used. Indeed, we found no apparent differences in the levels (data not shown) or percentages of spleen CD4^+ and CD8^+ cells expressing activation (CD69, CD25) and memory (CD44, CD62L^low^) markers (Fig. 6B), nor were changes observed in the percentage of CD4^+ (12 ± 0.6%; 15.4 ± 1.6%) or CD8^+ cells (8.8 ± 0.4%; 11.15 ± 0.1%) in vehicle- and lovastatin-treated mouse spleens.

To further address whether lovastatin affected the priming phase of the DTH response, T cells were isolated from vehicle- or lovastatin-treated mice 1 wk after priming with *C. albicans*, and adoptively transferred to naive, untreated mice; 1 day after cell transfer, mice were injected with *C. albicans*, and the increment in swelling was measured as a readout of the DTH response. We detected a similar increment in footpad swelling after the challenge, independently of the origin of transferred T cells (Fig. 6C). These results suggest that, in our conditions, lovastatin did not markedly affect primary T cell activation.

**Lovastatin induces changes in cytokine and chemokine expression**

We used real-time quantitative RT-PCR to analyze changes in mRNA levels of representative cytokines, chemokines, adhesion molecules, and proteases isolated from total cell lysates from inflamed footpads of vehicle- and lovastatin-treated mice. Compared with the PBS-injected control, *C. albicans* challenge induced a >2-fold up- or down-modulation in 40 (80%) of the 50 genes analyzed (Table I); 15 (37.5%) of these 40 genes were differentially regulated by lovastatin. Among the most important changes observed, lovastatin increased mRNA levels for the Th2 cytokines IL-4 and IL-5, and reduced levels for the Th1-chemokines associated with Th1 responses (CCL4, CCL5) and up-regulated Th2-associated chemokines (CCL11, CCL1) (Fig. 8A and Table I). Lovastatin also up-regulated CCL27, whose receptor (CCR10) is expressed on both Th1 and Th2 cells (41), and CXCL15, whose receptor remains to be identified. Lovastatin reduced mRNA levels for the CXCR2 ligands CXCL1, CXCL2, and

![FIGURE 9](http://www.jimmunol.org/)

**Lovastatin increases the percentage of CD3^+FoxP3^+ Treg cells in the inflamed footpad and draining lymph node.** A. *x*-fold induction of FoxP3 mRNA levels in a fraction enriched in lymphoid cells isolated from the inflamed footpad of vehicle- and lovastatin-treated mice at the indicated days postchallenge. Data are mean ± SEM in one representative experiment of three (*n* = 3). B. Total cell extracts from the inflamed footpads of vehicle (V)- and lovastatin (Lov)-treated mice were resolved by SDS-PAGE and immunoblotted sequentially with anti-FoxP3 and anti-actin Abs. *Left panels,* Show representative immunoblots; *right panel,* shows the mean ± SEM of the FoxP3/actin ratio of the densitometric values obtained from four mice/group in two independent experiments. *C,* FACS detection of CD4^+FoxP3^+ T cells in the inflamed footpad at day 2 postchallenge. Data are mean ± SEM of the percentage of FoxP3^+ cells in the CD4^+ population in one representative experiment of two. Value of *p* was calculated by two-tailed Student’s *t* test using the square root of the data. *D,* Footpad sections from vehicle- or lovastatin-treated mice at day 2 postchallenge were stained with anti-CD3 (red) and anti-FoxP3 (green) Abs. Open and solid arrowheads indicate CD3^+ and CD3^+FoxP3^+ cells, respectively. Magnification, ×400. *E,* CD4^+FoxP3^+ T cells in draining PO-LN of *C. albicans* and PBS-injected footpads were analyzed by FACS at the indicated days postchallenge. Data show the mean ± SEM of the percentage of CD4^+FoxP3^+ cells in PO-LN of four mice/group in a representative experiment (*n* = 3).
CXCL5, which are chemoattractants for Gr1+ cells; this could explain the reduced number of Gr1+ cells in the inflamed footpads of lovastatin-treated mice. Other chemokines were only minimally affected by lovastatin (Table I). We found no lovastatin-induced differences in expression of the adhesion molecules ICAM-1, ICAM-2, E-selectin, or VCAM-1, or the metalloproteases matrix metalloproteases-2, -9, -13, or -14 (data not shown). Collectively, the results indicate that lovastatin produces a shift from Th1- to Th2-associated cytokines, as well as changes in the expression pattern of chemokines that can affect the trafficking of different leukocyte subsets.

