Regulation of Vascular Cell Adhesion Molecule-1 Expression by IL-4 and TNF-α in Cultured Endothelial Cells

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Abstract

Interaction between vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells and α4 integrins on leukocytes is thought to mediate the selective recruitment of eosinophils and lymphocytes that occurs in allergic diseases. IL-4 is associated with allergic conditions, and it has been shown to selectively increase expression of VCAM-1 on endothelial cells in vivo, suggesting that it could be responsible for VCAM-1 expression in allergic disease. Using a combination of immunofluorescence, flow cytometry, and Northern analysis, we compared the effect of TNF-α and IL-4 on VCAM-1 expression. TNF-α is also associated with allergic diseases, and it rapidly increases transcription of the VCAM-1 gene. The effect of IL-4 was relatively modest with prolonged kinetics: VCAM-1 was not detected until 72 h after treatment with IL-4. However, when TNF-α and IL-4 were combined, there was a synergistic increase in VCAM-1 expression and a dramatic prolongation of the appearance of VCAM-1 on the cell surface. This synergy results from a combination of transcriptional activation by TNF-α and the stabilization of resulting transcripts by IL-4. We propose that IL-4 allows subthreshold concentrations of TNF-α (concentrations that would not normally activate expression of adhesion molecules on the endothelium) to selectively increase VCAM-1 expression and to prolong its appearance on the surface of cells in allergic disease. (J. Clin. Invest. 1995. 95:264–271.) Key words: cell adhesion molecules • half-life • messenger RNA • inflammation • selective leukocyte recruitment

Introduction

Adhesion of leukocytes to the endothelium is mediated by several classes of cell adhesion molecules (1, 2). E-selectin (CD62E) interacts with oligosaccharide containing molecules on leukocytes mediating the initial rolling of the leukocyte in the slower moving circulation of the microvasculature (3, 4). This interaction allows the strong adhesion mediated by members of the Ig superfamily, including vascular cell adhesion molecule-1 (VCAM-1, ICAM-1, CD106) and intercellular adhesion molecule-1 (ICAM-1, CD54), which interact with integrins, a family of heterodimeric receptors on the leukocyte surface (1, 2). These same molecules have also been implicated in the subsequent transmigration across the endothelium. Many of the endothelial surface cell adhesion molecules, including E-selectin, VCAM-1, and ICAM-1, are increased by inflammatory cytokines, such as TNF-α, IL-1, and LPS (1, 2).

The differential expression and activation of cell adhesion molecules have been postulated to account for the selective recruitment of leukocytes seen in different inflammatory diseases (1, 2). For example, infiltrates containing neutrophils are characteristic of bacterial infections, whereas infiltrates of eosinophils and lymphocytes typify allergiclike conditions such as atopic dermatitis, allergic rhinitis, and allergic asthma (5). Two integrins, α4β1 (CD49d/CD29) and α4β7, are present on a subpopulation of leukocytes, including lymphocytes and eosinophils but not neutrophils. The α4 integrins bind VCAM-1 but not ICAM-1 or E-selectin (6, 7). The interaction between VCAM-1 and α4 integrins has been shown to mediate the adhesion of lymphocytes and eosinophils but not neutrophils to endothelial cells in culture, suggesting that the interaction between VCAM-1 and α4 integrins could mediate a selective recruitment of leukocyte subpopulations in vivo (1, 2).

In support of this possibility, several reports have demonstrated a correlation between VCAM-1 expression and the selective recruitment of lymphocytes and eosinophils in vivo (8). For example, VCAM-1 has been identified on the endothelium in correlation with the lymphocytic infiltration seen in the rejection process after human heart, liver, and pancreas allograft transplantation (9, 10). VCAM-1 has been identified on the endothelium in perennal allergic rhinitis where there is a selective infiltrate of eosinophils (11). In a recent report, VCAM-1/α4 integrin interactions mediated recruitment of lymphocytes and eosinophils to the trachea in an inhaled antigen mouse model of asthma (12). In this model inhalation of ovalbumin in sensitized mice resulted in the expression of VCAM-1 but not ICAM-1 on tracheal endothelium; this expression of VCAM-1 correlated with infiltration of T cells and eosinophils. Infusion of blocking antibodies to VCAM-1 or α4 but not ICAM or E-selectin prevented the antigen-induced infiltration of T cells and eosinophils, resulting in peripheral blood eosinophilia. In another study, VCAM-1 was upregulated on the endothelium of bronchial biopsies taken from asthmatic patients after bronchial antigen provocation. The intensity and extent of VCAM-1 expression correlated with the eosinophilic infiltrate (13). Together these studies suggest that VCAM-1 expression is selec-

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Received for publication 18 July 1994 and in revised form 27 September 1994.

