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Interleukin-12-Induced Adhesion Molecule Expression in Murine Liver

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Systemically administered interleukin (IL)-12 causes liver inflammation in mice characterized by Kupffer cell proliferation and hypertrophy, hepatocyte necrosis, and multifocal accumulations of leukocytes in the hepatic parenchyma and around portal tracts and central veins. We have used both immunohistochemical staining and radiolabeled antibody quantitation to examine adhesion molecule expression in the livers of mice dosed daily with murine IL-12. Cells infiltrating livers of IL-12-treated mice were primarily mononuclear leukocytes expressing LFA-1, VLA-4, MAC-1, and CD18 adhesion molecules but little L-selectin. Kupffer cells constitutively expressed LFA-1 and smaller amounts of MAC-1, and high levels of ICAM-1 were constitutively expressed by liver sinusoidal lining cells, portal tract, and central vein endothelia. With IL-12 treatment, existing ICAM-1 expression was up-regulated and *de novo* expression occurred along bile duct epithelia. VCAM-1 levels were dramatically increased, with induced expression occurring along portal tract and central vein endothelia and scattered bile duct epithelial cells and in aggregations of cells in perivascular areas and the liver parenchyma. Although constitutive expression of E- and P-selectin was negligible, IL-12 induced a moderate rise in E-selectin levels. These increases in adhesion molecule expression may have implications for the therapeutic use of IL-12, especially in patients with liver disease or autoimmune conditions where augmented adhesion molecule expression may be critical to disease pathogenesis. *(Am J Pathol 1998, 152:457–468)*

Interleukin (IL)-12 is a potent heterodimeric cytokine secreted by antigen-presenting cells and known for its ability to enhance the lytic capabilities of $CD8^+$ cytotoxic T lymphocytes and natural killer cells[1–3] and to regulate the balance between the two classes of T helper cells, T_{H1} and T_{H2}.[4,5] IL-12 also causes T cells and NK cells to proliferate and secrete interferon (IFN)-γ[6,7]; many of its most important effects probably occur through its potentiation of IFN-γ secretion.

IL-12 has demonstrated therapeutic efficacy in murine models of bacterial[8,9] and viral[10–12] infections, and the T_{H1} response promoted by IL-12 has proven critical in clearing a variety of intracellular and extracellular pathogens.[13–19] T_{H1} responses may also be beneficial in arming the immune system to fight tumors, and IL-12 has displayed potent anti-tumor effects in murine models of established subcutaneous tumors[20–22] and tumor metastasis.[20,22,23] The efficacy observed in these animal models supported a rationale for developing IL-12 as a therapeutic agent, and clinical trials in renal cell carcinoma patients are currently underway.

The pleiotropic immunomodulatory effects of IL-12 are not all positive. The cytokine can disrupt normal trafficking of cells, with marked changes occurring in leukocyte populations found in liver, lymphoid tissue, and peripheral blood.[24,25] Livers of IL-12-treated mice are characterized by leukocytic infiltration, widespread Kupffer cell activation and proliferation, and multifocal areas of megakaryocyte proliferation, erythropoiesis, and hepatocyte necrosis. Mononuclear cells invade skeletal muscle of IL-12-treated mice, causing a multifocal mineralization and progressively severe degeneration of the muscle fibers.

Spleens of IL-12-treated mice become enlarged due to extramedullary hematopoiesis and mononuclear cell infiltration in the red pulp. Splenic white pulp, thymus, and lymph nodes are depleted of lymphoid cells, and in peripheral blood, IL-12 caused dose-dependent decreases in erythrocytes, platelets, and lymphocytes.

Although circulating leukocytes may enter liver, spleen, and lungs through nonspecific trapping in the vasculature, retention of leukocytes in the parenchyma of these organs is governed by specific interactions between adhesion molecules expressed on both leukocytes and the vascular endothelium. Leukocyte migration into tissue is generally thought to occur through a cascade of molecular events[26–28] that begins with transient, low-affinity interactions dominated by the selectin adhesion molecules and their carbohydrate ligands. This brief tethering slows the transit of leukocytes through post-capillary venules and makes subsequent firm adhesion and transendothelial migration more likely to occur.[29,30] The selectins include L-selectin, expressed on all leukocytes,

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Table 1. Abs Ued for Immunohistochemical Detection of Murine Leukocyte Subsets and Adhesion Molecules

	Specificity	Isotype	Conjugation	Source
Primary mAbs				
30F11.1	CD45	Rat IgG_{2b}		Pharmingen
500A2	CD3ε	Hamster IgG		Pharmingen
F4/80	Mature macrophages	Rat IgG_{2b}		Harlan
GR-1 (RB6-8C5)	Granulocytes	Rat IgG_{2b}		Pharmingen
FD441.8	LFA-1	Rat IgG_{2b}		ATCC
M1/70	MAC-1	Rat IgG_{2b}		ATCC
2E6	CD18	Hamster IgG		ATCC
YN1	ICAM-1	Rat IgG_{2a}		ATCC
R1-2	VLA-4	Rat IgG_{2b}		ATCC
M/K1.9	VCAM-1	Rat IgG_1		ATCC
MEL-14	L-selectin	Rat IgG_{2a}		ATCC
Mu-Hu-E	E-selectin	Goat polyclonal		Pocono
10A10	P-selectin	Rat IgG_1		HLR
Polyclonal secondary Abs	Goat anti-hamster IgG (H&L)		Biotin	Vector
	Rabbit anti-rat IgG (H&L)		Biotin	Vector
	Horse anti-goat IgG (H&L)		Biotin	Vector
	Goat anti-rat IgG (γ)		Peroxidase	K & P

Harlan, Harlan Bioproducts for Science, Indianapolis, IN; ATCC, American Type Culture Collection, Rockville, MD; Pocono, Pocono Rabbit Farms, Canadensis, PA; HLR, Hoffmann-La Roche, Nutley, NJ; K & P, Kirkegaard & Perry, Gaithersburg, MD.

and the inducible endothelial cell adhesion molecules P-selectin and E-selectin.

