IL-12 Inhibition of Endothelial Cell Functions and Angiogenesis Depends on Lymphocyte-Endothelial Cell Cross-Talk

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IL-12 Inhibition of Endothelial Cell Functions and Angiogenesis Depends on Lymphocyte-Endothelial Cell Cross-Talk

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In vivo IL-12-dependent tumor inhibition rests on the ability of IL-12 to activate a CD8-mediated cytotoxicity, inhibit angiogenesis, and cause vascular injury. Although in vivo studies have shown that such inhibition stems from complex interactions of immune cells and the production of IFN-γ and other downstream angiostatic chemokines, the mechanisms involved are still poorly defined. Here we show that IL-12 activates an anti-angiogenic program in Con A-activated mouse spleen cells (activated spc) or human PBMC (activated PBMC). The soluble factors they release in its presence arrest the cycle of endothelial cells (EC), inhibit in vitro angiogenesis, negatively modulate the production of matrix metalloproteinase-9, and the ability of EC to adhere to vitronectin and up-regulate ICAM-1 and VCAM-1 expression. These effects do not require direct cell-cell contact, yet result from continuous interaction between activated lymphoid cells and EC. We used neutralizing Abs to show that the IFN-inducible protein-10 and monokine-induced by IFN-γ chemokines are pivotal in inducing these effects. Experiments with nude mice, nonobese diabetic-SCID mice, or activated spc enriched in specific cell subpopulations demonstrated that CD4+ T, CD8+ T, and NK cells are all needed to mediate the full anti-angiogenic effect of IL-12. The Journal of Immunology, 2001, 166: 3890–3899.

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2 Address correspondence and reprint requests to Dr. Federico Bussolino, Istituto Ricerca e Cura del Cancro, s.p. 142, Km. 3,95, 10060 Candiolo (To), Italy. E-mail address: fbussolino@ircc.unito.it

3 Abbreviations used in this paper: MMP, metalloproteinase; EC, endothelial cells; TSA, transplantable mammary adenocarcinoma; BrdU, 5-bromodeoxyuridine; IP-10, IFN-inducible protein-10; MIG, monokine induced by IFN-γ; spc, mouse spleen cell; VEGF, vascular endothelial growth factor; NOD, nonobese diabetic.

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cells were cultured in DMEM (Sigma, St. Louis, MO) supplemented with 2 mM glutamine (Sigma), 10% FBS (Life Technologies, Paisley, U.K.), 100 U/ml of penicillin, and 100 µg/ml of streptomycin (Sigma). Microplasma contamination was periodically checked with a PCR kit (Molec-Tech, La Jolla, CA) with negative results. Human EC were isolated from umbilical cord veins, characterized, and grown in medium 199 (Sigma) containing 20% FBS as previously described (31). They were used at early passages (I-IV).

**Activation of scp**

Spc were obtained from normal and IFN-γ−/− BALB/c, C57BL/6, CD1 nude mice (Charles River, Calco, Italy), and nonobese diabetic (NOD) Lnsx scid/scid mice (The Jackson Laboratory, Bar Harbor, ME). In selected experiments scp were obtained from mice bearing a s.c. transplantable mammary adenocarcinoma (TSA) (17) that were treated, or not treated, with 0.1 µg of recombinant murine IL-12 (kindly provided by Michael Brunda, Hoffmann-La Roche, Nutley, NJ) i.p. daily for 5 or 10 days. Spc (2 × 10⁵/ml) were activated by overnight culture in RPMI 1640 supplemented with 10% FBS, 100 U/ml of penicillin, 10 µg/ml of streptomycin, and 2.5 × 10⁻³ M 2-ME (Sigma) (RPMI 1640 complete medium) containing 2 µg/ml Con A (Sigma) in the absence (activated scp) or the presence (activated scp-IL-12) of 10 ng/ml murine IL-12 (kindly provided by Michael Brunda, Hoffmann-La Roche, Nutley, NJ). In a few experiments scp were enriched in T and NK cells through passage on a nylon wool column as previously described in detail (32). Column-emerging cells were activated with Con A or with IL-12 (10 ng/ml) in the presence or the absence of adherent BALB/c peritoneal macrophages obtained by peritoneal washes of BALB/c mice as previously described (33). In other experiments depletion of CD4⁺, CD8⁺, and NK cells was performed using the MiniMACS magnetic separation system (Miltenyi Biotec, Bergsch Gladbach, Germany). BALB/c activated scp were first labeled with anti-CD4 (L3T4 mAb) or anti-CD8 (Lyt-2 mAb) and then with indirect goat anti-rat IgG microbeads (Miltenyi Biotec). C57BL/6 activated scp were first labeled with anti-CD4 (clone XMG 1.2; final dilution 10⁻² g/ml; PharMingen), then starved in medium 199 (Sigma) containing 1% FBS for 24 h, and 2.5 × 10⁻³ M 2-ME (Sigma; RPMI 1640 complete medium) were added to the wells in medium containing 2.5% human EC/well and allowed to attach for 24 h. The cells were then starved in medium 199 containing 1% FBS for 24 h, and IL-12 or VEGF-A₁₆₅ (R&D Systems) were added to the wells in medium containing 2.5% FBS every 2 days. Cells were counted on day 5 as previously detailed.