J. Clin. Invest. © The American Society for Clinical Investigation, Inc. 0021-9738/95/01/0264/08 $2.00 Volume 95, January 1995, 264–271

1. Abbreviations used in this paper: BAL, bronchoalveolar lavage; CAT, chloramphenicol acetyltransferase; GAPDH, glyceraldehyde phosphate dehydrogenase; HUVECs, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.
tively upregulated on the endothelium and its expression correlates with the selective recruitment of lymphocytes and eosinophils.

This selective upregulation of VCAM-1 expression may involve the cytokine, IL-4. IL-4 is found at sites of allergic inflammation. Increased levels of IL-4 have been found in the blood and bronchoalveolar lavage (BAL) of allergic asthmatic patients (14). Increased IL-4 mRNA was found in T cells recovered from the BAL of allergic asthmatics (15). In a study of allergen-provoked rhinitis in hay fever sufferers, tissue eosinophilia in nasal biopsies correlated with CD4+ Th2 cells expressing IL-4 mRNA (16). Together, these studies suggest a role for IL-4 in allergic conditions.

Additionally, experimental evidence suggests a role for IL-4 in the selective recruitment of leukocytes. In a mouse model of allergic asthma, IL-4 was detected in the BAL in association with a lymphocytic and eosinophilic pulmonary infiltrate (17). When IL-4-deficient mice (IL-4−/−) and wild-type mice (IL-4+/+) were sensitized and then treated with ovalbumin, there was a substantial attenuation of the BAL eosinophilia and the selective peribronchial inflammation in the IL-4−/− mice compared with the wild-type mice (18). In nude mice injected with IL-4 either intraperitoneally or intradermally, there was a marked increase in recruitment of eosinophils but not neutrophils (19). Likewise, overexpression of IL-4 in transgenic mice resulted in allergic-like inflammatory disease with the selective recruitment of eosinophils and mononuclear cells (20).

Originally, IL-4 was thought to be restricted to lymphocytes; however, it is now clear that other cells also secrete IL-4 (21). For example, enhanced release of IL-4 has been measured in human mast cells from asthmatics (22). The mast cell is positioned among microvascular endothelium in the skin, gut, and lung, in a location ideally suited for antigen presentation and a subsequent inflammatory response (21). Likewise, human basophils, which are also thought to be important cellular components in allergic inflammation, reportedly secrete IL-4 (21).

IL-4 has been shown to increase the adhesiveness of endothelial cells for leukocytes that express α4 integrins (23–25). This increased adhesiveness results from an IL-4–mediated selective upregulation of VCAM-1 expression on the endothelial cell surface and is not mediated by an interaction with ICAM-1 or E-selectin (25, 26–30). Therefore, IL-4 could be responsible, at least in part, for the selective increase in VCAM-1 expression at sites of allergic inflammation, resulting in the selective recruitment of the subset of leukocytes that express α4 integrins.

IL-4 has also been shown to act with TNF-α to selectively increase VCAM-1 expression in cultured endothelial cells (25, 28, 31). Likewise, in a baboon model of acute dermal microvascular inflammatory response, subcutaneously administered IL-4 combined with low doses of TNF-α resulted in a selective recruitment of lymphocytes. In this model there was increased expression of VCAM-1, but neither ICAM-1 nor E-selectin were detected on high endothelial venules (32). Interestingly, increased TNF-α has also been detected in asthmatic patients (33, 34), raising the possibility that TNF-α and IL-4 could mediate selective recruitment of lymphocytes and eosinophils through the synergistic upregulation of VCAM-1 expression on the endothelium.