Stable adhesion and transendothelial migration are generally governed by interactions between members of the integrin and immunoglobulin (Ig) superfamilies of adhesion molecules.[31–33] The β2 integrins are a family of adhesion molecules expressed exclusively on leukocytes. Its members have in common the CD18 β-chain, but they express different α-chains. This family consists of LFA-1, MAC-1, and p150, 95. One of the principal ligands of the β2 integrins is ICAM-1, an inducible Ig superfamily member that may be expressed on endothelial cells and mononuclear cells.[34]

VLA-4 is a β1 integrin family member that is expressed on lymphocytes and monocytes but not on neutrophils. It governs interactions with endothelial cells, other lymphoid cells, and the extracellular matrix.[32,35] VLA-4 binds both fibronectin in the extracellular matrix and VCAM-1, another Ig superfamily member expressed primarily on activated endothelial cells.[36] Both VLA-4 and the β2 integrins require an activation step to efficiently bind their receptors.[37]

In this study, we investigated the expression of adhesion molecules in the livers of CD-1 mice dosed systemically with IL-12. Although high levels of ICAM-1 are constitutively expressed in mouse liver, IL-12 was found to induce expression of VCAM-1 in the hepatic vasculature. Leukocytes migrating into the livers of IL-12-treated mice showed strong expression of the ICAM-1 ligands LFA-1 and MAC-1 and the VCAM-1 ligand VLA-4. This striking pattern of hepatic adhesion molecule expression may have implications for clinical therapies relying on IL-12 for the amelioration of infectious disease or cancer.

Materials and Methods

Animals

Six- to eight-week-old male and female CD-1 mice were purchased from Charles River Laboratories (Raleigh, NC). Six-week-old male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME).

Antibodies

Antibodies used in the immunohistochemical detection of mouse leukocyte subsets and adhesion molecules are listed in Table 1. Radiolabeled monoclonal antibodies (MAbs) used to quantify mouse liver endothelial cell markers included M/K2.7, a rat IgG_1 specific for murine VCAM-1 (Pharmacia-Upjohn, Kalamazoo, MI); 10E6, rat IgG_{2b} to mouse E-selectin (Hoffmann-La Roche, Nutley, NJ); and RB40.34, a rat IgG_1 against murine P-selectin (Pharmingen, San Diego, CA). P-23, a murine IgG_1 specific for human P-selectin, was used as the nonbinding MAb in this assay.

Experimental Protocol

Recombinant murine IL-12 was diluted in a vehicle of phosphate-buffered saline (PBS) containing 100 μg of mouse serum albumin/ml and delivered intraperitoneally in a volume of 0.2 ml/mouse/day. Recombinant IL-12 was produced in Chinese hamster ovary cells and was obtained from Dr. Maurice Gately, Hoffmann-La Roche.

Livers for immunohistochemistry were obtained from male and female CD-1 mice dosed from 1 to 11 days with 5 μg of recombinant murine IL-12 daily, a dose equivalent to 200 μg/kg for a 25-g mouse. Livers for radiolabeled MAb quantitation of endothelial cell adhesion molecules were obtained from male C57BL/6 mice dosed from 1 to 5 days with 1 μg of IL-12 daily. C57BL/6 mice require less IL-12 than CD-1 mice to produce a similar pattern of adhesion molecule expression.

Immunohistochemistry

Livers from IL-12- and vehicle-dosed mice were excised, and slices from two lobes were positioned in a mold

containing OCT solution before being snap frozen in a slurry of isopentane and dry ice. Frozen sections were cut at 5 to 6 μm, air dried overnight, and fixed for 10 minutes in cold acetone before immunostaining. Antibodies used for detecting adhesion molecules and liver-infiltrating leukocytes are listed in Table 1. Optimal concentrations and incubation times were chosen during preliminary titrations on liver and known positive tissues.

For peroxidase-based immunostaining protocols, endogenous peroxidase activity was blocked by a preliminary 30-minute incubation of sections with 0.3% H_2O_2 in absolute methanol. Any endogenous liver avidin and biotin was blocked using reagents supplied in a kit from Vector Laboratories (Burlingame, CA), and nonspecific protein binding was blocked during a 15- to 30-minute incubation with 10% serum obtained from the same species used to produce the secondary antibody. The tissue was then incubated for 30 to 60 minutes with one of the primary antibodies, followed by subsequent processing with either a peroxidase-conjugated secondary antibody or a combination of biotinylated secondary antibody followed by avidin/biotinylated peroxidase complex (Vectastain Elite ABC reagent, Vector Laboratories). 3,3′-Diaminobenzidine was used as the chromogen.

In an alkaline-phosphatase-based protocol, the H_2O_2/methanol incubation was omitted, but liver avidin/biotin and nonspecific binding were blocked as detailed above. Tissue was then incubated for 60 minutes at room temperature with primary antibody, followed by 30 minutes with biotinylated secondary antibody and then 30 minutes with avidin/biotinylated alkaline phosphatase complex (Vectastain ABC/AP kit, Vector Laboratories). Endogenous alkaline phosphatase activity was blocked by the addition of 1 mmol/L levamisole to the substrate buffer. Alkaline phosphatase substrate was purchased as a kit (Vector Red) from Vector Laboratories). All slides were counterstained with hematoxylin.

Immunostaining was performed on serial sections cut from livers obtained 1, 3, 5, 7, 10, or 11 days after daily intraperitoneal injections of IL-12 or vehicle. Livers from both males and females were examined, with no significant differences in adhesion molecule expression noted between the sexes. Tissue from three to five animals was examined for each time point. For all samples, negative controls consisted of substituting the primary antibody with the same concentration of an isotype-matched antibody of an irrelevant specificity and/or incubation with 0.1% bovine serum albumin (BSA)/PBS (buffer) instead of the primary antibody. When possible, concurrent staining on known positive tissue was performed.

Radiolabeled Quantitation of Liver E-Selectin, P-Selectin, and VCAM-1

Liver VCAM-1, E-selectin, and P-selectin expression was assayed by a technique using radiolabeled MAbs, as previously described.[38] Binding MAbs (M/K2.7, 10E6, and RB40.34) and nonbinding MAb (P-23) were labeled with ^{125}I and ^{131}I, respectively, using an iodogen-based method.[39]

Male C57BL/6 mice (n = 3 to 5 per group) were anesthetized, their left jugular vein and descending abdominal aorta were catheterized, and a mixture of binding and nonbinding MAbs was injected through the jugular vein catheter. After 5 minutes, the mice were heparinized and rapidly exsanguinated by perfusion of bicarbonate-buffered saline (BBS) through the jugular vein catheter and simultaneous blood withdrawal through the abdominal aorta catheter. This was followed by perfusion of 10 ml of BBS through the abdominal aorta catheter after severing the inferior vena cava.