Lovastatin increases the percentage of Treg cells in the inflamed footpad and draining lymph nodes

Of the chemokines whose expression was altered by lovastatin treatment, we focused on CCL1 because its receptor, CCR8, is associated with Th2 and Treg cell mobilization (26, 29, 42). We analyzed CCL1 protein levels by immunohistochemistry to validate the lovastatin-induced CCL1 mRNA up-regulation described above, using C. albicans-injected footpads of CCL1-deficient mice as a staining control (Fig. 8B). CCL1 staining was weaker in the inflamed footpads from Ag-injected, vehicle-treated mice than in those from lovastatin-treated animals; in both cases, CCL1 staining was largely confined to CD31+ cells (Fig. 8B). Quantification of the images indicated that the CCL1-stained area relative to that of CD31 (Fig. 8C), as well as CCL1 staining intensity (Fig. 8D) were significantly higher in inflamed footpads from lovastatin-treated than from vehicle-treated mice. At day 2 postchallenge, ELISA measurement of CCL1 levels in footpad cell lysates confirmed the lovastatin-induced increase in CCL1 expression in inflamed skin (Fig. 8D). We found no changes in CCL1 levels in other organs, such as noninflamed skin and lungs, as a consequence of lovastatin treatment (data not shown).

We next measured the levels of forkhead box P3 (FoxP3) transcription factor, a specific marker for the Treg cell lineage. FoxP3 mRNA (Fig. 9A) and protein levels (Fig. 9B) were augmented in the inflamed footpad of lovastatin-treated compared with vehicle-treated mice. The FoxP3 increase occurred at day 2 postchallenge, coinciding with the initiation of CD4+ T cell infiltration in the footpad. Accordingly, FACS analysis indicated that lovastatin treatment significantly increased the percentage of CD4+FoxP3+ cells in inflamed footpads (Fig. 9C). Immunohistochemistry showed a larger number of infiltrating CD3+FoxP3+ cells in the Ag-challenged footpad of lovastatin-treated mice compared with controls (Fig. 9D).

Lovastatin also increased the percentage of CD4+FoxP3+ Treg cells in the PO-LN draining the C. albicans-injected compared with vehicle-treated footpads at day 2 postchallenge (Fig. 9E). Notably, lovastatin treatment did not augment CD4+FoxP3+ cell percentages in naive mouse spleen (data not shown) or in the PO-LN draining the PBS-injected footpad (Fig. 9E, right). We detected no increase in the number or the percentage of CD4+FoxP3+ cells in the peritoneum of lovastatin-treated compared with control mice (data not shown). These results suggest that lovastatin might specifically affect Treg cell recruitment to inflamed areas and to the SLO that drain these sites.

CCL1 is a key element in the immunomodulatory activity of lovastatin

To study the functional relationship between lovastatin-induced CCL1 up-regulation and its anti-inflammatory effect, we analyzed the C. albicans-induced DTH response in vehicle- and lovastatin-treated CCL1-deficient mice. These mice did not exhibit an overt phenotype; when maintained in barrier isolation, they were healthy and bred according to Mendelian inheritance patterns. FACS analysis of CD4+, CD8+, and B220+ lymphocyte subpopulations from thymus, lymph nodes, spleen, and peripheral blood indicated no differences between CCL1-deficient and WT mice (our unpublished results).

