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Del-1 is an endogenous inhibitor of leukocyte-endothelial adhesion limiting inflammatory cell recruitment[&]

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Abstract

Leukocyte recruitment to sites of infection or inflammation requires multiple adhesive events. While numerous players promoting leukocyte-endothelial interactions have been characterized, functionally important endogenous inhibitors of leukocyte adhesion have not been identified. Here, we describe the endothelial-derived secreted molecule, developmental endothelial locus-1 (Del-1), as an anti-adhesive factor that interferes with the integrin LFA-1-dependent leukocyte-endothelial adhesion. Endothelial Del-1-deficiency increased LFA-1-dependent leukocyte adhesion *in vitro* and *in vivo*. Del-1^{-/-} mice displayed significantly higher neutrophil accumulation in LPS-induced lung inflammation *in vivo*, which was reversed in Del-1/LFA-1-double deficient mice. Thus, Del-1 is an endogenous inhibitor of inflammatory cell recruitment and could provide a basis for targeting leukocyte-endothelial interactions in disease.

Leukocyte extravasation is integral to the response to infection or injury and to inflammation and autoimmunity. Leukocyte recruitment comprises a well coordinated cascade of adhesive events including selectin-mediated rolling, firm adhesion of leukocytes to endothelial cells and

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their subsequent transendothelial migration. The interaction between LFA-1 (α L β 2, CD11a/CD18) and endothelial ICAM-1 is crucial during firm endothelial adhesion of leukocytes (1-5). Whereas numerous adhesion receptors promoting inflammatory cell recruitment have been identified, very little information exists about endogenous inhibitors of the leukocyte adhesion cascade (1-7). Developmental endothelial locus-1 (Del-1) is a glycoprotein that is secreted by endothelial cells and can associate with the endothelial cell surface and the extracellular matrix (8-10). Del-1 is regulated upon hypoxia or vascular injury and has been implicated in vascular remodelling during angiogenesis (10-12). Here, we sought to determine whether endothelial-derived Del-1 participates in leukocyte-endothelial interactions. RT-PCR analysis revealed Del-1 mRNA predominantly in the brain and lung, with no expression in liver, spleen, or whole blood (Fig. 1A and fig. S1A). Del-1 was expressed in WT but not in Del-1^{-/-} murine lung endothelial cells (Fig. 1B, 9). Immunohistochemistry of lung tissues demonstrated the presence of Del-1 in vessels, as observed by co-staining with the endothelial marker PECAM-1 (fig. S1B).

To determine whether Del-1 participates in leukocyte recruitment interactions, we studied adhesion of primary neutrophils to immobilized Del-1. Mouse neutrophils specifically bound to Del-1 under static conditions. Adhesion was inhibited by a blocking monoclonal antibody (mAb) to CD11a (α L-integrin subunit), but not by antibodies to α v-integrin or β 1-integrin (Fig. 1C), suggesting that LFA-1 mediates the interaction of neutrophils with Del-1. Consistently, LFA-1^{-/-} neutrophils displayed reduced adhesion to Del-1 (Fig. 1C). The residual, LFA-1-independent binding of neutrophils to Del-1 was blocked by mAb to Mac-1 (fig. S2A), consistent with the fact that LFA-1 and Mac-1 are closely related and share several ligands (13). In addition, α L-transfected but not vector-transfected J- β 2.7 cells specifically bound to immobilized Del-1 (Fig. 1D), whereas a direct interaction between Del-1 and the ligand-binding I-domain of LFA-1, locked in the open high-affinity conformation, was observed (Fig. 1E and fig. S2B). These findings indicate that Del-1 is a ligand of LFA-1 integrin.

To address whether Del-1 participates in leukocyte-endothelial interactions, we studied neutrophil and monocyte adhesion to WT and Del-1^{-/-} endothelial cells (14,15). Contrary to our prediction, Del-1^{-/-} endothelial cells promoted significantly higher neutrophil and monocyte adhesion. LFA-1-deficiency on leukocytes and mAb to LFA-1 abolished the enhanced adhesion to Del-1^{-/-} endothelium (Fig. 2A and fig. S3). Thus, enhanced inflammatory cell adhesion to Del-1^{-/-} endothelium is specifically mediated by LFA-1 on leukocytes.

To understand the unexpected inhibitory role of Del-1 in leukocyte-endothelial adhesion, we addressed whether soluble Del-1 interfered with the interaction of LFA-1 with its major ligand, ICAM-1. Mn²⁺-induced binding of ICAM-1-Fc to murine leukocytes in solution was significantly inhibited by soluble Del-1 (Fig. 2B). Moreover, soluble Del-1 inhibited the LFA-1-dependent adhesion of WT neutrophils to immobilized ICAM-1 under physiologic flow conditions, whereas soluble Del-1 did not affect the weaker adhesion of LFA-1^{-/-} neutrophils to ICAM-1 (Fig. 2C).