The method for calculating the expression of endothelial cell adhesion molecules has been described previously.[38] In brief, the ^{125}I (binding MAbs) and ^{131}I (nonbinding MAb) activities in liver were counted in a gamma counter, with automatic correction for background activity and spillover. The total injected activity in each experiment was calculated by counting a 2-ml sample of the radiolabeled MAb mixture. The radioactivity remaining in the tube used to mix the MAbs and the syringe used to inject the mixture was subtracted from the total injected activity and was on average less than 1% of the total activity. The accumulated activity of each MAb in liver was expressed as the percentage of the injected activity per gram of tissue. Adhesion molecule expression was calculated by subtracting the accumulated activity per gram of liver of the nonbinding MAb from the activity of a binding MAb. Previous studies have shown that MAbs retain their functional activity after radio-iodination.[40]

Image Analysis

Inflammatory cell foci in formalin-fixed, 4- to 5-μm hematoxylin and eosin (H&E)-stained liver sections were measured using NIH Image 1.55 b5 public domain image processing and analysis software on a Macintosh Quadra 800 computer interfaced to a Zeiss Axioskop light microscope (Zeiss, Thornwood, NY), a high-resolution Hitachi HV-C10 3-CCD color camera (Hitachi Denshi, Tokyo, Japan), and a Scion LG-3 image capture board (Scion Corp., Frederick, MD). The signals were obtained via three separate channels that could be displayed as gray scale representations and also integrated into a full-color image. Quantification involved manually outlining foci of inflammatory cells with a computer mouse, calculating the surface area of foci in square microns, and expressing this area as a percentage of the total liver section surface area (excluding large portal tract and central vein lumens). Tissue from each mouse examined consisted of 10 ×288 fields from the two largest lobes of the liver (five fields/lobe) selected by a structured algorithm that allowed sampling along the horizontal and vertical axes of the entire tissue section. Coded slides were analyzed by a single operator blind to the treatment.

Statistics

The image analysis data, radiolabeled MAb measurement of adhesion molecule expression, and each of the hematological values were evaluated by the *t*-test. For the

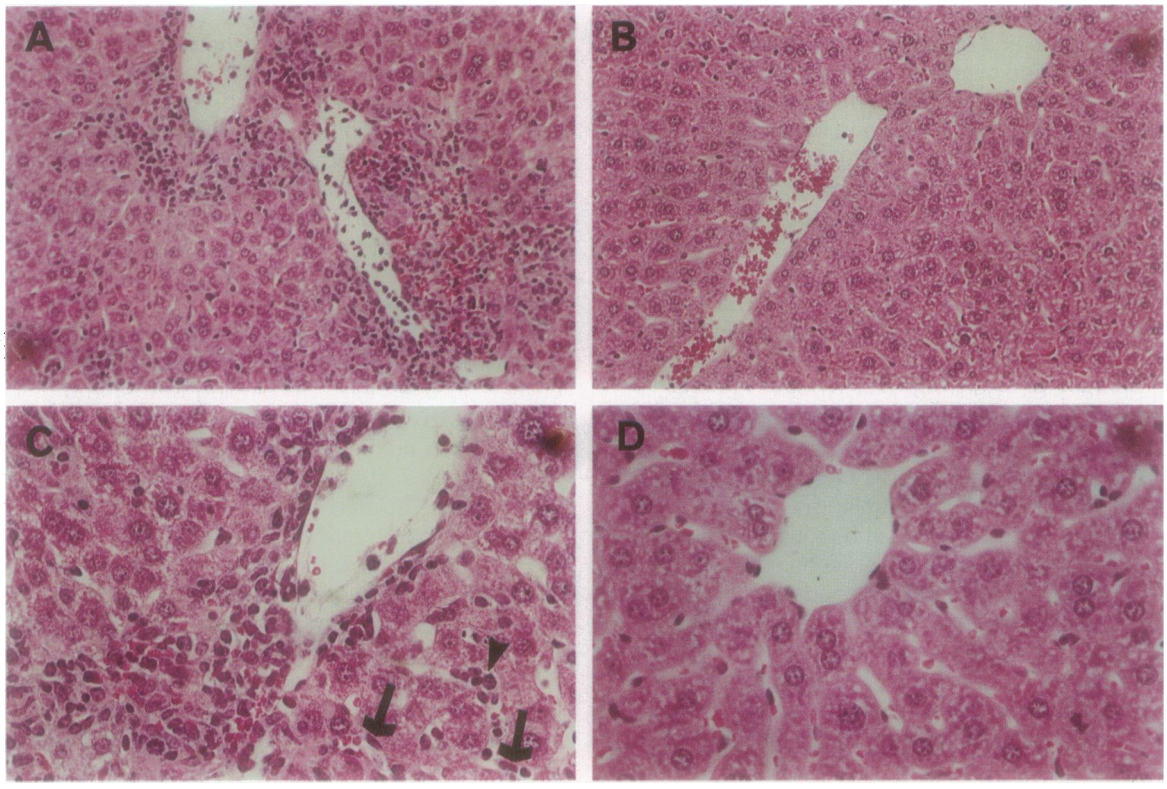

Figure 1. IL-12-induced hepatic lesion, as shown by H&E-stained sections from livers of CD-1 mice given four daily injections of 5 μg/day murine IL-12 (**A** and **C**) or vehicle (**B** and **D**). IL-12 caused a dose-dependent infiltration of lymphocytes and macrophages around central veins and portal tracts and in the liver parenchyma, as well as the activation of resident sinusoidal Kupffer cells (**arrows** in **C**) and proliferation of sinusoidal cells (**arrowhead** in **C**). Magnification, ×200 (**A** and **B**) and ×400 (**C** and **D**).

hematological data, when the variance between vehicle and IL-12-treated groups was homogeneous, a standard *t*-test was used. When the variance was not homogeneous, the Satterthwaite modified *t*-test was used. Differences were considered significant when $P \leq 0.05$.

Results

IL-12-Induced Pathology

IL-12 caused leukocytes to infiltrate murine liver (Figure 1). The infiltrate was dominated by irregular-shaped aggregates of lymphocytes and macrophages in the liver parenchyma and as cuffs surrounding portal tracts and central veins. Kupffer cells increased in both size and number in response to IL-12. Scattered areas of single-cell hepatocyte necrosis were accompanied by a random distribution of hepatocytes in mitosis. The serum enzymes alanine aminotransferase and aspartate aminotransferase were increased (data not shown), reflecting the ongoing hepatocyte necrosis. Increased numbers of megakaryocytes were present in the sinusoidal space, indicative of IL-12-induced extramedullary hematopoiesis.