In contrast to a significant lovastatin-induced reduction of inflammation in WT mice, we found that DTH responses to C. albicans were comparable in vehicle- and lovastatin-treated CCL1−/− animals (Fig. 10A). Compared with vehicle treatment, lovastatin did not increase the percentage of CD3+FoxP3+ cells infiltrating the inflamed footpads of CCL1−/− mice (Fig. 10B); this contrasted with the significant lovastatin-triggered increase in...
CD3+FoxP3+ cell infiltration of the WT mouse footpad (Fig. 10B). Moreover, we observed a reduction in the number of CCR8+FoxP3+ cells in the inflamed skin of CCL1−/− compared with WT mice, independently of the treatment (Fig. 10C). These results suggest that CCL1 deficiency impairs the immunomodulatory effect of lovastatin by affecting the infiltration of specific cell types into the inflamed footpad.

We also found that CCL1 deficiency affected Treg cell trafficking in the draining PO-LN. Whereas lovastatin administration induced a significant increase in the percentage of Treg cells in draining PO-LN in WT animals, the percentage of infiltrating Treg cells in draining PO-LN was unaffected by treatment in CCL1−/− mice (Fig. 10D). Nonetheless, the percentage of Treg cells in the draining PO-LN of vehicle-treated CCL1−/− mice was significantly higher than that in vehicle-treated WT animals (Fig. 10D). This suggests that CCL1 deficiency could affect Treg cell recruitment into or egress from the PO-LN in inflammatory conditions, independently of statin treatment. Collectively, our results indicate a major role for CCL1 in the in vivo immunomodulatory and anti-inflammatory activities of lovastatin in this system.

**Discussion**

Several studies have shown that statins are not only lipid-lowering drugs, but also are potent modulators of the immune system (16). Given the broad administration of these drugs in the population, it is extremely important to elucidate how in vivo statin treatment affects immune system function.

To define the mechanisms underlying the immunomodulatory activity of lovastatin, we used a C. albicans-induced DTH model in mice, which recapitulates the coordinated cell interactions that take place during Th1 immune reactions. In our system, lovastatin attenuated the acute inflammatory response triggered by C. albicans challenge. Concurring with studies in animal models of Th1-mediated autoimmune diseases (6, 9, 15), we found that lovastatin treatment triggered a decrease in production of proinflammatory cytokines and promoted a Th2-biased DTH response. We also detected a slight reduction in MHCI expression in CD11c+ cells in lovastatin-treated mice (Fig. 6A). This decrease appears not to affect the inductive phase of the DTH response, however, because splenocytes isolated from lovastatin- or vehicle-treated mice and adoptively transferred to naive recipients responded equally well to antigenic challenge (Fig. 6C). The lovastatin dose used neither altered homeostatic T cell homing to SLO, in contrast to other studies (22), nor affected in vivo mobilization of myeloid cells to the peritoneum (Fig. 5). Nonetheless, lovastatin treatment inhibited CCL21- and CCL1-induced chemotaxis of T cells in vitro (Fig. 5A, and data not shown).

In addition to the reported effects of statin administration, our model showed that lovastatin promoted an increase in the percentage of CD3+FoxP3+ cells at the inflammation site and in the draining PO-LN. The lovastatin-induced increase in Treg cells at the inflamed area was dependent on expression of CCL1, a chemokine locally up-regulated by statin administration. Strikingly, the anti-inflammatory effect of lovastatin was abrogated in CCL1-deficient mice, suggesting that the CCL1-mediated increase in Treg cells in the inflamed footpad might be critical for the anti-inflammatory activity of lovastatin.

