

# Interleukin 12 Induces T-Cell Recruitment Into the Atherosclerotic Plaque

Xiaoyu Zhang,\* Alexander Niessner,\* Takako Nakajima, Wei Ma-Krupa, Stephen L. Kopecky, Robert L. Frye, Jörg J. Goronzy, Cornelia M. Weyand

**Abstract**—CD4 T cells, through the release of cytokines as well as direct effector functions, have been implicated in promoting inflammation of the atherosclerotic plaque. Plaque-infiltrating CD4 T cells include a specialized subset of CD4<sup>+</sup>CD28<sup>−</sup> T cells that express a unique profile of regulatory receptors and are responsive to novel microenvironmental cues. Here we report that CD4<sup>+</sup>CD28<sup>−</sup> T cells, either isolated from the plaque tissue or from the blood of patients with acute coronary syndrome (ACS), spontaneously express interleukin (IL)-12 receptors, even in the absence of antigenic stimulation. CD4<sup>+</sup>CD28<sup>−</sup> IL-12R<sup>+</sup> cells responded to IL-12 stimulation with the upregulation of the chemokine receptor CCR5 and the C-type lectin receptor CD161, both implicated in regulating tissue homing of effector T cells. IL-12 treatment of CD4<sup>+</sup>CD28<sup>−</sup> T cells enhanced their chemotaxis and transendothelial migration toward the chemokine CCL5. In vivo relevance for the role of IL-12 in regulating the recruitment of CD4<sup>+</sup>CD28<sup>−</sup> T cells into the atheroma was examined in human atheroma-SCID mouse chimeras. Exposure of nonstimulated CD4<sup>+</sup>CD28<sup>−</sup> T cells to IL-12 was sufficient to amplify T-cell accumulation within the inflamed plaque, and coadministration of anti-CCR5 antibodies blocked T-cell recruitment into the plaque. Thus, CD4<sup>+</sup>CD28<sup>−</sup> T cells functionally resemble NK cells, which have proinflammatory activity even in the unprimed state and respond to any IL-12-inducing host infection with a shift in tissue trafficking and accrual in inflammatory lesions. (*Circ Res.* 2006;98:524-531.)

**Key Words:** inflammation ■ interleukins ■ cytokines ■ T lymphocyte ■ vascular inflammation

The cellular and biological events causing acute coronary syndrome (ACS) have been linked to the process of plaque rupture and superimposed thrombosis, leading to vessel occlusion and ischemia.<sup>1</sup> Tissue disruption in the atheroma has multiple underlying causes; yet the presence of plaque inflammation is an excellent predictor of vulnerability.<sup>2</sup> Plaque-infiltrating cells are composed of macrophages, dendritic cells (DC), and T cells, all of which contribute to the ultimate outcome of tissue injury. T cells mediate inflammatory damage through 2 pathways: either by regulating the functional activity of macrophages,<sup>3</sup> endothelial cells, and vascular smooth muscle cells or via direct cytotoxicity.<sup>4</sup> Little is known about signals that regulate T-cell recruitment to and retention in the plaque and their functional activity in the nonlymphoid microenvironment of the vessel wall.

Plaque-infiltrating T cells are enriched for a particular subset, CD4<sup>+</sup>CD28<sup>−</sup> T cells.<sup>5</sup> Such CD4<sup>+</sup>CD28<sup>−</sup> T cells are proinflammatory lymphocytes, which produce high amounts of interferon (IFN)- $\gamma$ <sup>6</sup> and tumor necrosis factor (TNF)- $\alpha$ .<sup>7</sup> They also are efficient killer cells, expressing perforin and granzyme B, and lyse endothelial target cells.<sup>8</sup> CD4<sup>+</sup>CD28<sup>−</sup>

T cells have an unusual chemokine receptor profile, including expression of CX<sub>3</sub>CR1, a receptor typically expressed by NK cells and cytotoxic CD8 T cells. Thus, the tissue trafficking pattern of the CD4<sup>+</sup>CD28<sup>−</sup> T-cell subset may be fundamentally different from that of other CD4 T-cell populations.

T-cell function is primarily regulated by antigen recognition, and clonality within CD4<sup>+</sup>CD28<sup>−</sup> T cells has been cited as evidence of chronic antigen stimulation.<sup>5</sup> However, CD4<sup>+</sup>CD28<sup>−</sup> T cells from ACS patients also respond to heat shock protein 60 (HSP60),<sup>9</sup> suggesting a possible role for autoantigens and nonclassical triggers. Accordingly, CD4<sup>+</sup>CD28<sup>−</sup> T cells bypass T-cell receptor (TCR) triggering if they have acquired killer immunoglobulin-like receptors and the appropriate adaptor DAP12.<sup>4</sup> Thus, plaque-residing CD4 T cells are regulated by signals beyond antigen recognition, with some of these signals specific for the atheroma microenvironment.

Besides HSP60 and KIR ligands, several other mediators could enhance the activities of proinflammatory T cells in the plaque. Candidates include cytokines produced by DC and activated macrophages which colocalize with CD4 T cells in

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the plaque infiltrate.<sup>10</sup> Interleukin (IL)-12, a heterodimeric cytokine released by activated macrophages and DC, is abundantly produced in plaque tissues.<sup>11</sup> IL-12 plays a critical role in atherogenesis as indicated by reduced plaque burden in apolipoprotein E IL-12 knockout mice.<sup>12</sup> IL-12 is a potent regulator of T-helper cell differentiation.<sup>13</sup>

Two chains, IL-12 receptor (IL-12R)  $\beta$ 1 and IL-12R $\beta$ 2, form the membrane receptor complex for IL-12. Optimal IL-12R expression in T cells requires prior activation through the TCR.<sup>14</sup> One of the major functions of IL-12 is IFN- $\gamma$  induction.<sup>13</sup> IL-12 also induces a chemokine receptor switch, equipping activated T cells to enter peripheral sites of infection or injury.<sup>15</sup> Specifically, IL-12 upregulates CCR5 expression through which differentiated effector T cells are recruited to tissues that attract them via CCL5 (also called RANTES)<sup>16</sup> and macrophage inflammatory protein (MIP)-1 $\alpha$ . Another IL-12-inducible gene is CD161, a C-type lectin receptor facilitating T cell–endothelial cell interactions.<sup>17</sup>