**Activation of human PBMC**

Human PBMC were from buffy coats of healthy blood donors obtained through the courtesy of Centro Transfusionale AVIS (Torino, Italy). Blood was washed once with cold PBS at 400 × g to remove plasma and platelets and then was centrifuged on Histopaque 1077 (Sigma) at 600 × g at 30 min at room temperature. Cells were collected at the interface, washed twice with PBS, and resuspended in RPMI 1640 complete medium. Cells (1.5 × 10⁶/ml) were activated for 24 h with Con A (1.5 µg/ml) in the absence (activated PBMC) or the presence (activated PBMC-IL-12) of 10 ng/ml human IL-12 (R&D Systems, Wiesbaden-Nordenstadt, Germany). Activated PBMC and activated PBMC-IL-12 were then, respectively, resuspended in RPMI 1640 complete medium alone or supplemented with 10 ng/ml IL-12.

**Coculture of EC and scp or human PBMC**

Coculture experiments were performed in six-well plate Transwell systems (0.4 µm; Falcon, Plymouth, U.K.) with mouse H.end cells (3 × 10⁶) or EC (6 × 10⁵) plated at the bottom of the wells. Murine scp (2 × 10⁵/ml) and human PBMC (1.5–3 × 10⁶/ml) were seeded onto the inserts. Activated scp and human PBMC were cultured in RPMI 1640 complete medium. Activated scp-IL-12 and activated PBMC-IL-12 were cultured in complete medium supplemented with 10 ng/ml IL-12. In a few cases cocultures were established up to 1 × 10⁶ cells and then starved in medium 199 supplemented with 1% FBS (31). Indicated concentrations of IL-12 or VEGF-A₁₆₅ were placed in the lower compartment of a Boyden chamber, and 1.25 × 10³ H.end or human EC were seeded in 48-well plates (1 × 10⁶/ml and 2.5 × 10⁻³ M 2-ME) and 2.5 × 10⁻³ M 2-ME (Sigma) were added to the wells in medium containing 2.5% human EC/well and allowed to attach for 24 h. The cells were then starved in medium 199 containing 1% FBS for 24 h, and IL-12 or VEGF-A₁₆₅ (R&D Systems) were added to the wells in medium containing 2.5% FBS every 2 days. Cells were counted on day 5 as previously detailed.

**Proliferation assay**

Proliferation of H.end and human EC in the cocultured system was measured by evaluating cell number by crystal violet staining (31). Briefly cells were washed twice with PBS, fixed with 2.5% glutaraldehyde for 20 min at room temperature, and stained with 0.5 ml crystal violet (0.1% in 20% acetic acid and read at 540 nm on a microplate reader (model HTS7000; Perkin-Elmer, Boston, MA). A calibration curve was set up with known numbers of cells, and a linear correlation between absorbance and cell counts was established up to 1 × 10⁶ cells. To assay mitogenic activity of IL-12, 10⁵/ml murine or human EC were seeded in 96-well plates (1 × 10⁶/ml and 2.5 × 10⁻³ M 2-ME) and 2.5 × 10⁻³ M 2-ME (Sigma) were added to the wells in medium containing 2.5% FBS every 2 days. Cells were counted on day 5 as previously detailed.

**Adhesion assay**

EC adhesion was assayed as previously described (34). Suspended cells in medium 199 containing 1% FBS were plated (3 × 10⁵) in 96-well plates coated with vitronectin (Sigma; overnight at 4°C, 25 µg/ml), washed, and then incubated at room temperature with 1% human serum albumin (Sigma) for 2 h. After 1-h incubation at 37°C cells were washed, fixed, stained with crystal violet, and processed as above.

**Chemotaxis assay**

Cell migration assay was performed using a 48-well microchemotaxis chamber (Neuroprobe, Pleasanton, CA). Polysixinylpolyrionidone-free polycarbonate filters (Nucleopore, Corning Costar, Cambridge, MA) with a pore size of 5 µm were coated with 1% gelatin for 10 min at room temperature and equilibrated in medium 199 supplemented with 1% FBS (31). Indicated concentrations of IL-12 or VEGF-A₁₆₅ were placed in the lower compartment of a Boyden chamber, and 1.25 × 10³ H.end or human EC were seeded in the upper compartment. Cells were allowed to migrate for 7 h at 37°C in a humidified atmosphere with 5% CO₂. The filter was then removed, and cells on the upper side were scraped off with a rubber policeman. Migrated cells were fixed in methanol, stained with Giemsa solution (Diff-Quick; Baxter Diagnostics, Rome, Italy), and counted from five random high power fields (magnification, ×100) in each well.

**Matrigel morphogenetic assay**

Matrigel (Collaborative Biomedical Products, Becton Dickinson, Milan, Italy; not purified from contaminant growth factors; 0.2 ml) was added to the insert compartment of a Boyden chamber, and 1.25 × 10³ H.end or human EC were seeded in the upper compartment. Cells were allowed to migrate for 7 h at 37°C in a humidified atmosphere with 5% CO₂. The filter was then removed, and cells on the upper side were scraped off with a rubber policeman. Migrated cells were fixed in methanol, stained with Giemsa solution (Diff-Quick; Baxter Diagnostics, Rome, Italy), and counted from five random high power fields (magnification, ×100) in each well.

