

SUPPLEMENTAL MATERIAL

Supplemental Methods and Materials

Plasmid and Adenoviral Vector Generation

ECs that overexpress IL8RA and/or IL8RB were generated using adenoviral vectors that contain human IL8RA and/or IL8RB cDNAs and the green fluorescent protein (GFP) gene using the AdEasy™ Adenoviral Vector System (Stratagene)¹⁰ as summarized in **Supplemental Figure 1**. Full length human IL8RA (IL8 receptor alpha, Cat# MGC-40015, ATCC) and IL8RB (IL8 receptor beta, Cat# MGC-46215, ATCC) cDNAs in pCMV-SPORT6 vectors were purchased from American Type Culture Collection (ATCC). The shuttle vector pAdTrack-CMV which has the GFP gene controlled by the CMV promoter was purchased from Addgene (Cat# 16405). To construct the recombinant vectors, pAdTrack-IL8RA-GFP or pAdTrack-IL8RB-GFP, the IL8RA or IL8RB cDNAs were cut out from pCMV-SPORT6 host vectors by *Sal* I/*Xba* I or *Kpn* I/*Xba* I, respectively. The isolated IL8RA or IL8RB cDNAs were then subcloned into pAdTrack-CMV plasmids at the corresponding restriction sites. Expression of IL8RA, IL8RB and GFP is controlled by separate CMV promoters. An empty vector pAdTrack-Null-GFP that does not contain IL8RA or IL8RB cDNAs was also generated and served as a control. The pAdTrack-IL8RA-GFP, pAdTrack-IL8RB-GFP, and pAdTrack-Null-GFP plasmids were subcloned into pAdEasy-1 adenoviral backbone in BJ5185 cells and then amplified in XL10-Gold ultra-competent cells following the manufacturer's instruction (AdEasy™ Adenoviral Vector System, Stratagene Cat# 240010). The positive recombinant DNAs (pAd-IL8RA-GFP-, pAd-IL8RB-GFP and pAd-Null-GFP) were harvested and purified using the Qiagen® Plasmid Purification System.

The Ad-293 cells were used to generate adenoviral vectors containing the IL8RA or IL8RB cDNAs. Briefly, Ad-293 cells were plated at $7-8 \times 10^5$ cells per 60-mm tissue culture dish in 10% FBS DMED medium 24 hrs prior to transfection. The ~70% confluent cells were

transfected with *Pac* I-linearized pAd-IL8RA-GFP, pAd-IL8RB-GFP or pAd-Null-GFP recombinant DNAs using Lipofectamin LTX and PLUS transfection reagents from Invitrogen (Cat# 15338-100) and cultured in 10% FBS DMED medium for 7-10 days. When most of the cells became GFP+, the primary adenovirus was harvested by four-rounds of freezing/thawing. The adenoviral stocks were stored at -80°C until use.

Cell Migratory Activity and Chemotaxis assays.

EC migratory and chemotactic activity was assayed in a 96-well modified Boyden chamber (Millipore, Billerica, MA) using IL8RA, IL8RB, IL8RA/RB, AdNull and vehicle (not transduced) ECs. To test the IL8 dependence of EC chemoattractant activity, the bottom wells of the chamber were filled with endothelial growth medium contained 0, 20, 100, or 500 ng/ml of IL8. A polyvinylpyrrolidone-free polycarbonate filter plate with 3- μ m pores was placed over the samples, and 100 μ l of the EC suspension (2×10^6 cells/ml) were placed into the upper wells. The chambers were incubated in humidified air with 5% CO₂ at 37°C for 12 hrs. The upper portion was then removed, and four photomicrographs (200x) per well were digitally recorded using an Olympus IX70 microscope and Perkin-Elmer Ultraview image capture equipment. Cell counts were made from these images.