The current study explored whether IL-12 modulates functional activity of CD4<sup>+</sup>CD28<sup>−</sup> T cells that preferentially accumulate in the inflamed atheroma. CD4<sup>+</sup>CD28<sup>−</sup> T cells were found to be constitutively IL-12 responsive, even in the absence of antigen stimulation. IL-12 induced enhanced chemotaxis and transendothelial migration of CD4<sup>+</sup>CD28<sup>−</sup> T cells in response to CCL5 in vitro and recruitment of CD4<sup>+</sup>CD28<sup>−</sup> T cells into the plaque in a mouse chimera model in vivo, suggesting that IL-12 production during infectious episodes could enhance T-cell influx into the atheroma.

## Materials and Methods

### Blood Samples of ACS Patients and Carotid Artery Specimens

ACS patients admitted to the hospital were enrolled into the study. Diagnostic criteria have previously been described.<sup>12</sup> Peripheral blood mononuclear cells (PBMC) were isolated from 44 patients using Ficoll-Hypaque (Amersham Biosciences, Piscataway, NJ). Distal embolization protection devices from 11 patients undergoing coronary artery stenting and carotid artery specimens from 68 patients (75% male, 70.9 $\pm$ 9.8 years) undergoing endarterectomy were collected in sterile saline and processed without delay.

The Institutional Review Board approved all protocols, and appropriate consent was obtained.

### T-Cell Lines and T-Cell Clones

Tissue fragments were immediately washed off the distal embolization protection devices, spun and cultured in RPMI medium supplemented with 50 U/mL recombinant human (rh) IL-2 (Proleukin Chiron, Emeryville, Calif), and stimulated with 1 $\times$ 10<sup>6</sup>/mL irradiated PBMCs, 1.5 $\times$ 10<sup>5</sup>/mL irradiated EBV-transformed B cells, 30 ng/mL anti-CD3 monoclonal antibodies (Ortho Diagnostics, Raritan, NJ), and 50 U/mL rh IL-2 after 7 days. CD4 T-cell lines were separated by fluorescence-activated cell sorting (FACS). CD4<sup>+</sup>CD28<sup>+</sup> and CD4<sup>+</sup>CD28<sup>−</sup> clones were established from peripheral blood T cells by limiting dilution as previously described.<sup>4</sup> T-cell lines or clones were stimulated with 10 ng/mL rh IL-12 (R&D Systems, Minneapolis, Minn) for 48 hours.

For adoptive transfer experiments, T cells were labeled with 4 $\times$ 10<sup>−6</sup> molar PKH26 red fluorescent dye in 2 mL of PKH26 diluent C (both from Sigma, St Louis, Mo); labeling efficiency was confirmed by flow cytometry. Cell viability as determined by trypan blue exclusion was >95%.

### Flow Cytometry

For multicolor flow cytometry, T cells were stained with PerCP-labeled anti-CD4, fluorescein isothiocyanate-labeled anti-CD28, anti-CD161, and anti-CCR5 and phycoerythrin-labeled anti IL-12R $\beta$ 1 chain monoclonal antibodies (all Becton Dickinson, Franklin Lakes, NJ). Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson). Frequencies and mean fluorescence intensities were determined using WinMDI software (Joseph Trotter, Scripps Research Institute, La Jolla, Calif).

### Reverse-Transcriptase Polymerase Chain Reaction

RNA from carotid artery tissue or T-cell clones was extracted using TRIzol (Invitrogen Life Technologies, Grand Island, NY), reverse transcribed, and amplified by PCR with primers specific for  $\beta$ -actin, TCR  $\alpha$ -chain, CCL5, MIP-1 $\alpha$ , and IL-12 $\beta$ 1 and  $\beta$ 2 chains (supplemental Table I, available online at <http://circres.ahajournals.org>). Transcripts for  $\beta$ -actin, TCR, CCL5, and MIP-1 $\alpha$  were quantified using the Mx 4000 PCR instrument (Stratagene, Cedar Creek, Tex).<sup>18</sup> Transcripts were normalized to 2 $\times$ 10<sup>6</sup>  $\beta$ -actin copies.

### Transwell and Transendothelial Migration System

Transendothelial migration assays were performed in 12-well plates with 12-mm, 3- $\mu$ m pore, collagen-coated polytetrafluoroethylene transwell inserts (Corning Costar, Acton, Mass). Human umbilical vein endothelial cells (HUVEC) (American Type Culture Collection, Manassas, Va) were plated at 5 $\times$ 10<sup>5</sup> to 1 $\times$ 10<sup>6</sup> cells/insert. After 2 days, 5 $\times$ 10<sup>5</sup> T cells were placed in the upper chamber and CCL5 (R&D Systems) was added to the lower chamber. After 4 hours of incubation at 37°C, T cells that migrated into the lower chamber were recovered, counted, and expressed as the percentage of the total cell number.

In the chemotaxis assays, T cells were added to the upper chamber of transwell plates with polycarbonate membranes (12-mm, 3- $\mu$ m pore; Corning Costar), and CCL5 was added to the lower chambers. After 2 hours, T cells in the lower chamber were recovered, and the percentage of migrated T cells was determined.