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**Table 1. Effect of IL-12 on EC functions**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Proliferation (10⁵ cells)</th>
<th>Migration (cell number)</th>
<th>V-CAM-1 (positive)</th>
<th>ICAM-1 (positive)</th>
<th>E-Selectin (positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H.end</td>
<td>EC</td>
<td>H.end</td>
<td>EC</td>
<td>H.end</td>
</tr>
<tr>
<td>None</td>
<td>2.2 ± 1.2 ² ³</td>
<td>3.6 ± 1.4</td>
<td>24 ± 6</td>
<td>28 ± 5</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>VEGF-A₅</td>
<td>11.3 ± 4.1 ³ ⁴</td>
<td>15.4 ± 2.4</td>
<td>89 ± 15</td>
<td>124 ± 19</td>
<td>NT</td>
</tr>
<tr>
<td>TNF-α</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>IL-12</td>
<td>2.0 ± 1.8 ³ ⁴</td>
<td>3.5 ± 2.6</td>
<td>30 ± 9</td>
<td>16 ± 10</td>
<td>45 ± 7</td>
</tr>
</tbody>
</table>

² Quiescent cells were stimulated every 2 days with VEGF-A₁₆₅ or with murine or human IL-12, and cells were counted at day 5.
³ Cell migration was evaluated after 7-h stimulation by Boyden’s chamber technique.
⁴ Adhesion molecule expression was evaluated after 24 h (VCAM-1 and ICAM-1) or 6-h cell stimulation.
⁵ Mean ± SD of three experiments done in triplicate.

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3891

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FIGURE 1. Cocultures between EC and spc and human PBMC. The growth of H.end cells (A) and of human EC (B) was evaluated after 72 h of cocultures with differently treated spc or PBMC or with conditioned medium (C.M.) from cultures ofactivated spc-IL-12 and activated PBMC-IL-12. The mean ± SD of five experiments performed in triplicate are shown. * and §, p < 0.05 vs cocultures of EC with nonactivated spc and PBMC and with activated spc and activated PBMC, respectively, evaluated by Student-Neuman-Keuls test performed after ANOVA (A, F = 10.75; B, F = 17.08).

each well of a 48-well plate and incubated at 37°C for 30 min to allow gel formation. Human EC (2 × 10⁵/well) were plated onto Matrigel and cocultured with human PBMC. After 48-h incubation in 5% CO₂ humidified atmosphere at 37°C, the cell three-dimensional organization was examined under an inverted phase contrast photomicroscope (DM-IBM model; Leica Microsystems, Wetzlar, Germany) and then photographed. This Matrigel allows spontaneous in vitro angiogenesis (35).

* Gel zymography

MMPs activity was assayed by zymography of EC proteins (36). EC were lysed in 50 mM Tris, 300 mM NaCl, and 1% Triton X-100, pH 7.5, and 10 μg of proteins were separated by 10% SDS-PAGE impregnated with gelatin (1 mg/ml; Sigma) in nonreducing conditions. Gels were washed twice for 20 min each time with 50 mM Tris, and 2.5% Triton X-100, pH 7.5, and incubated overnight at 37°C in 40 mM Tris, 200 mM NaCl, 10 mM CaCl₂, and 0.02% NaN₃, pH 7.5, with or without 5 mM EDTA. Clear bands, identifying the positions of 72- and 92-kDa gelatinases, were visualized on the blue background after staining with 0.25% Coomassie blue R250 and destaining with 50% methanol and 10% acetic acid.

Expression of adhesion molecules

Single-cell suspensions of H.end or human EC (1 × 10⁵/ml) recovered from cocultures or adherent cell cultures stimulated with IL-12 or human or murine TNF-α (both from R&D Systems; 10 ng/ml) for appropriate times were respectively incubated in PBS containing 1% FBS (30 min in ice) with rat anti-mouse mAb against VCAM-1 (Clone M/K-2; 0.1 μg/sample), ICAM-1 (Clone KAT-1; 0.1 μg/sample; R&D Systems), E-selectin (Clone 10E9; 0.5 μg/sample; provided by E. Dejana, Istituto Mario Negri, Milan, Italy), or mouse anti-human mAbs against VCAM-1 (Clone 51-10C9; 0.1 μg/sample), ICAM-1 (Clone HA58; 0.1 μg/sample; PharMingen), and E-selectin (Clone 10C10; 0.2 μg/sample; R&D Systems). Cells were then washed twice with ice-cold PBS and labeled with appropriate FITC-conjugated secondary Abs (R&D Systems). The secondary Ab alone was used as a control. FACS analysis was performed with a FACSscan flow cytometer (FACS Calibur, Becton Dickinson).

Apoptosis assays

H.end and human EC apoptosis was studied by exposure in the outer membrane of phosphatidylserine assayed using an annexin V-FITC kit (Bender Medsystems, Wien, Austria) and propidium iodide staining by FACS analysis. The two annexin V-positive quadrants of the analysis were taken as histone-associated DNA fragments with a Cell Death Detection ELISA kit (Roche, Mannheim, Germany). The absorbance was measured at 405 nm with a microplate reader (Perkin-Elmer). DNA laddering was studied on 3% agarose gel (Bio-Rad, Hercules, CA) containing ethidium bromide (1.8%) to visualize DNA fragmentation.