Mouse thymus, spleen, and lymph nodes exhibited lymphoid depletion in response to IL-12 treatment. Splenomegaly occurred because of erythropoiesis, granulopoiesis, megakaryocytosis, and mononuclear cell infiltration into the red pulp. In peripheral blood, murine IL-12 induced a statistically significant anemia, thrombocytopenia, and leukocytopenia (Table 2). The leukocytopenia was driven by a reduction in lymphocytes.

Leukocyte Subpopulations in IL-12-Treated Livers

The number of leukocytes migrating into IL-12-treated livers more than tripled after 3 days of dosing with 5 μg of IL-12 daily (data not shown). When the IL-12-induced hepatic infiltrate was quantified with image analysis software, a statistically significant, time- and dose-dependent increase in the number of inflammatory foci was observed (Table 3).

Immunohistochemical staining of IL-12-treated livers revealed a time-dependent influx of various leukocyte subpopulations (Figure 2). CD45, the leukocyte common antigen, is expressed on the surface of all leukocytes.[41,42] T cells, macrophages, and neutrophils detected by immunostaining with MAb specific for murine CD45 peaked after approximately 5 days of daily IL-12 administration (Figure 2A). MAb to the CD3ε chain, present on murine T cells, stained a major population of cells in livers of IL-12-treated mice (Figure 2B). The $CD3\varepsilon^+$ cells accumulated in thick perivascular cuffs and as single cells and aggregations of cells in the paren-

Table 2. IL-12-Induced Changes in Peripheral Blood: Mean Hematological Values

Treatment	Erythrocytes (10^6/mm^3)	Platelets (10^3/mm^3)	Leukocytes (10^3/mm^3)	PMNs (10^3/mm^3)	Lymphocytes (10^3/mm^3)	PMNs (% WBCs)	Lymphocytes (% WBCs)
Vehicle (n = 6)	9.36	1192.5	8.82	1.40	7.18	16.2	81.0
1 μg of IL-12 for 4 days (n = 50)	8.48	603.8	3.10	0.77	2.18	25.5	69.6
1 μg of IL-12 for 14 days (n = 6)	6.49	623.0	3.02	1.40	1.60	46.2	53.0

Female CD-1 mice were dosed for 14 days with vehicle or for 4 or 14 days with 1 μg of murine IL-12 daily. PMNs, polymorphonuclear neutrophils; WBCs, white blood cells.

Table 3. IL-12-Induced Increase in Liver Inflammatory Foci

Treatment	% Inflammatory foci	
	Group average	Individual mice
Control	0.6	0.5
		0.7
1 μg for 4 days	3.7	3.4
		3.6
		4.1
5 μg for 4 days	5.4	3.0
		7.9
1 μg for 7 days	9.7	8.2
		11.2

Female CD-1 mice were dosed daily with the indicated amount of IL-12. H&E-stained paraffin sections were obtained from two liver lobes from each mouse. Inflammatory foci were measured using image analysis software as described in Materials and Methods. Data represent the percentage of measured surface area occupied by inflammatory foci. Ten fields were examined to obtain mean values for each mouse (five fields/lobe).

chyma. The increase in CD3ε$^+$ cells was apparent as early as 24 hours after IL-12 treatment.

Based on immunostaining with MAb F4/80, mice treated with vehicle had more macrophages than T cells in their livers. Numerous F4/80$^+$ macrophages, many of them presumably Kupffer cells, were found in the liver parenchyma and sinusoids, but treatment with IL-12 up-regulated F4/80 expression, generally after 3 days of daily treatment (Figure 2C).

Neutrophils were much less numerous in livers of both normal and IL-12-treated mice. The number of neutrophils stained by MAb GR-1[43] did increase in response to IL-12 treatment, but positively staining cells remained fairly evenly distributed throughout the liver parenchyma (Figure 2D).

Constitutive Expression of ICAM-1 in Murine Liver

Vehicle-treated mice constitutively expressed ICAM-1 along portal tract veins and arteries, central vein and sinusoidal endothelia, and weakly in hepatocytes (Figure 3B). IL-12 treatment tended to elevate existing ICAM-1 expression (Figure 3A) and caused *de novo* ICAM-1 expression along bile duct epithelia. At later time points in the course of IL-12 treatment, ICAM-1$^+$ cuffs of perivascular cells were apparent.

VCAM-1 Up-Regulation in Response to IL-12

In contrast to the constitutive expression of ICAM-1 in livers of CD-1 mice, little VCAM-1 was detected in livers of vehicle-treated mice, but a sharp increase occurred in response to IL-12 (Figure 4, A, C, and E). Increased VCAM-1 expression occurred within 24 hours, and after 5 days of IL-12 administration, VCAM-1$^+$ cells were present along portal tracts, bile duct epithelia, and some of the larger central veins and as both single cells and foci of cells in the liver parenchyma. By day 11, most single-cell staining had disappeared, but large parenchymal and perivascular accumulations of VCAM-1$^+$ cells remained. Among endothelial cells, VCAM-1 expression tended to be stronger in the portal tracts than the central veins, with portal tract arterioles staining especially intensely.

Adhesion Molecule Expression on Liver Leukocytes

IL-12 caused a dramatic and dose-dependent influx of leukocytes positive for the adhesion molecules LFA-1, VLA-4, and MAC-1 into livers of CD-1 mice (Figures 3 and 4). Cells expressing these adhesion molecules occurred in cuffs surrounding portal and central vein endothelia and as foci and singly-staining cells within the liver parenchyma. Perivascular cuffing was pronounced after 5 days of IL-12, and by 7 or 11 days, the number of singly-staining cells in liver parenchyma tended to decrease somewhat, with more positively stained cells gathered instead into foci.

VLA-4, LFA-1, and MAC-1 exhibited different patterns of constitutive expression in the mouse liver. VLA-4 expression in vehicle-treated livers was low (Figure 4B), but constitutive LFA-1 and MAC-1 expression occurred in cells scattered throughout the liver parenchyma (Figure 3, D and F). Immunostaining for LFA-1 and MAC-1 produced a pattern consistent with Kupffer cell expression of these adhesion molecules, although constitutive levels of LFA-1 expression were much higher than constitutive levels of MAC-1. IL-12 treatment boosted expression of VLA-4, LFA-1, and MAC-1 (Figures 4, D and F, and 3, C and E), but less MAC-1 was expressed than LFA-1 or VLA-4, especially at later time points.