### CD4 T Cells Transfer Into Carotid Artery Plaque-SCID Mouse Chimeras

Six- to 8-week-old NOD.CB17-Prkdc<sup>scid</sup>/J mice or NOD-129S7 (B6)-Rag1 (*tm1Mom*) J mice (Jackson Laboratories, Bar Harbor, Maine) were anesthetized with 50 mg/kg pentobarbital (Abbott Laboratories, North Chicago, Ill) IP and engrafted with human carotid artery plaque tissue placed into a subcutaneous pocket on the midback.<sup>18</sup> All procedures were approved by the Animal Care and Use Committee. On day 7 after implantation, mice were injected intravenously with 1 $\times$ 10<sup>7</sup> CD4 T cells or PBS. Forty-eight hours after cell transfer, the human carotid artery grafts were explanted and shock frozen for mRNA analysis or embedded in OCT compound (Sakura Fine-Tek, Torrance, Calif). Tissue-infiltrating cells were counted in 5 nonserial sections. In each section, 25 fields were analyzed by fluorescence microscopy.

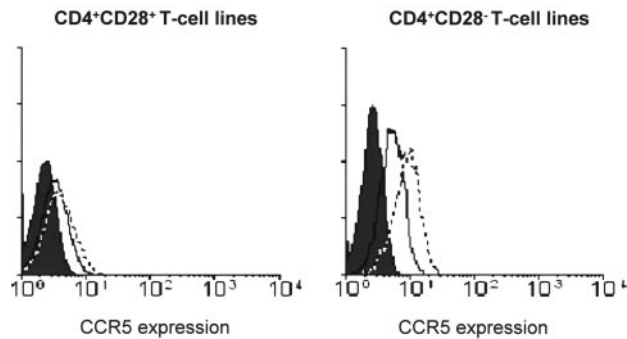
### Statistical Analysis

Statistical analyses were performed with Mann–Whitney *U* test, Student *t* test,  $\chi^2$  test, and Fischer exact test where appropriate. We compared the response of cell lines to different stimulation conditions using 2-way repeated measures ANOVA (SigmaStat; SPSS, Chicago, Ill).

## Results

### IL-12 Regulates the Function of Plaque-Derived CD4 T Cells

To evaluate whether plaque-infiltrating T cells are amenable to cytokine regulation, we devised a method to sample tissue-derived T cells from atherosclerotic lesions. Tissue debris captured in distal embolization protection devices from



**Figure 1.** CD4<sup>+</sup>CD28<sup>−</sup> T cells isolated from plaque fragments are IL-12 responsive. T-cell lines, established by culturing tissue debris captured in distal embolization protection devices from 5 different patients, were stimulated with IL-12 (10 ng/mL), and expression of the IL-12-inducible gene CCR5 was analyzed by multicolor FACS. Representative results are shown for CD4<sup>+</sup>CD28<sup>+</sup> and CD4<sup>+</sup>CD28<sup>−</sup> T cells before (solid line) and after (dashed line) IL-12 stimulation for 48 hours. Shaded areas represent staining with isotype-matched control antibody.

11 patients was cultured; T-cell lines grew out of the tissue debris from 5 patients. No difference in risk factor profile, medication, or clinical presentation between patients with or without outgrowing tissue lines was found. Expanding T-cell lines contained CD4 and CD8 T cells and, consistent with prior reports, the cell populations were enriched for CD4<sup>+</sup>CD28<sup>−</sup> T cells.

To test for IL-12-mediated effects, the upregulation of CCR5 on the T-cell surface was determined. Plaque-derived T-cell lines responded promptly to IL-12 (Figure 1), even without prior TCR triggering, indicating constitutive expression of functional IL-12 receptors. Phenotyping of IL-12-responsive T cells revealed that CCR5 upregulation was limited to CD4<sup>+</sup>CD28<sup>−</sup> T cells (Figure 1), suggesting that IL-12 had a particular role in regulating this T-cell subset.

### Circulating CD4<sup>+</sup>CD28<sup>−</sup> T cells Constitutively Express IL-12R and IL-12-Inducible Genes

CD4<sup>+</sup>CD28<sup>−</sup> T cells are rare in healthy individuals but are elevated in the blood of ACS patients.<sup>4,5,8</sup> To study whether spontaneous expression of IL-12R is a characteristic of CD4<sup>+</sup>CD28<sup>−</sup> T cells, we collected peripheral blood T cells from 44 ACS patients (see supplemental Table II) and compared the CD4<sup>+</sup>CD28<sup>+</sup> and CD4<sup>+</sup>CD28<sup>−</sup> populations for IL-12R expression. CD4<sup>+</sup>CD28<sup>+</sup> T cells had very low levels of IL-12Rβ1 chains. In contrast, IL-12Rβ1 was consistently detected on CD4<sup>+</sup>CD28<sup>−</sup> T cells ( $P<0.0001$  compared with CD4<sup>+</sup>CD28<sup>+</sup> T cells). Essentially the entire subset of CD4<sup>+</sup>CD28<sup>−</sup> T cells stained positive for IL-12Rβ1 expression.

To obtain evidence for *in vivo* functional relevance of IL-12R expression on CD4<sup>+</sup>CD28<sup>−</sup> T cells, we evaluated freshly harvested T cells for the presence of IL-12-inducible genes. Expression of IL-12R on CD4<sup>+</sup>CD28<sup>−</sup> T cells was closely correlated with the presence of 2 IL-12-dependent membrane receptors, CD161 and CCR5 (Figure 2). The C-type lectin receptor CD161 was present in low levels on CD4<sup>+</sup>CD28<sup>+</sup> T cells, whereas the entire population of circulating CD4<sup>+</sup>CD28<sup>−</sup> T cells was positive. Similarly,

CD4<sup>+</sup>CD28<sup>+</sup> and CD4<sup>+</sup>CD28<sup>−</sup> T cells displayed a clear difference for CCR5 expression. Differences in expression of CD161 and CCR5 on the 2 CD4 subpopulations were highly significant, with  $P=0.0006$  and  $P<0.0001$ , respectively.