Cell cycle analysis

Mice bearing TSA tumor were treated with vehicle or with 0.1 mg of propidium iodide (100 μg/ml; Sigma) containing 5 μg/ml pancreatic RNase (Stratagene) for 20 min at 37°C. After gating out cellular aggregates and debris, propidium iodide fluorescence was measured using a FACSscan flow cytometer, and cell cycle analysis was performed with the Mod Fit LT program (Verity Software House, Topsham, ME).

DNA synthesis was measured by 5-bromodeoxyuridine (BrdU) uptake and compared with DNA content as determined by propidium iodide uptake (38). Briefly, adherent cells were incubated with 30 μM BrdU (Sigma; 1 h at 37°C). Detached cells were fixed as described above, washed, and suspended in PBS. Cells were then counted in 1 ml of 2 N HCl for 20 min at room temperature and then neutralized with 1 ml of 0.1 M Na₂B₄O₇ for 5 min at room temperature. The cells were centrifuged and incubated (10 min at 45°C) in 2 ml of PBS containing 5% Tween 20 and 0.1% BSA (Sigma), then centrifuged and incubated with 10 μg of fluoresceinated mAb anti-murine antibody (7.5 M; pH 7.5). Ethanol-precipitated DNA pellets were treated with pancreatic RNase (200 ng for 15 min at 37°C) in Tris-borate EDTA buffer. Samples were electrophoresed on an agarose gel (Bio-Rad, Hercules, CA) containing ethidium bromide (1.8%) to visualize DNA fragmentation.

FIGURE 2. Growth of murine H.end cells cocultured with spc from TSA mammary adenocarcinoma-bearing BALB/C mice treated with systemic IL-12. Mice bearing TSA tumor were treated with vehicle or with 0.1 mg of murine IL-12 i.p. daily for 5 or 10 days. The results from 72-h cultures are expressed as the mean ± SD of three animals. Student’s t test was used for evaluation of statistical significance.
Both cells, while the addition of TNF-α abated by activated PBMC IL-12.

B by coculturing human PBMC with human EC (Fig. 1 shown) and peaked at 72 h (Fig. 1 in this case, inhibition was already evident after 24 h of coculture (data not found in the presence of 10 ng/ml IL-12. In this case, stronger inhibition was directly proportional to the amount of IL-12. The stronger inhibi-

ion of spc from mice bearing TSA tumor and treated systemically with IL-12 to reduce H.end proliferation was tested. A significant im-

pairment of proliferation was found in cocultures with spc from mice bearing TSA adenocarcinoma that received 10 daily injections of 100 pg of IL-12 (Fig. 2).

Activated spc-IL-12 and activated PBMC-IL-12 arrest the cell cycle of EC

The observed inhibition of EC by mouse activated spc-IL-12 and human activated PBMC-IL-12 could depend either on cell death or cell cycle arrest. As no toxic effect in the cocultures (i.e., lactate dehydrogenase release and trypan blue exclusion) were observed, it was first evaluated whether the reduced number of EC was due to an increase in their apoptosis. In contrast with etoposide (40), which markedly increased human EC apoptosis (positive control), no enhanced EC apoptosis was found after 72-h culture with hu-

man activated PBMC-IL-12 (Table II). This result was assessed with four independent methods, namely by evaluating the binding of fluoresceinated annexin V to phosphatidylserine expressed in the outer membrane, the presence of nucleosome by measuring histone-associated DNA fragments, the presence of a hypodiploid

Table II. Effect of human PBMC cells stimulated by IL-12 on EC apoptosis

<table>
<thead>
<tr>
<th>EC Cocultured with</th>
<th>Annexin V staining (%) positive cells</th>
<th>Nuclease ELISA (OD at 405 nm)</th>
<th>Propidium iodide staining (% hypodiploid cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.61 ± 0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.401 ± 0.04</td>
<td>3.19 ± 1.20</td>
</tr>
<tr>
<td>PBMC</td>
<td>0.96 ± 0.50</td>
<td>0.352 ± 0.05</td>
<td>5.70 ± 1.89</td>
</tr>
<tr>
<td>Activated PBMC</td>
<td>1.23 ± 0.54</td>
<td>0.36 ± 0.02</td>
<td>7.81 ± 2.54</td>
</tr>
<tr>
<td>Activated PBMC IL-12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.74 ± 0.77</td>
<td>0.34 ± 0.07</td>
<td>8.06 ± 1.77</td>
</tr>
<tr>
<td>Etoposide 5 μM</td>
<td>17.4 ± 1.56</td>
<td>2.68 ± 0.12</td>
<td>53.12 ± 9.50</td>
</tr>
</tbody>
</table>

<sup>a</sup> Human EC were cocultured with 2 × 10<sup>6</sup> human PBMCs for 72 h in transwells.

<sup>b</sup> Mean ± SD of three measures in one experiment representative of three independent experiments.

<sup>c</sup> IL-12 was used at 10 ng/ml.

Although conditioned medium from activated spc-IL-12 was not sufficient to mimic the inhibition observed in the cocultures (Fig. 1), arrest of either mouse H.end or human EC proliferation did not require direct cell contacts because it occurred when the two cell types were separated by a semipermeable membrane.