In contrast, staining with MAb 2E6 specific for the common β-chain (CD18) present in both LFA-1 and MAC-1 molecules was especially intense (Figure 3G). In vehicle-treated mice, 2E6 stained single leukocytes scattered throughout the parenchyma and occasional aggregates of leukocytes. IL-12 treatment of mice produced a sharp increase in 2E6 expression observable as early as 24 hours and increasing markedly by day 3 of IL-12 dosing. As was true with VLA-4 immunostaining, more

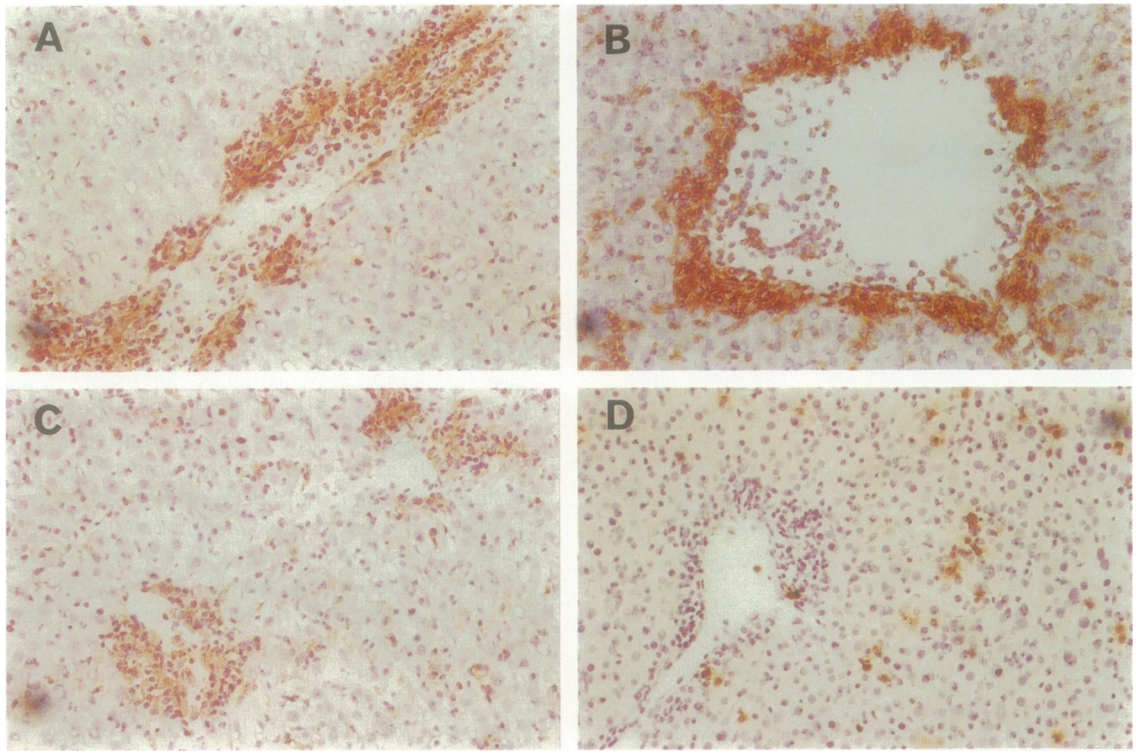

Figure 2. Leukocyte subsets in IL-12-treated liver, as shown by immunoperoxidase staining of livers of CD-1 mice dosed for 7 days with 5 μg of IL-12 daily. Sections were incubated with MAbs to murine pan-leukocyte Ag (α-CD45; **A**), mouse T cells (α-CD3ε; **B**) mature mouse macrophages (F4/80; **C**), and granulocytes (GR-1; **D**). Although livers of vehicle-treated mice contained numerous cells weakly positive for the F4/80 Ag but few $CD3\varepsilon^+$ and $GR\text{-}1^+$ cells, IL-12 treatment caused a dramatic increase in all three subsets. The T cells and macrophages tended to accumulate in foci, but the $GR\text{-}1^+$ cells (mostly neutrophils) did not. Magnification, ×200.

positively staining perivascular cuffs were noted around portal endothelium than around central vein endothelium early in the course of treatment.

Immunohistochemical Detection of Selectin Expression in IL-12-Treated Livers

$L\text{-selectin}^+$ leukocytes were not a major component of the hepatic leukocyte population in either IL-12- or vehicle-treated mice. Positively staining cells had a lymphocyte-like morphology, but the number of these cells was quite low, especially when L-selectin staining was compared with serial sections stained with MAbs specific for T cells or neutrophils, two cell types that have the capability of expressing L-selectin but that apparently do not in this case.

MAbs to the endothelial cell adhesion molecule P-selectin did not stain sinusoidal or central vein endothelium of either IL-12- or vehicle-treated mice but occasionally stained small sections of portal tract endothelium. This expression did not increase with IL-12 treatment. P-selectin expression was intense, however, in megakaryocyte-like cells found in the parenchyma of IL-12-dosed mice but largely absent from normal mice. These cells, frequently more than twice the size of hepatocytes, contained complex, multi-lobed nuclei (Figure 3H) and peaked in number by day 7 of IL-12 treatment.

Detecting E-selectin expression by immunohistochemistry was problematic. MAbs to E-selectin suitable for immunostaining liver tissue were unavailable. A polyclonal antibody was tried, and although it revealed E-selectin staining along scattered portal vein and arterial endothelia in both normal and IL-12-treated mice, nonspecific background staining levels were high enough to make these results questionable. Therefore, an alternate strategy was devised.

Radiolabeled MAb Quantification of Endothelial Cell Adhesion Molecules

Radiolabeled MAbs had been used in previous studies to quantify adhesion molecule expression in a variety of mouse tissues.[38,40] This technique was both sensitive and specific, so it was used to compare levels of E-selectin, P-selectin, and VCAM-1 expression in IL-12- and vehicle-treated mice. The results confirm the immunohistochemical findings of low levels of P-selectin expression and a rise in VCAM-1 expression during the course of IL-12 treatment. This technique also uncovered a surprising peak in E-selectin expression after 5 days of daily IL-12 treatment (Figure 5). A statistically significant increase in E-selectin also occurred after 24 hours of dosing.