The finding that endothelial Del-1 antagonizes LFA-1-dependent adhesion (Fig. 2A) appeared to be discordant with the finding that immobilized Del-1 promoted leukocyte adhesion under static conditions (Fig. 1C). We therefore assessed the ability of Del-1 and ICAM-1 to promote adhesion when co-immobilized with P-selectin and the chemokine MIP-2 under physiologic flow conditions at low and high shear rate (0.8 and 2 dynes/cm²). In this system leukocytes first roll on selectin and then arrest on the integrin ligand. Whereas ICAM-1 promoted robust firm adhesion of neutrophils under both shear rates, Del-1 promoted only weak adhesion under the lower shear rate and almost none at the higher shear rate (Fig. 2D). We then analyzed how the presence of plate-bound Del-1 would affect adhesion of neutrophils to ICAM-1 under flow. Increasing concentrations of Del-1 co-immobilized with ICAM-1, P-selectin and MIP-2

significantly inhibited neutrophil adhesion to ICAM-1 (Fig. 2E). Thus, although being a ligand of LFA-1, Del-1 does not promote firm leukocyte adhesion under flow but interferes with leukocyte adhesion to endothelial ICAM-1.

We then assessed the ability of soluble Del-1 expressed as an Fc fusion protein to inhibit neutrophil recruitment *in vivo* in acute thioglycollate-induced peritonitis (14). Intravenous administration of Del-1-Fc 30 min prior to thioglycollate injection significantly reduced neutrophil accumulation, as compared to Fc control protein (Fig. 2F). Similarly, ICAM-1-Fc reduced neutrophil recruitment into the peritoneum (Fig. 2F).

To provide further evidence for the role of Del-1 in inflammatory cell recruitment *in vivo*, we performed intravital microscopy using the dorsal skinfold chamber model (16). Del-1^{-/-} mice displayed increased numbers of leukocytes adherent to postcapillary venules both at baseline and upon TNF- α stimulation (Fig. 3A and B). Besides firm arrest, the interaction between LFA-1 and ICAM-1 contributes to slow rolling processes (17). A significant decrease in rolling velocity accompanied by an increase in the fraction of slow rolling leukocytes was observed in Del-1^{-/-} mice (Fig. 3C and 3D).

We further studied whether Del-1 could regulate inflammatory cell recruitment *in vivo*, by performing LPS-induced lung inflammation. Del-1^{-/-} mice displayed significantly higher accumulation of neutrophils in the bronchoalveolar lavage (BAL) fluid, as compared to WT mice (Fig. 4A). LFA-1^{-/-} mice displayed reduced neutrophil accumulation in the BAL upon LPS-induced lung inflammation (Fig. 4A), consistent with a previous report (18). The increased neutrophil recruitment *in vivo* due to Del-1 deficiency required the presence of LFA-1, as neutrophil accumulation in the BAL in Del-1^{-/-}LFA-1^{-/-} mice equaled accumulation of these cells in LFA-1^{-/-} mice (Fig. 4A). The increased leukocyte recruitment due to Del-1 deficiency could not be attributed to an alteration in peripheral blood counts, since constitutive leukocyte numbers were comparable in WT and Del-1^{-/-} mice (fig. S4). In addition, i.v. administration of soluble Del-1 efficiently reversed the increased neutrophil recruitment in Del-1^{-/-} mice (Fig. 4B). Furthermore, we found that Del-1-deficiency resulted in an upregulation of baseline ICAM-1 protein expression by lung endothelial cells, which was overridden upon TNF- α stimulation, whereas VCAM-1 expression was unaffected (fig. S5). No significant increase in ICAM-1 expression, under baseline or inflammatory conditions, was found in Del-1^{-/-} lungs (fig. S6), suggesting that altered ICAM-1 expression is not involved in the increased leukocyte recruitment to Del-1^{-/-} lungs. Moreover, whereas the increased neutrophil recruitment to the lung upon Del-1-deficiency was completely reversed by leukocyte LFA-1-deficiency (Fig. 4A), inhibition of ICAM-1 by a blocking mAb (18,20) decreased neutrophil recruitment by the same extent in both WT and Del-1^{-/-} mice (Fig. 4C), implying an involvement of other LFA-1 ligands. These findings suggest that Del-1 deficiency enhances LFA-1-dependent leukocyte recruitment *in vivo*.

We found Del-1 to act in an anti-inflammatory fashion, however the expression of Del-1 in inflammation has not been previously elucidated. We therefore analyzed Del-1 mRNA expression in the lung and in endothelial cells upon inflammatory stimulation. Upon LPS administration, lung Del-1 mRNA was significantly reduced (Fig. 4D). Likewise, TNF- α stimulation of endothelial cells induced a significant decrease in Del-1 expression (fig. S7).