IL-12 impairs the in vivo growth of several murine tumors as well as that of TSA adenocarcinoma by inhibiting angiogenesis and causing vascular necrosis (16, 17, 39). To evaluate whether this in vivo situation is mirrored by our culture system, the ability of spc from mice bearing TSA tumor and treated systemically with IL-12 to reduce H.end proliferation was tested. A significant im-

pairment of proliferation was found in cocultures with spc from mice bearing TSA adenocarcinoma that received 10 daily injections of 100 pg of IL-12 (Fig. 2).

Activated spc-IL-12 and activated PBMC-IL-12 arrest the cell cycle of EC

The observed inhibition of EC by mouse activated spc-IL-12 and human activated PBMC-IL-12 could depend either on cell death or cell cycle arrest. As no toxic effect in the cocultures (i.e., lactate dehydrogenase release and trypan blue exclusion) were observed, it was first evaluated whether the reduced number of EC was due to an increase in their apoptosis. In contrast with etoposide (40), which markedly increased human EC apoptosis (positive control), no enhanced EC apoptosis was found after 72-h culture with hu-

man activated PBMC-IL-12 (Table II). This result was assessed with four independent methods, namely by evaluating the binding of fluoresceinated annexin V to phosphatidylserine expressed in the outer membrane, the presence of nucleosome by measuring histone-associated DNA fragments, the presence of a hypodiploid

Table III. Analysis of cell cycle in murine microvascular H.end cells and human EC<sup>a</sup>

| Cell cocultured with | Cell cycle analysis were performed on H.end cells or human EC cells respectively cocultured with murine or human lymphomononuclear cells for 72 h. At the end of cocultures cells were labeled with BrdU, stained with fluorosceinated Ab anti-BrdU and propidium iodide, and then analyzed by FACS.

<sup>b</sup> Mean ± SD of three samples from one typical experiments of three.

<sup>c</sup> spc and PBMC were respectively used with H.end and human EC.

<sup>d</sup> Human or murine IL-12 were used at 10 ng/ml.
cell population by propidium iodide staining (Table II), and cytosolic DNA laddering (data not shown). No apoptosis increase was also observed after 24- and 48-h cocultures of activated spc-IL-12 and H.end cells (data not shown). DNA synthesis in human EC and H.end cells was measured by evaluating BrdU uptake during S phase compared with DNA content by FACS analysis. Table III shows that a reduced number of human EC entered S phase after coculture for 72 h with human activated PBMC-IL-12. Similar results were obtained with H.end cells cocultured with activated spc-IL-12 (Table III).

Activated spc-IL-12 and activated human PBMC-IL-12 inhibit EC adhesion

As adhesion to extracellular matrix is crucial for survival of EC and nascent vessels (41), and impairment of integrin-mediated endothelial adhesion may even result in tumor regression (42, 43), the effect of activated spc-IL-12 and PBMC-IL-12 on adhesive properties of EC was next evaluated. A dramatically reduced capacity of murine and human EC to adhere to vitronectin, the ligand of αvβ3 integrin (44) was found; by contrast, no modulation of EC adhesion ability was found following their culture with conditioned medium from activated spc-IL-12 or PBMC-IL-12 cultured alone (Fig. 3).

Activated spc-IL-12 and activated human PBMC-IL-12 regulate adhesion molecule expression

In mice treated with systemic IL-12, the expression of VCAM-1 and, to a lesser extent, of ICAM-1 is consistently increased on EC lining tumor capillaries (13, 17). Our cocultures confirm these results. Factors released by both human and murine activated spc-IL-12 and activated PBMC-IL-12 increase the expression of VCAM-1 and ICAM-1 (Fig. 4), but not that of E-selectin (data not shown) on human EC and mouse H.end cells, respectively.

Activated PBMC-IL-12 impair in vitro angiogenesis of human EC

To gain an insight into the effect of IL-12 on angiogenesis, human EC were cultured on a three-dimensional matrix protein gel (Matrigel) (45–47), where they gave rise spontaneously to an organized meshwork of anastomosing cord-like structures (Fig. 5A). Although coculture with PBMC or activated PBMC did not modify the EC angiogenic ability (Fig. 5, B and C), their coculture with activated PBMC-IL-12 dramatically inhibited it. By failing to establish contacts to neighboring cells, EC were unable to form continuous tubes (Fig. 5D).
Production of MMPs by EC is a crucial event during the progression phase of angiogenesis. It is characterized by cell sprouting and matrix remodeling and regulates in vitro angiogenesis (10, 48). To assess the production of MMPs by cocultured human EC, the presence of the bioactive proteases was assessed by zymography in EC lysate. We found that EC did not show constitutive production of MMP-9 (97 kDa). The gelatinolytic activity appeared after co-culture with PBMC, but was not modified by activated PBMC. In contrast, the proteolytic activity was consistently reduced in co-culture with activated PBMC-IL-12. MMP-2 (72 kDa) was present in untreated EC, but its production was not affected by the cocultures (Fig. 6).