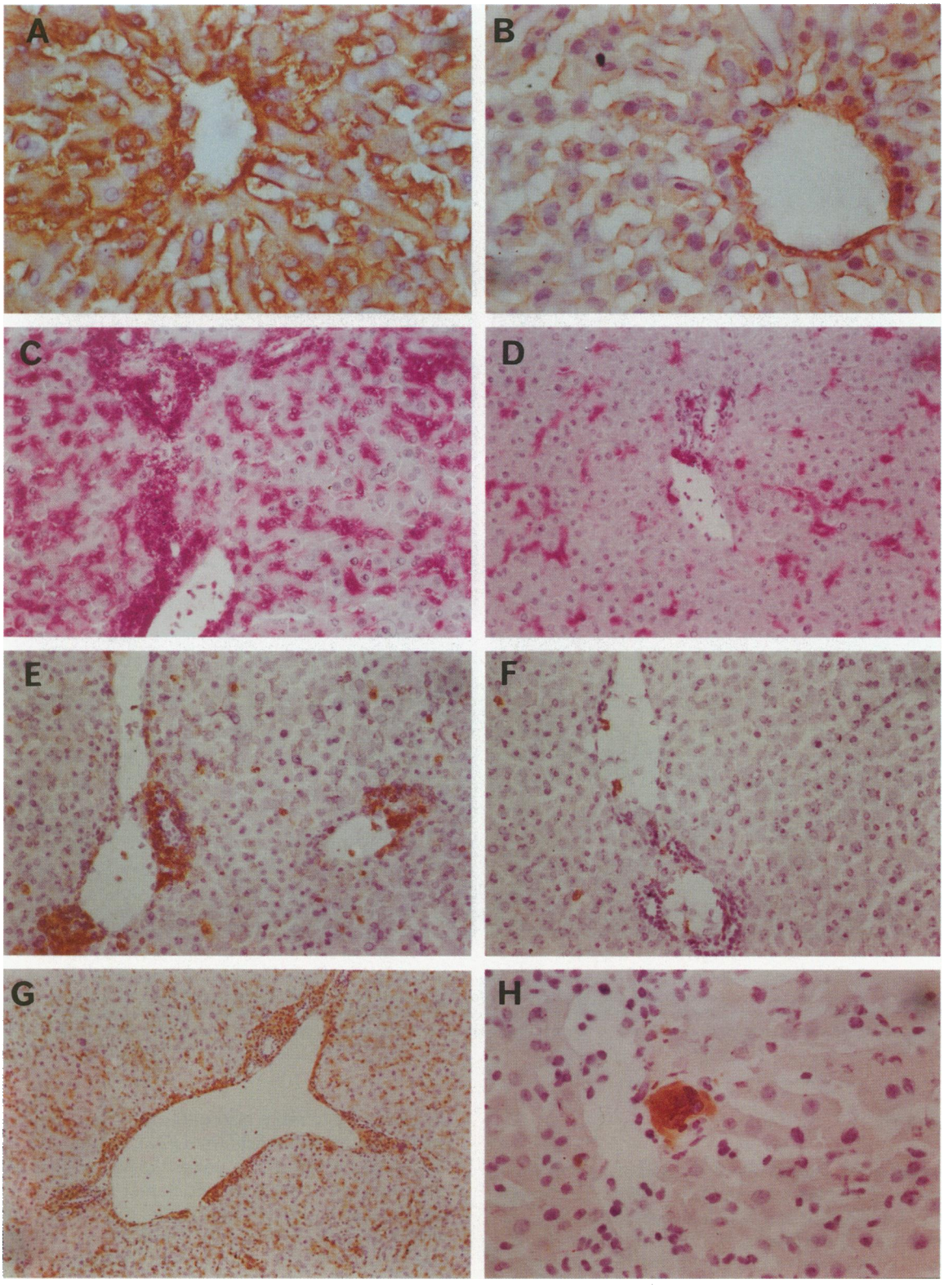

Figure 3. ICAM-1, LFA-1, MAC-1, CD18, and P-selectin expression in IL-12-treated murine liver. All mice were dosed for 5 or 7 days with either 5 μg of IL-12 daily or with vehicle. **A** and **B**: Peroxidase immunostaining using MAb YN1 specific for murine ICAM-1 in IL-12-treated liver (**A**) or vehicle-treated liver (**B**). **C** and **D**: Alkaline phosphatase immunostaining using MAb FD441.8 specific for murine LFA-1 in IL-12-treated liver (**C**) or vehicle-treated liver (**D**). **E** and **F**: Peroxidase immunostaining using MAb M1/70 specific for murine MAC-1 in IL-12-treated liver (**E**) or vehicle-treated liver (**F**). **G**: Peroxidase immunostaining of IL-12-treated liver using MAb 2E6 specific for murine CD18, the common β-chain of the leukocyte integrin family. **H**: Peroxidase immunostaining of IL-12-treated liver using MAb 10A10 specific for murine P-selectin. Magnification, ×400 (**A**, **B**, and **H**), C-F, ×200 (**C**), and ×100 (**G**).