The mechanism of action of IL-4 has been best studied in leukocytes where IL-4 has been shown to up-regulate or down-regulate the expression of a number of proteins. In the complex process of isotype switching to IgE in B cells, IL-4 increases γ gene transcription through an IL-4 responsive element in the promoter (35, 36). Likewise, the MHC Eo gene in B cells is also activated by IL-4 through an IL-4 responsive element (37). In contrast, IL-4 down-regulates the expression of IL-2 by inhibiting the transcription factor complex, NFIL-2B, which is required for efficient transcription (38). IL-4–regulatory elements have been also identified in other leukocyte genes (39, 40). In addition to its effect on transcription, IL-4 has been shown to destabilize several mRNAs in leukocytes (41–43).

IL-4 also functions in nonhematopoietic cells. It has been shown to increase the transcription of genes for fibronectin and collagen types I and II in cultured fibroblasts; however, IL-4 responsive elements have not been identified in these genes (44). In endothelial cells, IL-4 inhibits the IFN-γ–mediated increase in ICAM-1 mRNA (45), but the mechanism of action has not been examined.

Here, we examine the molecular mechanisms through which IL-4 and the combination of TNF-α and IL-4 selectively activate VCAM-1 expression. We demonstrate that IL-4 acts to increase the half-life of VCAM-1 mRNA and that the synergy between TNF-α and IL-4 results from TNF-α mediated transcriptional activation of the VCAM-1 gene combined with IL-4–mediated stabilization of the resultant transcripts.

Methods

Cell culture and cytokines. Human umbilical vein endothelial cells (HUVECs) were grown and propagated at low passage according to the supplier’s directions (Clonetics, San Diego, CA) except tissue culture plastic was precoated with gelatin 1 mg/ml (ICN Pharmaceuticals, Cleveland, OH). IL-4 was obtained from three sources: Promega (Madison, WI), R&D (Minneapolis, MN), and Monsanto (St. Louis, MO). All preparations yielded similar results. TNF-α was obtained from R&D.

Immunofluorescence and flow cytometry. Mouse anti–human monoclonal antibodies BBA-5 (R&D, diluted 1:1000), BBA-2 (R&D, diluted 1:1000), and LB2 (diluted 1:1000, Dr. E. A. Clark, Seattle, WA) all from a 1 mg/ml stock were used to detect human VCAM-1, E-selectin, and ICAM-1, respectively. Rabbit anti–human polyclonal antibodies NFκB p65 (LSI), NFκB p52 (K27), and c-rel (C) were used to detect the NFκB/Rel family of transcription factors (Santa Cruz Biotechnology, Santa Cruz, CA, diluted 1:500 from a 1 mg/ml stock). Immunofluorescent labeling was done as described (46) except cells were fixed with methanol at −20°C for 6 min and 1% Teleostean gelatin (Sigma Chemical Co., St. Louis, MO) was used as a nonspecific blocking agent. Single color flow cytometry using an Epics Elite flow cytometer (Coulter Cytometry, Hialeah, FL) was done as described (47). Initial gating was done using forward and side scatter to eliminate debris and identify a uniform population of endothelial cells. For each sample ≥1 × 10⁶ previously gated events were analyzed.

RNA analysis. Total RNA was isolated using RNASTAT-60 (Tel-Test “B”, Inc., Friendswood, TX) and subjected to Northern blot analysis. Probes for VCAM-1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNAs were described previously (46). ICAM-1 mRNA was detected with a 1.4-kb Xhol restriction enzyme fragment of ICAM-1 cDNA in pCD1.8 (48). For mRNA half-life measurements, HUVECs were grown to confluence and treated with cytokines for 16 h and then actinomycin D (Sigma Chemical Co.) was added at 3 μg/ml. Quantification of RNA was done both by directly measuring the radioactivity with an Ambus Radioanalytical Imaging System (Ambus Systems, San Diego, CA) and by densitometry. For densitometric analysis, multiple exposures of autoradiograms were scanned along with standard curves. Quantification of densitometric images was performed on a Macintosh computer using the public domain NIH Image program (written by Wayne Rasband from anonymous ftp from zippy.nlm.nih.gov).
Transient transfections. Transient transfections were done in HUVECs using the calcium phosphate technique (49). Confluent monolayers of cells on 10-cm plates were cotransfected for 8 h with 18 μg of reporter plasmid and 1 μg of pRSV/L, which contains the Rous sarcoma virus long terminal repeat fused to the firefly luciferase gene (49), as an internal control. Luciferase activity was measured using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Equal amounts of luciferase activity were added in each assay of chloramphenicol acetyltransferase (CAT) activity. Experiments were also done in the absence of pRSV/L to ensure that there was no promoter competition between pRSV/L and the VCAM-1 constructs. 36 h after transfection, protein extracts were made and CAT activity was determined (49). Approximately 20 μg of protein was used and assays were for 5 h. Acetylation of [14C]chloramphenicol was quantified by thin-layer chromatography followed by liquid scintillation counting.