To date, CCL1 is the only known mammalian ligand for CCR8 (43), a receptor preferentially expressed on Th2 compared with Th1 effector cells (29, 42). Although the role of CCR8 in mediating Th2 cell recruitment is debated (43–45), two reports assign a major role to CCL1 in CCR8+ Th2 cell trafficking to the skin in homeostatic and inflammatory conditions (27, 46). More importantly, CCR8 is expressed in a large proportion of murine FoxP3+ T cells, and Treg cells are hyperresponsive to CCL1 (26, 42). Several studies propose that the receptors for inflammatory chemokines (CCR2, CCR4, CCR5, and CCR8) on regulatory cell subsets would enable these cells to enter inflamed tissues (24, 26, 47, 48). Our observations suggest that statin-induced up-regulation of CCL1 reduces the inflammatory reaction by controlling the trafficking of specific CCR8+ T cell subsets.

In normal skin, CCL1 is undetectable (46) or is barely detected in CD31+ dermal microvessels (27). A recent report showed that C. albicans triggers expression of proinflammatory genes, including a wide array of chemokines, in primary human endothelial cells (49); notably. C. albicans did not induce CCL1 expression in endothelial cells in this study. Accordingly, we found no increase (<2-fold) in CCL1 mRNA expression in vehicle-treated C. albicans-injected footpads compared with PBS-injected controls, whereas CCL1 expression increased markedly in CD31+ cells from lovastatin-treated, yeast-injected footpads (Table I). The selective transcriptional regulation of specific chemokines by statin treatment could explain the CCL1 requirement for lovastatin activity, despite the redundancy of the chemokine system. Indeed, of the receptors for inflammatory chemokines reported in Treg cells (CCR2, CCR4, CCR5, and CCR8), CCL1 was the only ligand clearly up-regulated by in vivo lovastatin treatment; in contrast, CCR4 ligands (CCL17 and CCL22) were only minimally affected, and CCR5 agonists (CCL3, CCL4, and CCL5) were down-regulated in the footpad of lovastatin-treated mice (Table I). We observed FoxP3+ cells in the inflamed footpad of CCL1-deficient mice, indicating that other chemokine-receptor pairs also contribute to Treg cell infiltration independently of statin treatment.

CCR8-expressing FoxP3+ cells have been found not only in inflamed tissues, but also in peripheral lymph nodes (42). In our model, lovastatin treatment increased Treg cells both in the draining PO-LN and in the footpad of WT mice. The percentage of Treg cells in the inflamed footpad did not increase following statin treatment in CCL1-deficient mice, suggesting a role for the CCL1-CCR8 axis. In contrast, the Treg cell increase in statin-treated mouse lymph nodes is probably CCL1-CCR8 independent. Indeed, CCL1 deficiency enhanced the percentage of Treg cells in the draining PO-LN, independently of lovastatin administration. These results suggest that Treg cell homing to PO-LN involves other chemokine/receptor pairs (50, 51). Although further research is needed to understand the mechanism underlying these differences, CCL1 deficiency might impair Treg cell trafficking between lymph nodes and inflamed tissue, as reported for CCR7 (52), or CCL1 may have a role in Treg cell emigration from lymphoid organs.

In a recent report, Mausner-Fainberg et al. (53) suggested that certain statins enhance differentiation of human CD4+CD25+ FoxP3+ T cells to CD4+CD25+FoxP3+ Treg cells. This raised the question as to whether the lovastatin-induced increase in Treg cells in PO-LN and inflamed tissue in our DTH model is a consequence not only of differential trafficking, but also of a direct effect of statin treatment on Treg cell expansion. Nonetheless, these authors did not find statin-induced conversion of CD4+CD25+FoxP3+ to Treg cells when T cells were isolated from C57BL/6 mice (53), suggesting that lovastatin would not induce Treg cell expansion/differentiation in our system. Consistent with this idea, we found no changes in natural Treg cell numbers in the spleens of naive mice or in the draining PO-LN of PBS-injected footpads of lovastatin-treated mice.
In summary, statins would thus modulate the immune system through coordinated activity in various arms of the immune reaction, including terminal differentiation of immune cells, as well as the regulation of the molecules that steer these cells to the sites at which they exert their immunomodulatory activity.

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