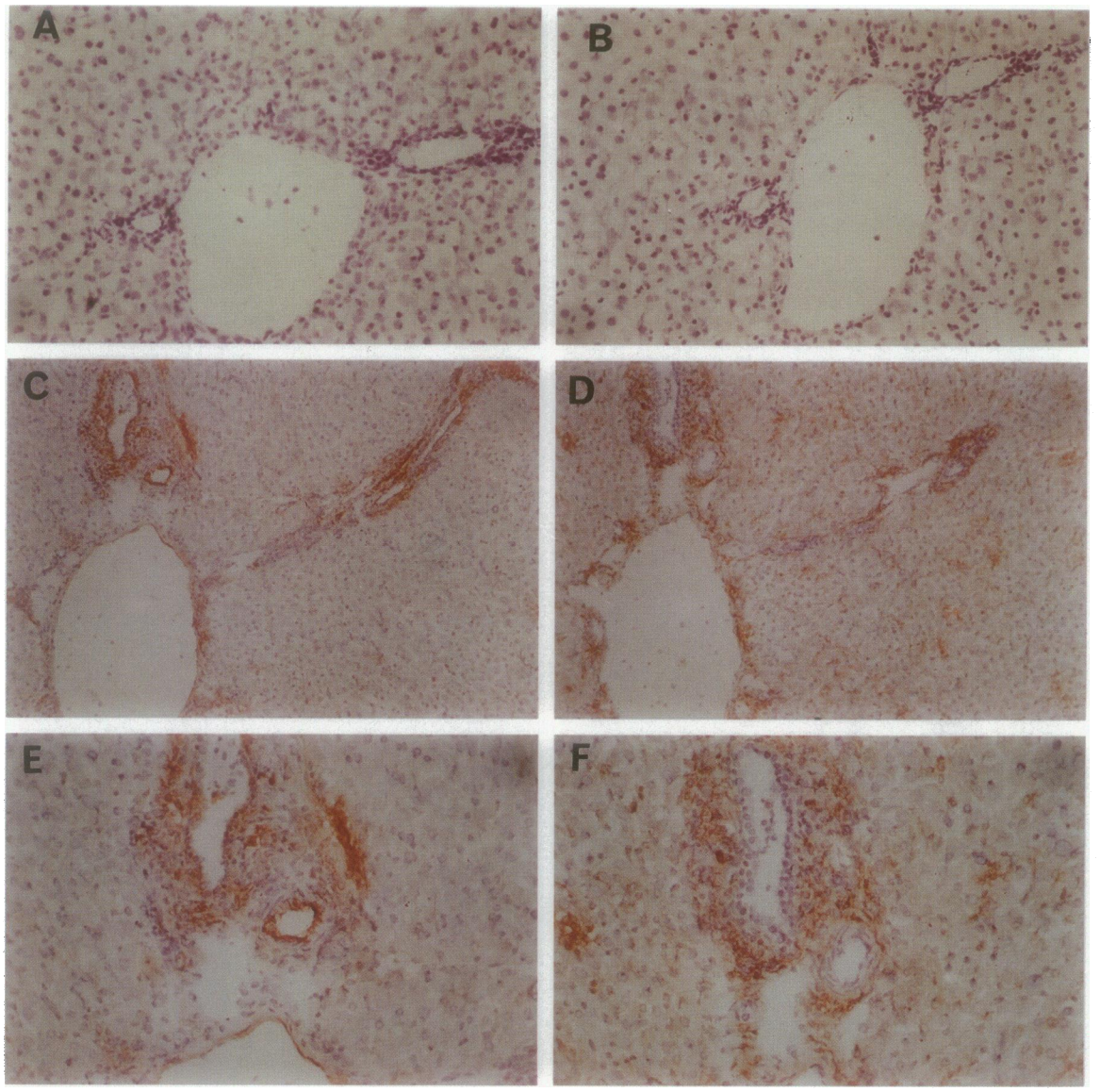

Figure 4. VCAM-1 and VLA-4 in IL-12-treated liver. Immunoperoxidase staining of livers of CD-1 mice dosed for 5 days with vehicle (**A** and **B**) or with 5 μg of IL-12 daily (**C** to **F**). Serial sections were incubated with MAb M/K1.9 specific for murine VCAM-1 (**A**, **C**, and **E**) or MAb R1-2 specific for murine VLA-4 (**B**, **D**, and **F**). IL-12 up-regulated VCAM-1 expression along portal tract and central vein endothelium, portal tract arterioles, and some bile duct epithelial cells. Both VCAM-1^+ and VLA-4^+ cells are present in the parenchyma as foci and singly staining cells and as perivascular cuffs. Magnification, ×200 (**A**, **B**, **E**, and **F**), and ×100 (**C** and **D**).

Discussion

Systemically administered IL-12 caused a murine liver inflammation the salient features of which included Kupffer cell activation, mononuclear cell infiltration, and the widespread up-regulation of adhesion molecule expression. The induction or up-regulation of individual adhesion molecules is summarized in Table 4, but several points are worth noting.

Leukocytes in IL-12-treated livers expressed abundant LFA-1, VLA-4, MAC-1, and CD18, but little L-selectin, suggesting an activated state and concomitant L-selectin shedding.[44,45] Although liver Kupffer cells are capable of expressing these adhesion molecules, migrating macrophages are probably also part of this positively stained population. The constitutive LFA-1 expression seen in Figure 3D occurred in a pattern consistent with Kupffer cell expression, but a quite different pattern of staining was detected using MAb to another macrophage marker, MAC-1 (Figure 3, E and F). The staining produced by MAb F4/80 (Figure 2C) cannot distinguish between Kupffer cells and peripheral blood monocytes/macrophages.[46]

A MAb suitable for immunohistochemical detection of natural killer (NK) cells in CD-1 mice was not available, but a previous study[24] indicated that the lymphocytes isolated from IL-12-treated mouse liver were predominantly NK cells and $CD8^+$ T cells. NK-like cells may

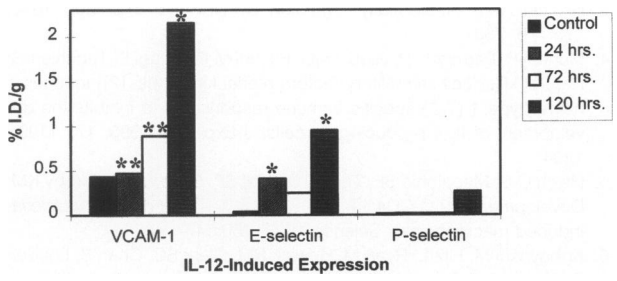

Figure 5. Radiolabeled MAb quantitation of IL-12-induced adhesion molecule expression in murine liver. Male C57BL/6 mice were injected daily with 1 μg of IL-12 or vehicle, and expression of liver VCAM-1, E-selectin, and P-selectin was measured at the indicated times using a radiolabeled MAb technique outlined in Materials and Methods. Control mice were injected with vehicle, and their livers were harvested after 24 hours. Values correspond to the percentage of injected dose per gram of liver tissue. *Statistically significant compared with control value; **statistically significant compared with 120-hour value.

contribute to the strong staining pattern noted with MAb specific for the T cell marker CD3ε (Figure 2B). In mouse liver, up to 50% of the αβ TCR^+ population may co-express murine NK cell markers.[47–50] These so-called natural T cells ($NK1.1^+TCR^{INT}$) are induced in mouse liver by IL-12 and can have potent cytotoxic activity.[49,51] NK cells may also express LFA-1, MAC-1, and VLA-4[24,52,53] and so could contribute to the positively stained populations of cells seen in Figures 3, C and E, and 4, D and F.