### IL-12 Enhances CCL5-Induced Chemotaxis and Transendothelial Migration of CD4<sup>+</sup>CD28<sup>−</sup> T Cells

The antigen-independent IL-12 responsiveness of circulating CD4<sup>+</sup>CD28<sup>−</sup> T cells raised the question of whether IL-12 modulated their tissue trafficking patterns. CCR5 is typically expressed on effector memory T cells and guides memory T cells away from secondary lymphoid tissues into peripheral inflammatory lesions.<sup>19</sup> The chemotactic responsiveness of CD4<sup>+</sup>CD28<sup>−</sup> T cells to CCL5 was explored in a transwell system. Stable CD4<sup>+</sup>CD28<sup>−</sup> T-cell clones were generated from the peripheral blood of ACS patients.<sup>4</sup> A total of 46 CD4 T-cell clones were screened by RT-PCR for IL-12Rβ1 chain expression. Forty-one had abundant mRNA for IL-12Rβ1 and 23 of these coexpressed IL-12Rβ2 sequences; 5 CD4<sup>+</sup>CD28<sup>−</sup> T-cell clones lacked transcripts for IL-12Rβ1. IL-12Rβ1 could not be induced in these T-cell clones (data not shown). Thus clones did not express CCR5 in response to IL-12 and served as control cells in functional assays testing IL-12 effects (Figure 3).

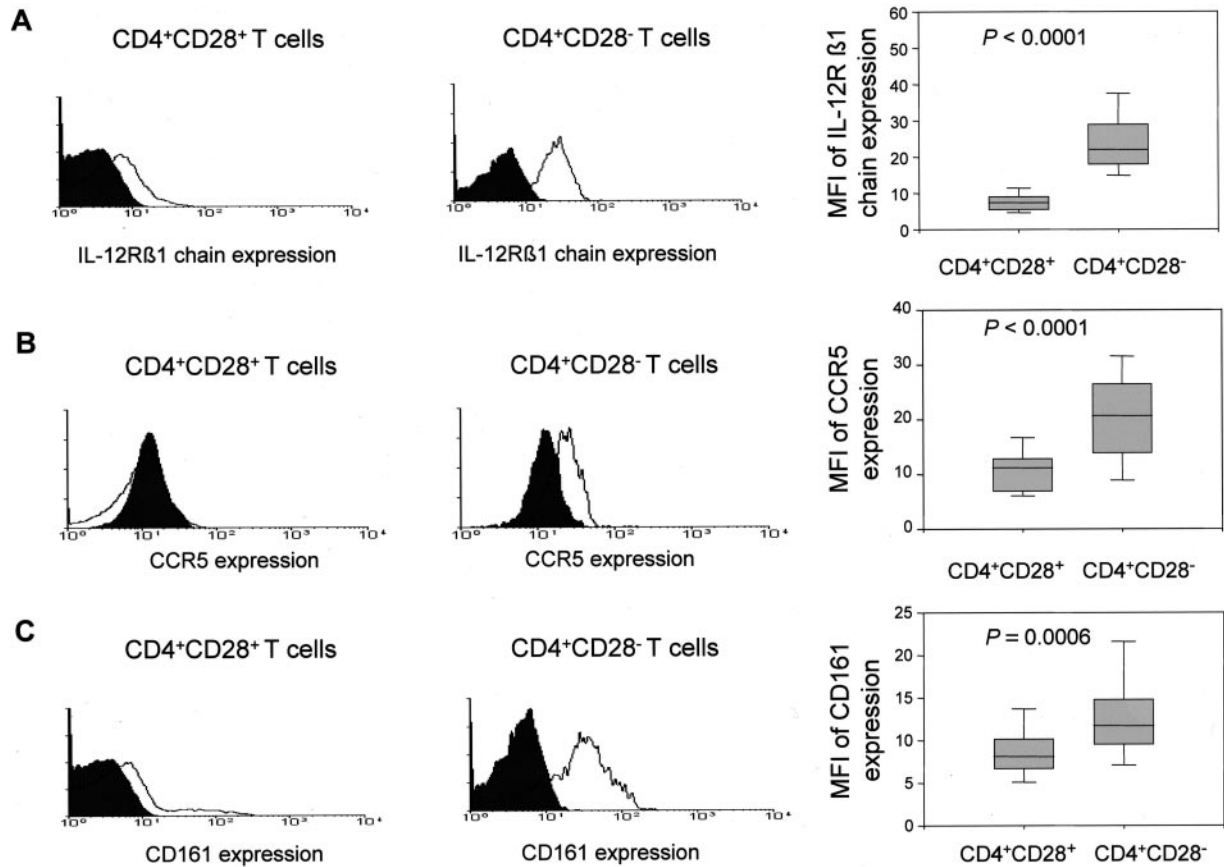
CD4<sup>+</sup>CD28<sup>−</sup> T cells responded to CCL5 by enhanced transmigration toward the chemokine source. The proportion of migrating CD4 T cells increased in a dose-dependent fashion and reached 20% for CCL5 concentrations of 50 ng/mL (Figure 4A). When preconditioned with IL-12, frequencies of CCL5 responsive T cells increased to 33%. Also, transendothelial migration was enhanced in the presence of CCL5. Pretreatment with IL-12 increased passage through the endothelial layer at all CCL5 concentrations. IL-12 had no effect on the trafficking pattern of CD4<sup>+</sup> T-cell clones lacking expression of IL-12R (Figure 4B).

Similar to T-cell clones, CD4 T-cell lines isolated from unstable plaque responded to IL-12 stimulation with a marked increase in CCL5-directed migration (Figure 5). With increasing doses of CCL5, a maximum of 6% of T cells could be attracted. When pretreated with IL-12, the rate of migrating T cells doubled to almost 12%.