Endogenous inhibitors exist in many aspects of inflammation and immunity (21,22), attenuating exuberant inflammatory and immune activation. To date, no endogenous inhibitor was known in the leukocyte adhesion cascade, a central paradigm of inflammation and immunity. Here, endothelial-derived Del-1 was identified to intercept LFA-1-dependent leukocyte-endothelial interactions. Given the importance of LFA-1-dependent leukocyte recruitment in several inflammatory and autoimmune disorders (13,23-25), Del-1 may provide

a platform for designing novel attractive therapeutic modalities to target leukocyte-endothelial interactions in disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References and Notes

1. Springer TA. Cell 1994;76:301. [PubMed: 7507411]
2. Hogg N, Laschinger M, Giles K, McDowall A. J Cell Sci 2003;116:4695. [PubMed: 14600256]
3. Vestweber D. Immunol Rev 2007;218:178. [PubMed: 17624953]
4. Imhof BA, Aurrand-Lions M. Nat Rev Immunol 2004;4:432. [PubMed: 15173832]
5. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Nat Rev Immunol 2007;7:678. [PubMed: 17717539]
6. Weber C, Fraemohs L, Dejana E. Nat Rev Immunol 2007;7:467. [PubMed: 17525755]
7. Chavakis T, Preissner KT, Herrmann M. Trends Immunol 2007;28:408. [PubMed: 17681885]
8. Hidai C, Kawana M, Kitano H, Kokubun S. Cell Tissue Res 2007;330:83. [PubMed: 17701220]
9. Hidai C, et al. Genes Dev 1998;12:21. [PubMed: 9420328]
10. Ho HK, et al. Circulation 2004;109:1314. [PubMed: 14981004]
11. Penta K, et al. J Biol Chem 1999;274:11101. [PubMed: 10196194]
12. Zhong J, et al. J Clin Invest 2003;112:30. [PubMed: 12840057]
13. Gahmberg CG, et al. Cell Mol Life Sci 1998;54:549. [PubMed: 9676574]
14. Materials and Methods are in the supporting online material.
15. Choi EY, et al. Blood 2008;111:3607. [PubMed: 18239087]
16. Fiedler U, et al. Nat Med 2006;12:235. [PubMed: 16462802]
17. Zarbock A, Lowell CA, Ley K. Immunity 2007;26:773. [PubMed: 17543554]
18. Basit A, et al. Am J Physiol Lung Cell Mol Physiol 2006;291:L200. [PubMed: 16461431]
19. Ding ZM, et al. J Immunol 1999;163:5029. [PubMed: 10528208]
20. Kumasaka T, et al. J Clin Invest 1996;97:2362. [PubMed: 8636417]
21. Parry RV, Riley JL, Ward SG. Trends Immunol 2007;28:161. [PubMed: 17336157]
22. Greenwald RJ, Freeman GJ, Sharpe AH. Annu Rev Immunol 2005;23:515. [PubMed: 15771580]
23. Leebwohl M, et al. N Engl J Med 2003;349:2004. [PubMed: 14627785]
24. Luster AD, Alon R, von Andrian UH. Nat Immunol 2005;6:1182. [PubMed: 16369557]
25. Yonekawa K, Harlan JM. J Leukoc Biol 2005;77:129. [PubMed: 15548573]

Figure 1. Del-1 is expressed in endothelial cells and interacts with leukocyte LFA-1

(A) Real-time RT-PCR demonstrating the expression of Del-1 mRNA in adult mouse tissues. Del-1 mRNA was normalized against 18S rRNA. (B) RT-PCR in primary lung endothelial cells from WT and Del-1^{-/-} mice. (C) Static adhesion of PMA-stimulated WT (open) or LFA-1^{-/-} (filled) neutrophils to immobilized BSA or mouse Del-1 is shown in the absence (-) or in the presence of mAbs to CD51 (α v-integrin), to CD29 (β 1-integrin), or to CD11a (LFA-1). Adhesion is presented as % adherent cells. Data are mean $\pm$ SD (n=3). **, P<0.01. (D) Adhesion of J- β 2.7 transfectants expressing LFA-1 (J- β 2.7/ α_L) or vector (J- β 2.7/vector) to immobilized Del-1 or ICAM-1. Adhesion is presented as % adherent cells. Data are mean $\pm$ SEM (n=3). **, P<0.01.

P<0.01. (E) Binding of the LFA-1 I-domain to immobilized Del-1 or ICAM-1. Specific binding is expressed as absorbance at 450 nm. Data are mean $\pm$ SEM (n=3).