Results

IL-4 alone induces only a low level of VCAM-1 expression with delayed kinetics. As a first step in examining the mechanism of action of IL-4 on VCAM-1 expression, we examined the time course of IL-4-mediated induction of VCAM-1 expression by immunofluorescent labeling in HUVECs. Surprisingly, we saw no evidence of VCAM-1 expression until 72 h after treatment with IL-4 and the level of expression appeared to be substantially less than with TNF-α (Fig. 1). IL-4 was used from three sources and HUVECs were obtained from several donors. Our results indicate that IL-4 alone had only a limited effect on VCAM-1 expression and the kinetics of its induction were delayed compared with TNF-α.

TNF-α and IL-4 act synergistically to increase VCAM-1 expression. It has been demonstrated that combining IL-4 with TNF-α increases VCAM-1 expression (25, 28, 31). Here, we show clear synergy between TNF-α and IL-4. When the concentration of TNF-α was reduced from 10 to 0.2 ng/ml, there was little or no apparent immunolabeling for VCAM-1 on permeabilized HUVECs (Fig. 2). Likewise, treatment with IL-4 at 100 U/ml for 24 h did not increase the expression of VCAM-1. However, when IL-4 was combined with TNF-α at 0.2 ng/ml, VCAM-1 expression increased dramatically. This synergistic expression appeared to be significantly greater than with TNF-α alone at 10 ng/ml.

To quantitate surface immunofluorescence, HUVECs were analyzed by flow cytometry. The results show that in response to 10 ng/ml of TNF-α, VCAM-1 expression peaks at ~24 h after treatment and is significantly decreased after 2 h (Fig. 1).
3). As was seen with immunolabeling, VCAM-1 does not appear until 72 h after treatment with IL-4 at 100 U/ml and the level of VCAM-1 is low; it is similar to the low level of VCAM-1 that persists after 72 h of treatment with TNF-α. When TNF-α and IL-4 were combined, there was a significant increase in expression of VCAM-1 over what was seen with TNF-α or IL-4 alone. This synergistic effect was seen as early as 16 h after treatment. However, it was most striking at 72 h, when the level of VCAM-1 in the presence of TNF-α alone had already decreased (Fig. 3). Therefore, IL-4 not only acts to increase the level of VCAM-1, it also affects the kinetics of expression, prolonging the presence of VCAM-1 on the cell surface. In contrast to the effect of IL-4 on VCAM-1 expression, IL-4 had no effect, either alone or in combination with TNF-α, on the expression of ICAM-1 or E-selectin after 10, 24, or 72 h of cytokine treatment (Fig. 4 and results not shown).

**TNF-α and IL-4 synergistically upregulate VCAM-1 mRNA.** Northern blot analysis was used to determine whether the combination of TNF-α and IL-4 increases the expression of VCAM-1 at the level of the mRNA. VCAM-1 mRNA was not detected in untreated HUVECs (Fig. 5). However, treatment of HUVECs with 10 and 0.2 ng/ml of TNF-α alone for 16 h resulted in a cytokine concentration-dependent increase in VCAM-1 mRNA. Treatment with either 100 or 2,000 U/ml of IL-4 alone for 16 h resulted in only a trace of VCAM-1 mRNA. When IL-4 was added along with TNF-α, there was a synergistic increase in the level of VCAM-1 mRNA. These results with VCAM-1 mRNA parallel those observed with the protein, indicating that TNF-α and IL-4 act at the level of VCAM-1 mRNA. The electrophoretic mobility of all cytokine-induced VCAM-1 mRNA was the same, suggesting that the form of VCAM-1 mRNA produced in response to the cytokines is similar.