### IL-12 Enhances Tissue Homing of CD4 T Cells *In Vivo*

The chemokine receptor expression profile and the trafficking behavior of IL-12-stimulated CD4<sup>+</sup>CD28<sup>−</sup> T cells suggested that IL-12 profoundly affects migration and tissue localization of IL-12-sensitive T cells. To establish a role for IL-12 in guiding T cells to inflammatory lesions *in vivo*, we examined the trafficking pattern of CD4 T-cell clones into atherosclerotic plaque. For these experiments, a novel animal model was used. Atherosclerotic plaques harvested by carotid endarterectomy were implanted into immunodeficient mice. Engraftment of the human tissue is accomplished after 7 days when mouse capillaries form anastomoses with human capillaries on the graft surface.<sup>18</sup> Although human CCL5 is cross-reactive on murine CCR5, mouse macrophages are mostly found in a granulation tissue around the human tissue,



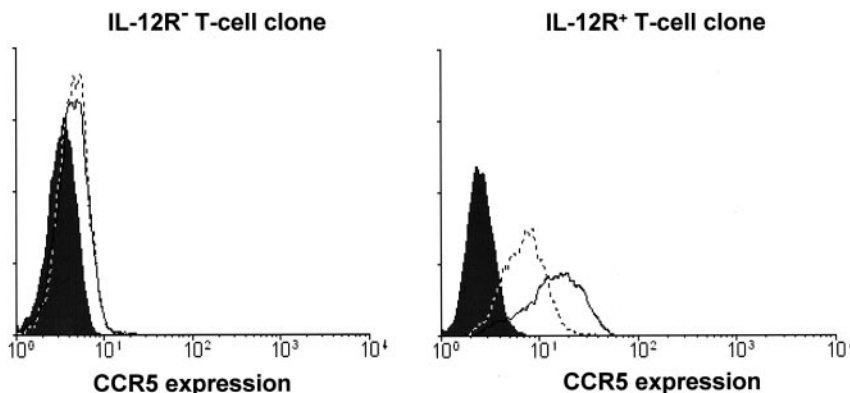


**Figure 2.** Expression of IL-12R and the IL-12-inducible molecules CCR5 and CD161 on circulating CD4<sup>+</sup> T cells in ACS. Freshly harvested peripheral blood T cells from 44 ACS patients were analyzed by multicolor flow cytometry for the expressions of CD4, CD28, and IL-12Rβ1 chain (A), CCR5 (B), and CD161 (C). In the representative histograms, isotype control stains are given as shaded areas; marker expressions on T cells are shown as solid lines (left and middle). Surface expression for each marker was determined as mean fluorescence intensity (MFI) and compared on CD4<sup>+</sup>CD28<sup>+</sup> and CD4<sup>+</sup>CD28<sup>-</sup> T cells (right). Results are given as box plots displaying medians with 25th and 75th percentiles as boxes and 10th and 90th percentiles as whiskers.

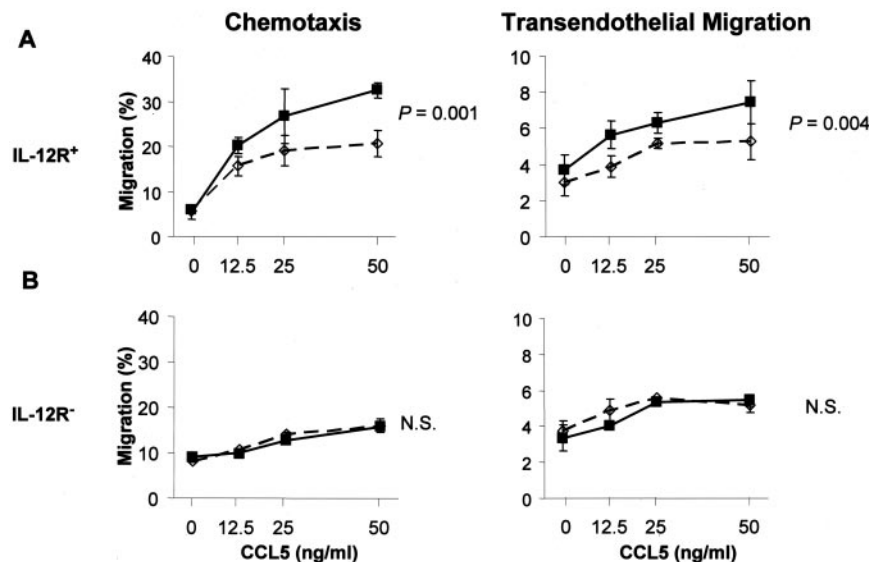
but not within the tissue graft, possibly because of incompatibility with human endothelial adhesion molecules. ED<sub>50</sub>s are approximately tenfold lower for human CCR5 responding to human RANTES as compared with mouse RANTES, giving human T cells a clear advantage for being recruited into the human graft. Although the model is a no-flow model, it is highly informative to study homing and effector functions of adoptively transferred cells.

To choose appropriate tissues for implantation, a total of 68 carotid plaque tissues were screened for the presence of

an inflammatory infiltrate. Thirty-two percent of the endarterectomies contained TCR-specific sequences indicating a T-cell infiltrate. Real-time PCR was used to quantify the transcription of CCL5 and MIP-1α, 2 CCR5-binding chemokines known to be produced in plaque tissue. As shown in supplemental Figure I, both chemokines correlated highly with the TCR load in the lesion ( $r=0.79$  for CCL5;  $r=0.68$  for MIP-1α). These data suggested that CCR5 expression on CD4 T cells may be a critical determinant in regulating their recruitment into the ather-



**Figure 3.** IL-12 responsiveness of CD4 T-cell clones depends on the combined expression of IL-12Rβ1 and β2 chains. A panel of 46 CD4<sup>+</sup>CD28<sup>-</sup> T-cell clones was established from 3 ACS patients. Individual T-cell clones were examined for the transcription of IL-12Rβ1 and IL-12Rβ2 genes, and biological responsiveness to IL-12 was analyzed by upregulation of CCR5. A representative example for a CD4 T-cell clone lacking transcripts for both IL-12R chains (left) and a CD4 T-cell clone expressing a combination of both IL-12Rβ chains (right) are shown. Shaded areas represent isotype staining controls. Dashed lines show CCR5 expression without IL-12 stimulation; solid lines show CCR5 expression with IL-12 stimulation for 48 hours.