**Cell populations responsible for the inhibition of EC proliferation**

To define the cell population mostly responsible for the inhibition of EC proliferation, activated spc-IL-12 from variously immunodeficient mice were first tested. Activated spc-IL-12 from nu/nu mice lacking T cell functions and displaying enhanced NK activity (49) displayed a reduced ability to inhibit mouse H.end growth (36% inhibition) compared with normal mice (70% inhibition). Activated spc-IL-12 from NOD-SCID mice exhibiting low NK

![Figure 5](http://www.jimmunol.org/)

**Figure 5.** Formation of tube-like structures by human EC cocultured with PBMC. Human EC (2 x 10^4) were plated onto Matrigel and cocultured with human PBMC. After 48-h incubation the cell three-dimensional organization was examined under an inverted phase contrast photomicroscope. A, EC alone; B, EC cocultured with PBMC; C, EC cocultured with activated PBMC; D, EC cocultured with activated PBMC-IL-12; E, EC cocultured with activated PBMC-IL-12 in presence of anti-MIG Ab; F, EC cocultured with activated PBMC-IL-12 in the presence of anti-IP-10 Ab; G, EC cocultured with activated PBMC-IL-12 in the presence of anti-MIG and anti-IP-10 Abs; H, EC cocultured with activated PBMC-IL-12 in the presence of irrelevant Ig. Magnification, x10. Results are representative of three independent experiments.

![Figure 6](http://www.jimmunol.org/)

**Figure 6.** MMP-2 and MMP-9 production by human EC cocultured with human PBMC. Cell lysates (30 μg) from EC alone or cocultured for 72 h with activated PBMC or activated PBMC-IL-12 were analyzed by gelatin zymography as detailed in Materials and Methods. The MMP-9 (upper band) and MMP-2 (lower band) were located at ~97 and 72 kDa, respectively. Results are representative of two independent experiments.
activity and defective for macrophage function (50) did not impair the H.end proliferation rate, while activated spc-IL-12 from NOD/Lt-β/β mice, displaying normal immune functions, inhibited H.end proliferation as did those from normal mice (Table IV).

In other experiments, activated spc-IL-12 were selectively depleted of CD4+ , CD8+ , or NK cells using specific mAbs and the MiniMACS magnetic separation system (Miltenyi Biotec; Table V). The absence of CD4+ , CD8+ , NK, and macrophages during cocultures partially decreased the inhibition of H.end proliferation. In contrast, spc passed through a nylon wool column had a reduced inhibitory effect on H.end proliferation, which was reversed by addition of peritoneal macrophages (Table V). Together these results strongly suggest that distinct activated spc-IL-12 subpopulations play a role in the inhibition of EC proliferation.

Factors leading to the inhibition of EC proliferation

In many experimental systems in vivo the anti-tumor activity of IL-12 appears to rest on its ability to induce downstream production of IP-10 and MIG as a consequence of its induction of IFN-γ (13, 17, 21, 23, 51). To evaluate the downstream mediators involved in the inhibition of EC in the cocultures with activated spc and activated PBMC, in all cases this inhibition was markedly and significantly amplified by the presence of activated spc-IL-12 from IFN-γ-activated mice (Table IV).

Discussion

The experiments presented here show that both spc and human PBMC activated by Con A in the presence of IL-12 markedly inhibit the proliferation, matrix adhesion, ability to differentiate into tube-like structure, and expression of VCAM-1 and ICAM-1 of human and murine EC. The strength of this inhibition makes it the in vitro equivalent of the marked anti-angiogenic and angio-static activity of IL-12 observed in vivo (13, 17, 18, 22–25). Use of a Transwell to impede physical contact between lymphoid cells and EC allowed investigation of the inhibiting factors released in the medium. IL-12 alone had no effect on EC functions, whose inhibition, in effect, stems from factors released by activated spc-IL-12 and activated PBMC-IL-12. Normal, nonactivated spc or PBMC are devoid of this inhibitory activity or even enhance EC proliferation. In a few cases a minor inhibition was observed in the presence of activated spc and activated PBMC. In all cases this inhibition was markedly and significantly amplified by the presence of IL-12, suggesting that this cytokine specifically induces an anti-angiogenic program in human and murine lymphoid cells. Murine H.end cells are a suitable model for investigating the activities of microvascular EC (28–30, 52). Their growth inhibition as well as that of human EC in the presence of factors released by activated spc-IL-12 and activated PBMC-IL-12 does not correlate with a major increase in their apoptosis, but with their delayed entry into S phase. This finding fits well in recent data showing that tumor vessels have a diminished proliferation index after treatment with IL-12 (25). Reduced proliferation of EC was associated with a decrease in their ability to adhere to vitronectin, the ligand of α5β1 integrin (44). In vivo adhesion of EC to this ligand provides anti-apoptotic signals to nascent vessels (42, 53) through suppression of p53 and p53-inducible cell cycle inhibitor p21WAF1/CIP1 and elevation of the Bcl-2:Bax ratio (54). Furthermore integrins containing αα subunit mediate the progression through G1 phase by protracted activation of Jun NH2-terminal kinase (55). Coculture of EC with activated spc-IL-12 and activated PBMC-IL-12 may first result in changes in α5β1 affinity for the ligand.