**The addition of IL-4 along with TNF-α results in persistence of VCAM-1 mRNA.** Treatment of HUVECs with TNF-α alone resulted in a peak of VCAM-1 mRNA at 8 h that diminished by 48 h (Fig. 6). Similar results were seen with ICAM-1.
mRNA. When IL-4 was added along with TNF-α, VCAM-1 mRNA also appeared at 8 h, but instead of peaking at 8 h and diminishing, it continued to increase until ≥ 48 h. Thus IL-4 not only increased the level of VCAM-1 mRNA when combined with TNF-α, it also dramatically prolonged the presence of VCAM-1 mRNA. In contrast, IL-4 had no effect on either the level or the kinetics of ICAM-1 mRNA when combined with TNF-α.

**IL-4 has only a limited effect on VCAM-1 gene promoter activity.** Previously, we have shown that the TNF-α-mediated increase in VCAM-1 expression results from increased transcription of the VCAM-1 gene (49). The 2180VCAMCAT construct (which contains the first 2.1 kb of the 5’ flanking region of the human VCAM-1 gene promoter) confers the proper tissue specificity and is responsive to TNF-α in transfection assays (49). In HUVECs, the activity of 2180VCAMCAT is not above background in the absence of cytokines. Treatment with TNF-α results in activation of this construct, which is mediated by two adjacent NF-kB–like sites at positions −63 and −77 bp of the VCAM-1 gene. As was the case with VCAM-1 mRNA, IL-4 alone had little or no effect on VCAM-1 gene promoter activity (Fig. 7). In contrast, TNF-α significantly increased VCAM-1 gene promoter activity, as we demonstrated previously (49). When combined with TNF-α, IL-4 had only a slight effect on promoter activity. Since IL-4 alone had no effect, it was possible that IL-4 acts to facilitate the activity of TNF-α. One mechanism through which this could occur is by increasing the level of the NF-κB proteins or by facilitating their translocation to the nucleus. In a series of immunolabeling experiments using antibodies to NF-κB/Rel family members, we observed translocation of p65 to the nucleus after treatment with TNF-α, constitutive nuclear expression of p50 and RelB, and constitutive cytosolic expression of c-rel as described previously (results not shown) (50, 51). However, there was no difference in either the level or the localization of the NF-κB/Rel family of proteins with the addition of IL-4. Because of its limited effect on VCAM-1 gene promoter activity, it was likely that the principle effect of IL-4 was to increase the half-life of VCAM-1 mRNA. This possibility would also be consistent with the IL-4–mediated prolongation of VCAM-1 expression described above.

**IL-4 increases the half-life of VCAM-1 mRNA.** Next, the effect of IL-4 on VCAM-1 mRNA half-life was examined. HUVECs were treated with TNF-α, IL-4, or both cytokines for 16 h, and then actinomycin D was added at 3 μg/ml to block transcription. To ensure this concentration of actinomycin D was sufficient to block transcription, actinomycin D was titrated into the media and its effect on TNF-α–mediated E-selectin was analyzed. Transcription of E-selectin is known to rapidly increase in response to TNF-α (52). RNA was isolated at various times after the addition of actinomycin D and subjected to Northern analysis. Actinomycin D had no effect on VCAM-1 mRNA in untreated cells (results not shown). VCAM-1 mRNA levels were quantified both by directly measuring radioactivity and by densitometry. Half-lives were determined by measuring...
the decay of VCAM-1 mRNA over time. Since there was no detectable VCAM-1 mRNA in IL-4–treated cells (Fig. 5), the difference in half-lives between TNF-α and the combination of TNF-α and IL-4 was examined. The half-life for VCAM-1 mRNA after TNF-α treatment was 5 h (Fig. 8). This contrasts to the markedly prolonged half-life seen when TNF-α and IL-4 were used in combination. As controls, TNF-α and IL-4 had no significant effect on the half-life of GAPDH mRNA and IL-4 had no effect on the half-life of ICAM-1 mRNA induced in response to TNF-α (Fig. 5 and results not shown).

Discussion
Here, we show that IL-4 increases the half-life of VCAM-1 mRNA in endothelial cells. In combination with TNF-α, IL-4 synergistically increases the level of VCAM-1 on the cell surface. This synergy is due to the combination of transcriptional activation by TNF-α and IL-4–mediated stabilization of the resultant transcripts. TNF-α also activates transcription of ICAM-1 and E-selectin genes, and this appears to occur through a common mechanism involving TNF-α–mediated translocation of members of the NF-κB/Rel family from the cytoplasm to the nucleus where they bind to κB sites in the gene promoters and activate transcription (49, 52–54). However, IL-4 has no effect on expression of these proteins in endothelial cells. Thus the combination of TNF-α and IL-4 is a potential mechanism for selectively increasing the expression of a single cell adhesion molecule, VCAM-1, on the endothelial surface.