Preparation of Activated Neutrophils

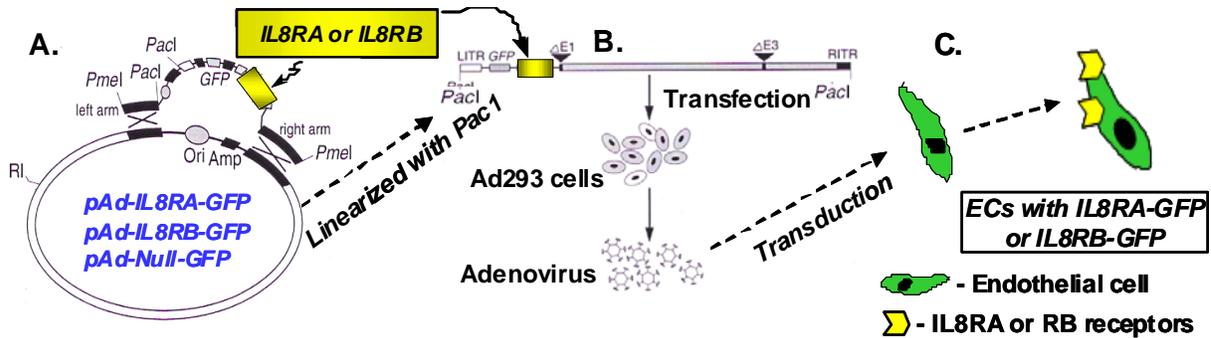
Human myeloid leukemia HL-60 cells (ATCC, Manassas, VA) were maintained in Iscove's modified medium (ATCC) supplemented with 10% fetal calf serum, 50 μ g/ml streptomycin, 2 U/ml penicillin, and 2 mM L-glutamine. For differentiation, cells (3×10^5 /ml) were incubated in the presence of 1.3% (vol/vol) DMSO for 4~6 days (Newburger PE) before using for transduced EC and neutrophil competition study.

Supplemental Table 1. Selective sense and antisense PCR primers for real-time quantitative RT-PCR Analysis

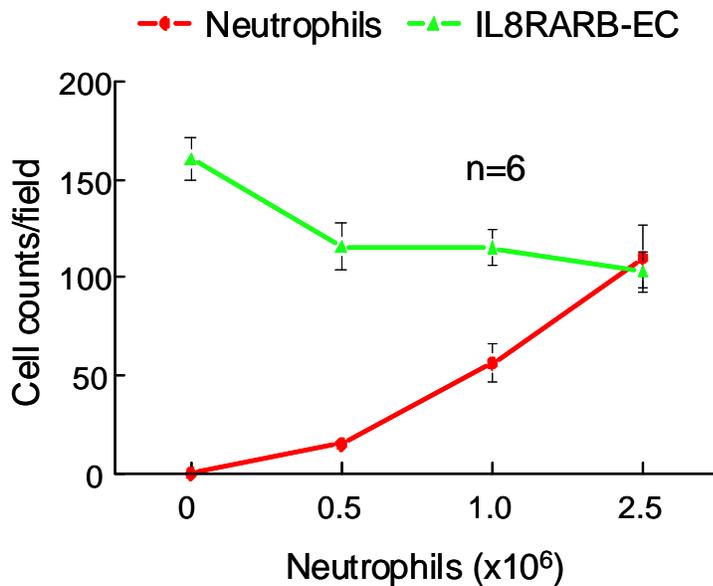
Gene	Primer Sense Sequence	Primer Antisense Sequence	PCR Product (bp)
CINC-2 β	TCAGGGACTGTTGTGG	TGACTTCTGTCTGGGTG	127
TNF- α	CTTATCTACTCCCAGGTTCTCTTCAA	GAGACTCCTCCCAGGTACATGG	200
MCP-1	ATGCAGGTCTCTGTCACGCT	GGTGCTGAAGTCCTTAGGGT	345
P-Selectin	AATGAAATCGCTCACCTC	TTATTGGGCTCGTTGTCT	152
VCAM-1	GGGGATTCCGTTGTTCT	CAGGGCTCAGCGTCAGT	136
IL-1 β	GGATGATGACGACCTGC	CTTGTTGGCTTATGTTCTG	146
IL-6	CAAAGCCAGAGTCCATTCAGAGC	GGTCCTTAGCCACTCCTTCTGT	151
IL-10	AAAGCAAGGCAGTGGAGCAG	TCAAACCTCATTGATGGCCTTGT	81
Ribosomal Protein S9	GCTGGATGAGGGCAAGAT	CGAACAATGAAAGATGGGAT	192

CINC-2 β - cytokine induced neutrophil chemoattractant-2-beta (equivalent to human IL-8).
TNF- α - tumor necrosis factor-alpha; **MCP-1** - monocyte chemotactic protein-1; **VCAM-1** - vascular cell adhesion molecule 1; **IL-1 β** - Interleukin-1 beta ; **IL-6** - Interleukin-6 ; **IL-10** - Interleukin-10