Table IV. Effect of spc from immunodeficient mice on H.end growtha

<table>
<thead>
<tr>
<th>Mice from Which spc Were Obtained</th>
<th>Activated spc</th>
<th>Activated spc-IL-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>20.0 ± 2.3a</td>
<td>6.3 ± 1.2a</td>
</tr>
<tr>
<td>BALB/c IFNγ-/-</td>
<td>22.2 ± 2.1</td>
<td>13.7 ± 1.1b</td>
</tr>
<tr>
<td>nu/nu mice</td>
<td>21.2 ± 1.5</td>
<td>14.7 ± 0.7a</td>
</tr>
<tr>
<td>NOD-SCID</td>
<td>18.3 ± 1.6</td>
<td>14.9 ± 1.5</td>
</tr>
<tr>
<td>NOD/Lt−/−</td>
<td>19.1 ± 2.0</td>
<td>8.3 ± 1.8</td>
</tr>
</tbody>
</table>

a Activated spc or activated spc-IL-12 (2 × 10⁶/ml) were cocultured for 72 h with 3 × 10⁶ H.end cells.

b EC number (× 10⁶) expressed as the mean ± SD of three independent experiments. ANOVA, F = 32.16, p < 0.05 vs activated spc (Student-Neuman-Keuls test).

c Anti-IFN-γ, anti-IP-10, and anti-MIG mAbs were added to the cocultures alone (Fig. 7) or in combination with anti-TNF-α or anti-GM-CSF mAbs (data not shown) at the beginning or every 12 h only partially abolished the inhibition. Moreover, a partial inhibition of H.end proliferative ability was still evident in the presence of activated spc-IL-12 from IFN-γ-activated mice (Table IV).

d Macrophages depleted by passage on nylon wool column (2 × 10⁶) were layered on mouse peritoneal macrophages (2 × 10⁶/cm²) and incubated overnight in RPMI 1640 complete medium containing 2 µg/ml Con A in the absence or in the presence of IL-12 as detailed in Materials and Methods.

Table V. Effect of spc sub-population on murine EC growth in coculturea

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>None</th>
<th>Con A + IL-12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC number</td>
<td>(1 × 10⁶)</td>
</tr>
<tr>
<td>spc</td>
<td>18.0 ± 2.3b</td>
<td>5.3 ± 1.2a</td>
</tr>
<tr>
<td>Nylon wool spc</td>
<td>17.0 ± 1.2</td>
<td>12.4 ± 0.3*§</td>
</tr>
<tr>
<td>Nylon wool spc supplemented with macrophagesd</td>
<td>20.4 ± 0.2</td>
<td>7.2 ± 3.0a</td>
</tr>
<tr>
<td>spc depleted of CD4+ cells</td>
<td>18.3 ± 1.8</td>
<td>16.4 ± 1.8§</td>
</tr>
<tr>
<td>spc depleted of CD8+ cells</td>
<td>17.2 ± 0.8</td>
<td>11.4 ± 2.1*§</td>
</tr>
<tr>
<td>spc depleted of NK cells</td>
<td>21.1 ± 1.1</td>
<td>13.0 ± 0.8*§</td>
</tr>
</tbody>
</table>

a spc (2 × 10⁶/ml) were activated as detailed in Materials and Methods and cocultured for 72 h with H.end cells (3 × 10⁶).
b EC number (× 10⁶) expressed as the mean ± SD of three independent experiments. ANOVA, F = 67.51. c and §, respectively, indicate p < 0.05 vs unstimulated cells and vs activated Ly-IL-12 (Student-Neuman-Keuls test).
c spc (2 × 10⁶) were layered on mouse peritoneal macrophages (2 × 10⁶/cm²) and incubated overnight in RPMI 1640 complete medium containing 2 µg/ml Con A in the absence or in the presence of IL-12 as detailed in Materials and Methods.
d Macrophages depleted spc by passage on nylon wool column (2 × 10⁶) were layered on mouse peritoneal macrophages (2 × 10⁶/cm²) and incubated overnight in RPMI 1640 complete medium containing 2 µg/ml Con A in the absence or in the presence of IL-12 as detailed in Materials and Methods.
the matrix ligand, followed by impairment of EC adhesion machinery and cell cycle arrest. Of interest, tumor-associated blood vessels of colon carcinoma cells transduced with IL-12 have reduced expression of α5β1 integrin even when injected in IFN-γ−/− mice (15). The inhibitory effect of activated PBMC-IL-12 on EC adhesion may explain their inability to differentiate into tube-like structures when grown on a three-dimensional gel of extracellular structures when grown on a three-dimensional gel of extracellular matrix. The failure of EC to engage extracellular matrix proteins through α5β1 integrin inhibits in vitro angiogenesis (56, 57).

Besides these effects on EC proliferation and matrix adhesion, activated spc-IL-12 and activated PBMC-IL-12 up-regulated the expression of VCAM-1 and ICAM-1 on EC surface. These adhesion molecules are involved in lymphocyte recruitment (58) and may be up-regulated in tumor vessels of mice receiving systemic IL-12 (13, 17). Additional impairment of tumor vasculature may also occur when lymphoid cells are allowed to touch endothelium, as occurs in vivo (59).