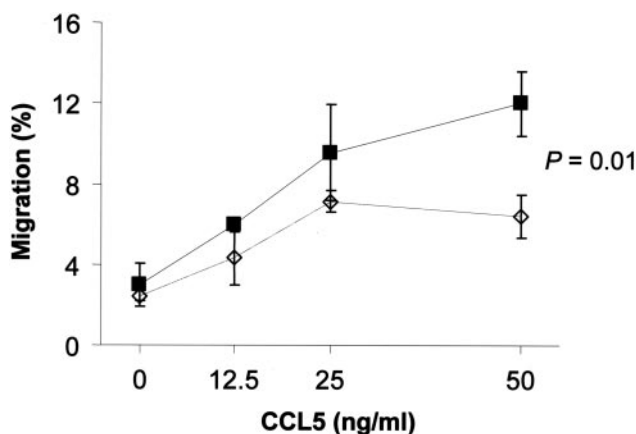
**Figure 4.** IL-12 enhances chemotactic responses and transendothelial migration of CD4 T cells toward CCL5. CD4<sup>+</sup>CD28<sup>-</sup> T-cell clones were established from ACS patients (see Figure 3), and the effects of IL-12 preconditioning (10 ng/mL) on CCL5-induced chemotaxis (left panels) and transendothelial migration (right panels) were tested. IL-12R-expressing (A) and IL-12R-negative (B) T-cell clones were compared. Results (mean±SD) for T cells preconditioned with IL-12 for 48 hours are given as solid lines/closed symbols, and T cells cultured without IL-12 are given as dashed lines/open symbols.

oma and that several chemokines participate in attracting CCR5<sup>+</sup>CD4<sup>+</sup> T cells into the plaque.

Endarterectomy tissues containing high levels of TCR and CCL5 transcripts from 5 patients were implanted into 10 mice. CD4<sup>+</sup>CD28<sup>-</sup> T-cell clones were labeled with a stable membrane dye, cultured with IL-12, and adoptively transferred into these human plaque mouse chimeras. After 48 hours, the human plaque tissues were explanted and analyzed for labeled T cells in the plaque. CCL5-negative plaque tissues from 5 donors were implanted into 10 mice and served as controls. Both sets of chimeras were injected with the identical CD4<sup>+</sup>CD28<sup>-</sup> T-cell clones labeled with PKH26. T-cell migration into the tissue was quantified by enumerating fluorescent T cells in tissue sections (Figure 6A). Labeled T cells consistently accumulated in CCL5<sup>+</sup> atheromas, mostly in tissue areas occupied by inflammatory infiltrate. Precon-

ditioning with IL-12 significantly enhanced the recruitment of adoptively transferred T cells, with doubling of the number of T cells invading the plaque tissue ( $P=0.008$ ). In plaques that lacked CCL5, adoptively transferred T cells were rarely detected. Likewise, IL-12 pretreatment could not enhance the migration of adoptively transferred T cells into such lesions ( $P=0.643$ ).

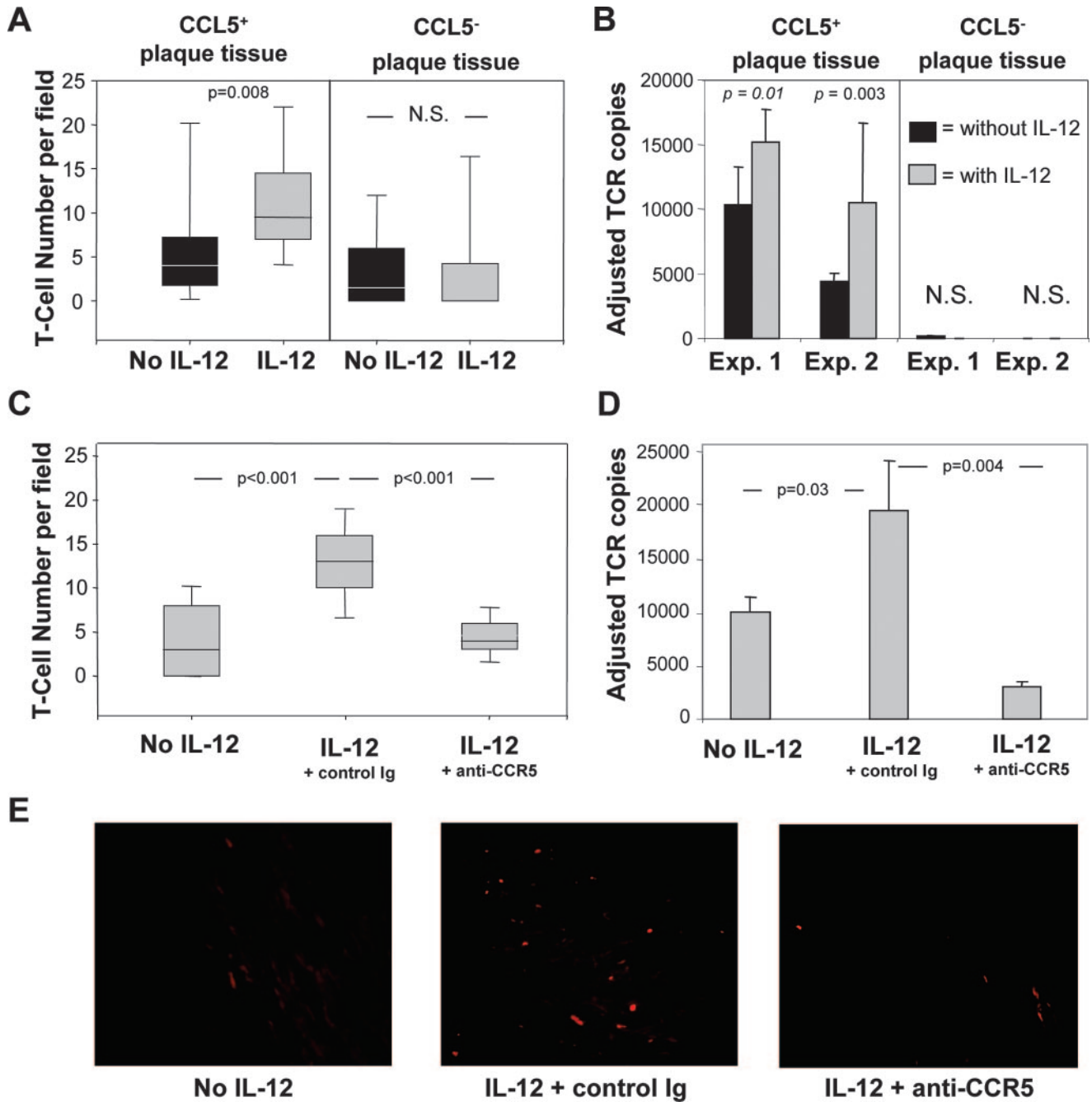
To provide further support for the role of IL-12 in regulating the tissue migration of plaque-infiltrating CD4 T cells, the density of adoptively transferred CD4 T cells in the atheroma was determined by quantitative PCR. Engrafted plaques were harvested 48 hours after T-cell injection. TCR-specific sequences were quantified by real-time PCR. Results from 4 representative experiments are shown in Figure 6B. TCR sequences were abundantly present in CCL5<sup>+</sup> atheroma. IL-12 preconditioning of transferred CD4 T-cell clones enhanced the tissue concentrations of TCR sequences significantly. CCL5<sup>-</sup> plaque tissues did not contain detectable levels of TCR sequences and were unable to attract any T cells, independent of IL-12 pretreatment. Blocking studies documented that the IL-12-mediated increase in homing to the atherosclerotic plaque was dependent on CCR5. Incubation of T cells with anti-CCR5 antibodies at the time of adoptive transfer completely inhibited the increase in T-cell accumulation (Figure 6C and 6E) and brought the tissue content of TCR mRNA even below baseline levels (Figure 6D). To add further support for IL-12 regulated T-cell trafficking to the inflamed atheroma, CD4<sup>+</sup>CD28<sup>-</sup> T-cell clones lacking IL-12R expression (see Figure 3) were adoptively transferred into chimeras engrafted with CCL5-rich atheroma tissue. As shown in supplemental Figure II, low numbers of adoptively transferred CD4<sup>+</sup>CD28<sup>-</sup>IL-12R<sup>-</sup> T cells were found in the grafts. IL-12 pretreatment left the trafficking pattern of such T cells unaffected. Together, these data confirmed the regulatory function of IL-12 in optimizing the tissue migration behavior of CD4 T cells into the atherosclerotic plaque.