The rather sharp increase in E-selectin expression in mice 5 days after IL-12 treatment (Figure 5) was a surprise in this study. E-selectin expression is generally thought to occur early in the course of an inflammatory cascade, and although there was a moderate rise in E-selectin expression after 24 hours of IL-12 treatment, the reason E-selectin expression spikes again at 5 days is unknown. It is conceivable that continued administration of IL-12 could cause serum levels of tumor necrosis factor (TNF)-α to rise to a level sufficiently high to induce a secondary peak in E-selectin expression. IL-12 is also known to cause a reproducible spike in serum IFN-γ levels after 5 days of daily administration.[24]

Table 4. Summary of IL-12-Induced Changes in Murine Liver Adhesion Molecule Expression

Adhesion molecule	Constitutive expression	IL-12-induced expression
LFA-1	++ KC	+++ KC, IL
MAC-1	+ KC	++ KC, IL
VLA-4	+/− (KC?)	+++ KC, IL
L-selectin	+/− (IL?)	+/− (IL?)
P-selectin	+/− PTE	+/− PTE
		+++ M
E-selectin	−	++ ND
ICAM-1	++ CVE, PTE, SE	+++ CVE, PTE, SE
	+ H	++ H
		+++ IL
		++ BDE
VCAM-1	+/− PTE	+++ PTE, CVE
		++ BDE, IL

H, hepatocytes; KC, Kupffer cells; IL, infiltrating leukocytes; M, megakaryocytes; CVE, central vein endothelia; PTE, portal tract endothelia; SE, sinusoidal endothelia; BDE, bile duct epithelia; ND, cell type not determined. +++, strong expression; ++, moderate expression; +, weak expression; +/−, sporadic and weak expression; −, no expression.

The results described here may have relevance in man as human livers that become inflamed because of infection, transplantation rejection, or reperfusion injury have features in common with the livers of IL-12-treated mice. These features include the expression of LFA-1, MAC-1, and VLA-4 on infiltrating leukocytes in inflamed human livers and the expression of a wide range of adhesion molecules by human Kupffer cells.[54] And, as was true for the mouse, ICAM-1 is a constitutively expressed molecule in human liver, whereas VCAM-1 expression tends to be inducible, particularly during transplant rejection, cholangitis, sepsis, and viral infection.[54,55]

An immunohistochemical approach to IL-12-induced adhesion molecule expression cannot ascertain whether this cytokine's effects on expression are direct or indirect. It is also difficult to ascertain from this study whether a particular endothelial cell adhesion molecule played a dominant role in attracting leukocytes into IL-12-treated liver. ICAM-1 is constitutively expressed in mouse liver, VCAM-1 is induced quite early in the course of treatment, and infiltrating leukocytes express ligands for both ICAM-1 and VCAM-1 (ie, LFA-1, MAC-1, and VLA-4). LFA-1 and VLA-4 expression patterns both increase in a roughly comparable fashion during the course of IL-12 treatment. Constitutive levels of LFA-1, however, were much higher than constitutive VLA-4 expression (Figures 3 and 4). This constitutive LFA-1 expression occurred largely on Kupffer cells, and it is conceivable that activated Kupffer cells could initiate the IL-12-induced inflammatory cascade in murine liver. Kupffer cell integrin activation could augment existing levels of liver ICAM-1, which could in turn attract circulating leukocytes expressing LFA-1 and MAC-1 and activate those integrins. IFN-γ secretion induced by IL-12 could conceivably up-regulate liver VCAM-1. IFN-γ up-regulates VCAM-1 expression in other tissues in humans,[56] and murine brain microvascular endothelial cells have been shown to synthesize VCAM-1 in response to either IFN-γ or TNF.[57] IL-12 induces NK and T cells to secrete both IFN-γ[6,7] and small amounts of TNF-α,[58–60] and the latter is a cytokine known to directly augment expression of ICAM-1 and VCAM-1.[61,62]

Experiments assessing IL-12-induced adhesion molecule expression and function in mice bearing disrupted genes for IFN-γ and the receptors for IL-1 and TNF are currently underway to further investigate the role of these cytokines in IL-12-induced cell adhesion. In a published report using IFN-γ receptor-deficient mice, however, IL-12 treatment still resulted in liver leukocyte accumulation and ICAM-1 up-regulation, but liver transaminase elevation did not occur.63

It is important to realize that the dose of IL-12 used to cause liver inflammation in this study (5 μg daily) was higher than doses normally required for *in vivo* immunomodulatory effects. A single injection of 1 μg of IL-12 significantly enhanced NK cell activity in liver,[24] 100 ng of IL-12 daily produced anti-metastatic and anti-tumor effects in a variety of tumor-bearing mouse models,[20,22] and only 30 ng of IL-12 daily was efficacious in a murine

model of malaria infection.[19] In squirrel monkeys, enhanced lymphocyte lytic activity was detected at a dose of human IL-12 that was 500-fold less than that which caused severe toxicity.[25] IL-12's bioactivity over a wide dose range should give this cytokine a therapeutic index sufficient for safely boosting the immune response during the treatment of certain cancers and infectious diseases.

As a therapeutic drug, however, IL-12 should probably be used with caution in patients with autoimmune disease or those undergoing bone marrow or solid organ transplantation. IL-12 can exacerbate disease severity in murine models of multiple sclerosis, Crohn's disease, and diabetes.[64–66] It can also stimulate the development of acute graft-*versus*-host disease in nonirradiated F1 mice injected with parental spleen cells,[67] and it has been shown to potentiate the development of gastrointestinal pathology after lethal irradiation.[68] IL-12's induction or augmentation of liver adhesion molecule expression should sound a cautionary note against its use in these and other clinical settings where augmented adhesion molecule expression are critical in pathogenesis.

The alterations in cell trafficking that occur during IL-12 therapy help underscore how critical cytokine balance is to tissue homeostasis. Profound alterations in normal cell trafficking occur with the systemic administration of other cytokines, including IL-2,[69,70] TNF,[71] and IL-1.[72] Gene-targeted cytokine disruption, on the other hand, may cause similar perturbations in normal cellular distribution in organs.[73,74] These examples of disrupted cell trafficking are no doubt also accompanied by alterations in adhesion molecule expression. It will be highly informative to decipher whether these alterations occur as a direct result of cytokine-induced changes in organ endothelia, whether the cytokines change adhesion molecule expression on particular non-endothelial cell populations native to certain organs, or whether adhesion molecule changes occur secondarily to other, as yet undefined, mechanisms that might alter fundamental cell trafficking patterns.

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