Investigation of the nature of the inhibitory factors responsible for these effects exerted by activated spc-IL-12 and activated PBMC-IL-12 showed that anti-IP-10 and anti-MIG Abs greatly reduced the inhibition of EC proliferation, adhesion, and in vitro angiogenesis. IP-10 and MIG are IFN-γ-induced chemokines that play a crucial role in the anti-tumor effect of IL-12. Their expression is up-regulated in tumors growing in IL-12-treated mice (13, 17, 23, 25), while their neutralization by specific Abs prevents the curative effect of IL-12 (21). They also inhibit the chemotaxis of EC and the angiogenesis induced by fibroblast growth factor 2 or by IL-8 in vivo (26). These effects appear to be mediated by their interaction with heparan sulfates (60) rather than by their ability to interact with CXC chemokine receptor 3. Several reports have demonstrated that this receptor is absent or very poorly expressed on human EC (61–64), while one study found evidence of its expression in murine EC (65). Human microvascular EC from dermis also express CXC chemokine receptor 3. However, the active concentration of IP-10 (5 × 10−7 M) is too high to be reached in vivo (64). Impairment of EC functions requires a continuous interaction between activated spc-IL-12 and activated PBMC-IL-12 and EC, as shown by the lack of inhibitory activity of conditioned medium from both activated spc-IL-12 and activated PBMC-IL-12 (Figs. 1 and 3) and of recombinant MIG and IP-10 in the absence of activated lymphoid cells (M. Strasly, unpublished observations).

In our murine cocultures established in the presence of IL-12 both lymphoid cells and EC expressed MIG and IP-10 (F. Cavallo, unpublished observations). This expression is mostly due to the high titers of IFN-γ elicited by IL-12 on lymphoid cells (66). The influence of lymphoid cells on EC functions thus rests on a ping-pong of soluble signals of lymphoid and endothelial origin. A possible scenario emerging from our data is that MIG and IP-10 do not directly cause the inhibition of EC growth. Instead, MIG and IP-10 of both endothelial and lymphoid origins appear to act primarily on T and NK cells. Following this additional signal, lymphoid cells activate a sustained release of factors that cause EC growth arrest. Depletion of T and NK cells abolishes the inhibitory activity of activated spc-IL-12. This scenario is also supported by in vivo data from Tosato’s group underlying the importance of NK and T cells in the anti-angiogenic loop triggered by IL-12 (63, 66). Removal of the inhibitory activity of activated spc-IL-12 by either anti-MIG or anti-IP-10 mAb suggests that these chemokines also have an independent effect, as reported in other systems (67). Besides their overlapping redundant functions, MIG and IP-10 may trigger unique intracellular signals that differentiate their activity. In vivo, the different patterns of MIG and IP-10 expression suggest that they may trigger nonredundant functions (67). Another possibility connected to the redundancy of the chemokine/cytokine system is that other molecules produced in our coculture system might interact with CXC receptor 3 and modify its sensitivity to MIG or IP-10.

Moreover, MIG and IP-10 do not seem to be the sole molecules that play a role in inducing the inhibition of EC functions in our cocultures. Actually, recombinant MIG and IP-10 are necessary, but not sufficient, for the elicitation of the anti-angiogenic activity, and other molecules appear to be needed.

The antitumor activity of IL-12, including inhibition of angiogenesis, is largely dependent on its ability to induce IFN-γ released by NK cells (22, 23, 25, 27) and to activate a perforin-mediated cytolytic pathway (68, 69). Surprisingly, our data obtained with neutralizing anti-IFN-γ mAb and with spc from IFN-γ−/− mice showed that in the absence of IFN-γ the inhibition of EC proliferation is not completely abrogated. This suggests that other molecules may replace IFN-γ in our system. Germane to our observation is the finding that in IFN-γ−/− mice, IL-12 is still able to induce an effective rejection of tumor cells engineered to release IL-12 (15). The presence of IL-12 during the activation of spc and PBMC is critical for the induction of their anti-angiogenic program. The way in which activated spc-IL-12 and activated PBMC-IL-12 inhibit EC is distinct from that of endotoxin-primed PBMC. Here, EC undergo cycle arrest and apoptosis through a pathway that requires PBMC-EC contact, expression of TNF-α on the lymphoid cell surface and TGF-α secretion (70). Similarly to endotoxin-primed PBMC, PBMC activated by Con A adhere to EC and induce their detachment from the matrix, but not their apoptosis (71).

The fact that IL-12 reduces angiogenesis in SCID mice, NK cell-deficient beige mice, and nude mice (22) indicates that its inhibition of neoangiogenesis is not necessarily mediated by a single immune cell population, although NK recruitment and cytotoxicity are needed (63). In our cocultures, total inhibition of EC growth required cooperation between two cell populations, each necessary but not in itself sufficient. Because both spc and human PBMC require prior activation by Con A before IL-12 priming, it would seem that the anti-angiogenic effect of IL-12 is not directly
activated by IL-12 on the cells that constitutively express the IL-12 receptor. Con A activation presumably up-regulates expression of the inducible IL-12 B2 receptor on T cells. Indeed, the T helper functions appear to form the basis of IL-12-mediated ischemic-hemorrhagic rejection of tumors in vivo. EC can no longer sustain neoangiogenesis and as-expression (72).

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These findings suggest that an early circuit triggered by IL-12 requires a ping-pong of soluble signals (such as MIP-10 and IP-10) between spc and EC, whose outcome is functional impairment of EC activities. EC can no longer sustain neoangiogenesis and assume a phenotype more prone to tissue invasion by leukocytes that amplify vascular damage in vivo (73). These alterations of EC functions appear to form the basis of IL-12-mediated ischemic-hemorrhagic rejection of tumors in vivo.

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