Figure 2. Del-1 interferes with LFA-1-dependent leukocyte adhesion

(A) PMA-induced adhesion of WT neutrophils in the absence (open) or presence of isotype control antibody (light gray) or mAb to LFA-1 (dark gray), or of LFA-1^{-/-} neutrophils (filled) to WT or Del-1^{-/-} lung endothelial cells is shown. Adhesion is presented as % adherent cells. Data are mean $\pm$ SD (n=4). *, P<0.05. (B) Binding of soluble ICAM-1-Fc to mouse bone marrow mononuclear cells in the presence of MnCl₂. Cells were preincubated with BSA or soluble Del-1. Data are mean $\pm$ SEM (n=3). *, P<0.05. (C) Adhesion of WT (open) or LFA-1^{-/-} (filled) neutrophils to immobilized P-selectin, MIP-2 and ICAM-1 under flow (0.8 dyn/cm₂) was studied in the presence of mAb to CD11a or isotype control antibody (each mAb 10 µg/ml), or in the presence of BSA or mouse soluble Del-1 (each at 20 µg/ml). Adhesion is shown

as % of control i.e. adhesion of WT neutrophils in the presence of control antibody. Data are mean $\pm$ SEM (n=3). *, P<0.05. (D) Adhesion of WT neutrophils to immobilized P-selectin, MIP-2 and ICAM-1 (open) or Del-1 (filled) was studied at indicated shear rates. Adhesion is shown as number of adherent cells/field. Data are mean $\pm$ SEM (n=4). *, P<0.05. (E) Adhesion of WT neutrophils to immobilized P-selectin, MIP-2 and ICAM-1 was studied in the presence of increasing concentrations of Del-1 that was coimmobilized. Adhesion is shown as % of control i.e. adhesion of WT neutrophils in the absence of immobilized Del-1. Data are mean $\pm$ SEM (n=6). **, P<0.01. (F) The numbers of neutrophils at 4 h after i.p. injection of thioglycollate in WT mice are shown. Mice were treated 30 min prior to thioglycollate injection with i.v. injection of control Fc protein, Del-1-Fc or ICAM-1-Fc. Data are expressed as absolute numbers of emigrated neutrophils. Data are mean $\pm$ SD (n=4 mice/group). *, P<0.05.

Figure 3. Slow rolling and firm adhesion of inflammatory cells *in vivo* are enhanced due to Del-1 deficiency in the dorsal skinfold chamber model

(A) The number of leukocytes adherent to the endothelium of postcapillary venules were assessed in WT (filled) or Del-1^{-/-} (gray) mice at baseline conditions as well as 2 h after TNF- α superfusion. Adherent leukocytes are shown as number of cells per vessel surface (mm²). Data are mean $\pm$ SD (n=5 mice/group). *, P<0.05. (B) Representative images of Rhodamine 6G labeled leukocytes adherent onto the endothelium of postcapillary venules of WT and Del-1^{-/-} mice. (C) The average rolling leukocyte velocities at 2 h after TNF- α superfusion in WT (filled) and Del-1^{-/-} (gray) mice are shown. Data are mean $\pm$ SD (n=5 mice/group). **,

$P < 0.01$. (D) The rolling flux fraction at 2 h after $\text{TNF-}\alpha$ superfusion in WT (filled circles) and $\text{Del-1}^{-/-}$ (gray squares) mice.

Figure 4. Increased inflammatory cell recruitment *in vivo* due to Del-1 deficiency

(A) The numbers of neutrophils in the BAL fluid in WT, Del-1^{-/-}, LFA-1^{-/-} or Del-1^{-/-}LFA-1^{-/-} mice are shown at 24 h after nasal administration of saline (open) or LPS (filled). Neutrophil recruitment upon saline inhalation was negligible. Data are expressed as absolute numbers and are mean $\pm$ SEM (n=11-16 mice/group). **, P<0.01; ns, not significant. (B) Thirty min prior to LPS administration WT or Del-1^{-/-} mice received i.v. injections of BSA (control, open bars) or Del-1-Fc (filled bar) (each at 90 $\mu\text{g}/\text{mouse}$). Data are expressed as absolute numbers and are mean $\pm$ SEM (n=4-11 mice/group). *, P<0.05. (C) Thirty min prior to LPS administration WT or Del-1^{-/-} mice received i.v. injections of isotype control IgG (open) or anti-ICAM-1 (filled) (each at 85 $\mu\text{g}/\text{mouse}$). Data are expressed as absolute numbers and are mean $\pm$ SEM (n=8-9 mice/group). *, P<0.05. (D) The expression of Del-1 mRNA in mouse lungs at 0 h, 10 h or 24 h post intranasal LPS administration was analyzed by semiquantitative RT-PCR. The data are shown as relative expression. The ratio of Del-1 mRNA / actin mRNA at 0 h was set as 1. Data are mean $\pm$ SEM (n=4). *, P<0.05 as compared to 0 h.