**Figure 5.** IL-12 regulates chemotactic responsiveness of plaque-derived T cells. CD4<sup>+</sup> T-cell lines were established from fragments of unstable plaque captured in distal embolization protection devices as described in Figure 1. Chemotactic responsiveness to CCL5 was measured in a transwell system. Results are given as mean±SD. IL-12 pretreatment for 48 hours (solid line/closed symbols) enhanced T-cell migration as compared with T cells cultured without IL-12 (dashed line/open symbols).

## Discussion

A specialized subset of CD4<sup>+</sup> T cells, CD4<sup>+</sup>CD28<sup>-</sup> T cells, are enriched in the culprit lesions of ACS patients. In contrast



**Figure 6.** IL-12 conditioning enhances T-cell recruitment into carotid atheromas. SCID mice were implanted with CCL5-producing or CCL5-negative carotid atherectomy tissues. After engraftment, the chimeras were injected with fluorescent-labeled CD4<sup>+</sup>CD28<sup>-</sup> T-cell clones that had either been cultured with IL-12 for 48 hours or with medium alone. Carotid artery tissues were explanted 48 hours later. Numbers of fluorescent T cells were measured in tissue sections. A, The expression of TCR transcripts was determined by real-time PCR in cDNA generated from extracts of shock-frozen explants. B, Representative results for four experiments each using CCL5-producing and CCL5-negative plaques are shown. C and D, CD4<sup>+</sup>CD28<sup>-</sup> T cells were left untreated or were treated with IL-12 for 48 hours followed by incubation with either control immunoglobulin or 200  $\mu$ g of neutralizing anti-CCR5 antibody. Cells were adoptively transferred into chimeras engrafted with CCL5-producing human carotid artery plaque. T-cell infiltration into the graft was estimated by enumerating the number of fluorescent T cells (C) and by quantifying TCR transcripts (D). Representative images show infiltration of PKH26-labeled T cells in graft tissues (200-fold magnification) (E).

to common CD4 T cells, CD4<sup>+</sup>CD28<sup>-</sup> T cells respond to different regulatory signals. Receptor ligands and soluble factors triggering the activation of such T cells outside and within the atherosclerotic plaque potentially play a critical role in plaque destabilization. Here we report that CD4<sup>+</sup>CD28<sup>-</sup> T cells are constitutively responsive to IL-12, a

proinflammatory cytokine produced by activated DC and macrophages. IL-12 enhanced chemotaxis and transendothelial migration by upregulating the chemokine receptor CCR5. In vivo relevance of this mechanism was tested in human atheroma SCID mouse chimeras in which carotid artery plaques were implanted into SCID mice, and the homing of

adoptively transferred T cells was determined. Pretreatment of CD4<sup>+</sup>CD28<sup>-</sup> T cells with IL-12 before adoptive transfer facilitated migration to the engrafted human atheroma.