Our results indicate that when IL-4 is present, the threshold concentration of TNF-α required for activation of the VCAM-1 gene is markedly reduced. Thus it is conceivable that in vivo one function of IL-4 is to reduce the threshold concentration of TNF-α required for activation of the VCAM-1 gene to a point where it is below the concentration required for activation of expression of other endothelial adhesion molecules such as ICAM-1 and E-selectin. This possibility is supported by studies where limiting concentrations of TNF-α were injected along with IL-4 into baboons, and VCAM-1 was selectively up-regulated (32).

Adhesion molecules on the endothelial cell surface recognize different ligands on leukocytes; however, these ligands are not uniformly distributed on the leukocyte population, suggesting that different adhesion molecules could recruit distinct subsets of leukocytes. For example, the α4 integrins, which are ligands for VCAM-1, are expressed on lymphocytes, eosinophils, and monocytes but not neutrophils (1, 2). Thus the selective expression of one of these endothelial cell adhesion molecules could control the type of leukocytes that are targeted to the endothelium in different disease processes (1, 2). In allergic-like diseases, TNF-α and IL-4 are evident and VCAM-1 expression is selectively up-regulated. Concomitantly, there is a selective recruitment of eosinophils and lymphocytes to the endothelium. We propose that IL-4 plays a pivotal role at such sites by allowing VCAM-1 to be induced by levels of TNF-α that would not normally activate expression of any of the endothelial adhesion molecules. Then, the subsequent interaction between VCAM-1 on the endothelium and α4 integrins on leukocytes is responsible, at least in part, for the selective recruitment of leukocyte subsets.

In addition to simply combining with TNF-α to increase the level of VCAM-1, IL-4 also dramatically prolongs the appearance of VCAM-1 on the endothelial surface. Therefore, IL-4 could also mediate selective expression of VCAM-1 at sites where the TNF-α concentration is high enough for a general induction of endothelial adhesion molecules by prolonging the expression of VCAM-1 on the cell surface beyond the time when the other adhesion molecules disappear. Such prolonged VCAM-1 expression could have particular significance in allergic-like diseases, where there is classically a chronic leukocyte infiltrate.

The combination of transcriptional activation with subsequent mRNA stabilization is an efficient mechanism for synergistic activation of mRNA levels. We have shown previously that a similar mechanism regulates expression of the extracellular matrix protein, fibronectin: cAMP increases transcription and glucocorticoids stabilize the resulting transcripts (55). Clearly, a number of cytokines are present during inflammatory processes, and it is likely that different cytokine combinations will be found that selectively regulate the expression of other endothelial adhesion molecules. Conceivably, it is the differential regulation of expression of endothelial cell adhesion molecules that is responsible, at least in part, for the distinctive patterns of leukocyte infiltration seen in many diseases.

The mechanism of action of IL-4 has been studied most extensively in leukocytes, where it can have pleiotropic effects stimulating expression of some proteins and inhibiting others. Generally, IL-4 activity in leukocytes falls into two categories: transcriptional regulation and mRNA destabilization. Our finding that IL-4 stabilizes an mRNA in endothelial cells is a unique activity for this cytokine. Presumably, IL-4–mediated stabilization will involve a unique sequence with the VCAM-1 mRNA.
It will eventually be interesting to determine whether there is any relationship between the mechanism of IL-4-mediated mRNA stabilization in endothelial cells and the IL-4-mediated mechanism of mRNA destabilization in leukocytes.

Acknowledgments

We thank A. Levine for the gift of IL-4; M. Dustin for pCD1.8; M. Holtzman for use of the flow cytometer; J. Billadello for use of the Ambus; and J. McQuillan, A. Postigo, and A. Sheppard for helpful comments.

These studies were supported by grants from the National Institutes of Health (HL-47518, CA-49438, and AR-41908). M. F. Iademarco was supported by a Career Investigator Development Award from the National Institutes of Health (HL-03083).

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