IL-12 is a powerful modulator of T-cell and NK cell function.<sup>20</sup> Bacterial infections and other means of DC activation rapidly induce IL-12.<sup>13</sup> On antigen recognition T cells upregulate IL-12R and thus become sensitive to DC modulation. By activating the STAT4 pathway,<sup>21</sup> IL-12 induces IFN- $\gamma$  production and guides the differentiation of T cells into potent Th1 effector cells.<sup>16</sup> Accordingly, genetic defects in the IL-12/IL-12R axis render individuals highly susceptible to infections, particularly intracellular pathogens.<sup>22</sup> Using its ability to regulate the differentiation and expansion of antigen-primed T cells, IL-12 has also been involved in promoting autoimmune responses.<sup>23</sup>

The surprising finding of this study is that CD4<sup>+</sup>CD28<sup>-</sup> T cells, in contrast to their CD4<sup>+</sup>CD28<sup>+</sup> counterparts, did not require antigen-priming to express functional IL-12R. Instead, they were constitutively IL-12 responsive. This rule held for T cells isolated from plaque debris captured in distal embolization protection devices and also pertained to circulating CD4 T cells from ACS patients where CD4<sup>+</sup>CD28<sup>-</sup> but not CD4<sup>+</sup>CD28<sup>+</sup> T cells were IL-12R<sup>+</sup>. Circumventing the need for antigen triggering to express IL-12R makes these cells rapidly responsive to activation of the innate immune system, comparable to NK cells, which are efficient effector cells even in an unprimed state. However, the expression of IL-12R on CD4<sup>+</sup>CD28<sup>-</sup> T cells also renders this T-cell subset dangerous, as they have escaped from tolerance control, set by the absolute need for antigen recognition before T cells invade peripheral tissue sites and release IFN- $\gamma$ .

The dichotomy between CD4<sup>+</sup>CD28<sup>-</sup> and CD4<sup>+</sup>CD28<sup>+</sup> T cells extends beyond homing patterns and includes several effector functions. CD4<sup>+</sup>CD28<sup>-</sup> T cells are delayed in cell cycle progression but are resistant to proapoptotic signals, produce high amounts of cytokines, in particular, IFN- $\gamma$  and TNF- $\alpha$ , and exhibit cytotoxic activity.<sup>24–26</sup> They are responsible for the increased constitutive IFN- $\gamma$  production seen in ACS patients.<sup>6</sup> The current study demonstrates the presence of IL-12-inducible genes CCR5 and CD161 on freshly harvested CD4<sup>+</sup>CD28<sup>-</sup> T cells. These data confirm that the T-cell pool in ACS is in a constant state of activation. This could be caused by a primary defect, eg, uncontrolled T-cell responsiveness, or a secondary defect, eg, excessive stimulation of the innate immune system. IL-12 is elevated in the circulation of ACS patients,<sup>27,28</sup> lending support to the model of chronic stimulation of hematopoietic phagocytic cells, such as DC and macrophages.

Whereas earlier studies had focused on T-cell effector function, the current study examined an alternative pathway that may have critical relevance for plaque instability, namely, the accumulation of effector T cells in the atheroma. The tissue trafficking of T cells is regulated through chemokine receptors. CCR5 is expressed on highly differentiated T cells of the memory effector type<sup>29</sup> and facilitates the transit from lymphoid organs to peripheral tissue sites.<sup>30–32</sup> CCL5 and MIP-1 $\alpha$ , both ligands for CCR5, are abundantly present in the plaque<sup>33</sup> and are produced by different cell types. In a series of carotid atherectomy samples, tissue CCL5 and

MIP-1 $\alpha$  expression correlated closely with the density of tissue-infiltrating T cells (supplemental Figure I), suggesting that the chemokine receptor CCR5 is a key regulator in bringing T cells to the inflamed atheroma.

In vivo, CD4 T cells could be exposed to IL-12 at 2 strategically important sites. IL-12 is abundantly expressed in the atheroma. However, IL-12 is also rapidly induced in phagocytic cells on host infection. IL-12 release within the plaque could regulate retention of CD4<sup>+</sup>CD28<sup>-</sup> T cells by upregulating CCR5. The plaque is an environment rich in the CCR5-binding chemokines CCL5 and MIP-1 $\alpha$ , and CCR5-expressing T cells would be trapped within the lesion. Also, it is possible that the binding of CCR5 ligands initiates signaling events that directly modulate T-cell function. Experiments shown here suggest that IL-12 exposure may even be more important when occurring outside of the atheromatous lesion; specifically, IL-12 pretreatment enhanced trafficking of CD4 T cells into engrafted human plaque. Classical sources of IL-12 are DC and other phagocytes after they have been triggered by macromolecules derived from infectious agents.<sup>34</sup> In this model, any infectious episode that causes IL-12 release by DC located in lymph nodes could immediately mobilize unstimulated CD4<sup>+</sup>CD28<sup>-</sup> T cells and redirect them from their storage place in the lymphoid tissue into CCL5- and MIP-1 $\alpha$ -producing peripheral tissue sites.

A large number of infectious microorganisms have been implicated as possible risk factors for coronary events.<sup>35,36</sup> Here we propose that IL-12 released from DC is 1 of the molecular mediators translating infection into increased plaque inflammation. In this model, a large spectrum of distinct infections could trigger plaque inflammation, even without being present in the vessel wall at all. The common denominator among such infectious triggers, cumulatively called “infectious burden,”<sup>35,36</sup> could be their ability to induce IL-12, leading to the detrimental redistribution of spontaneously IL-12-responsive effector T cells.

Data presented here support the notion that ACS patients have fundamental abnormalities in T-lymphocyte function. This is not limited to plaque-infiltrating T cells but extends to T cells stored in lymphoid tissues. The accumulation of CD4<sup>+</sup>CD28<sup>-</sup> T cells in these patients, with elevated frequencies easily detectable in the circulating blood,<sup>5</sup> signals defects in immunoregulation that have direct consequences for the process of plaque inflammation. The finding that CD4<sup>+</sup>CD28<sup>-</sup> T cells are spontaneously responsive to IL-12, reacting with enhanced transmigration into carotid lesions, places IL-12 in a key position for determining tissue trafficking patterns of end-differentiated effector lymphocytes. The central function of IL-12 lies in protecting the host from infections. In susceptible individuals, especially those who have high frequencies of CD4<sup>+</sup>CD28<sup>-</sup> T cells, this protective effect may paradoxically be associated with shifting CD4 T cells into peripheral inflammatory tissues, thereby threatening tissue integrity. These findings provide a rationale for exploring antigen-nonspecific means of immunosuppression to downregulate inflammatory responses in ACS.

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