Supplemental Figures and Figure Legends

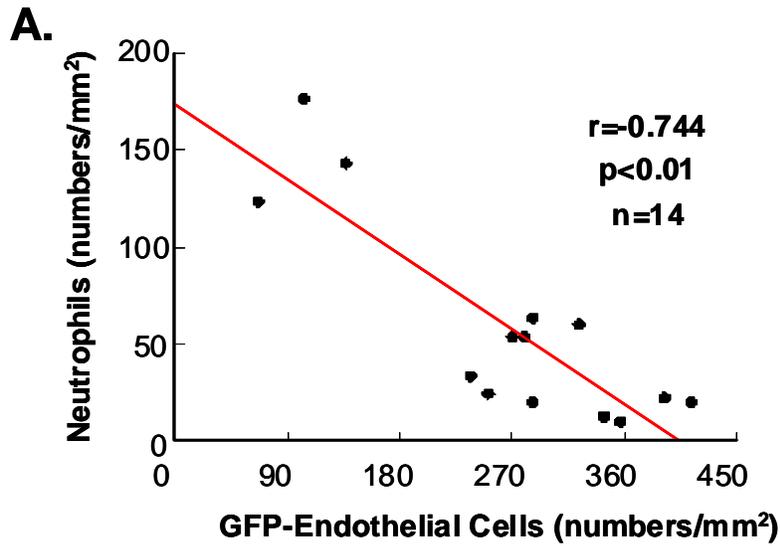


Online Supplemental Figure 1: Schematic illustration of the generation of rat aortic endothelial cells (ECs) that over-express IL8RA or IL8RB. Human IL8RA and IL8RB cDNAs (2,026 and 2930 bp, respectively) in the pCMV-SPORT6 plasmid were purchased from ATCC and subcloned to the shuttle plasmid pAdTrack-CMV (not shown here) containing green fluorescent protein (GFP) as a marker. The Pme I-linearized plasmid was transformed into BJ5183 cells (not shown here) carry the backbone of the replication deficient adenovirus vector. **(A)** Cells with the recombinant DNA were selected and amplified in XL10-Gold ultracompetent cells, which allowed for insert stability. **(B)** The Pac I-linearized plasmid was then transfected into the Ad293 cells, which provided the complementary adenoviral proteins necessary for replication. The adenovirus was subsequently amplified. After 7-10 days, the adenovirus was harvested from lysed Ad293 cells, and **(C)** used to transduce ECs, causing them to overexpress the IL8RA, IL8RB, or both (double transduction). pAd-Null-GFP is the empty vector used as a control.

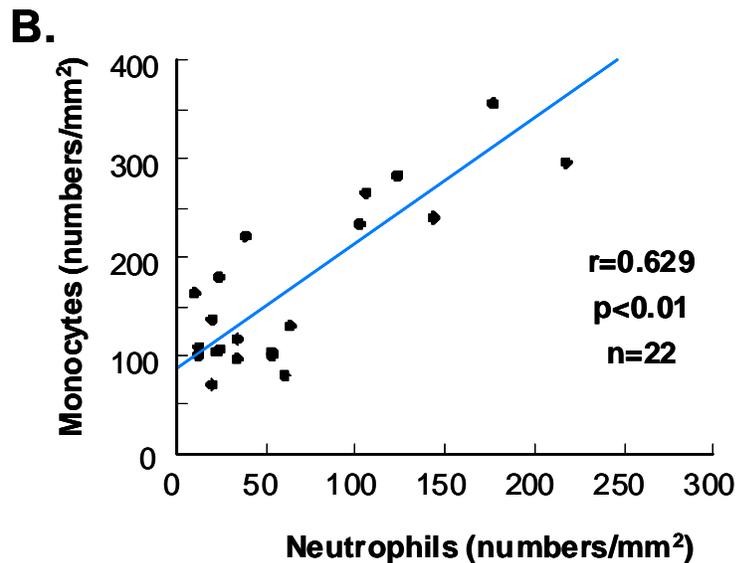


Online Supplemental Figure 2: Neutrophils inhibited binding of IL8RA/RB-ECs to endothelial monolayer in vitro. Endothelial monolayer was stimulated with TNF- α (10 ng/ml) for 16 hrs before various concentrations of neutrophils (labeled with CellTracker CM-Dil, red color) applied to the monolayer. IL8RA/RB-ECs (GFP color) were added 30 min after the application of neutrophils and incubated for additional 1 hr before cell (red neutrophils and green ECs) counting in the same well. Results are means \pm SEM; n=wells per group.

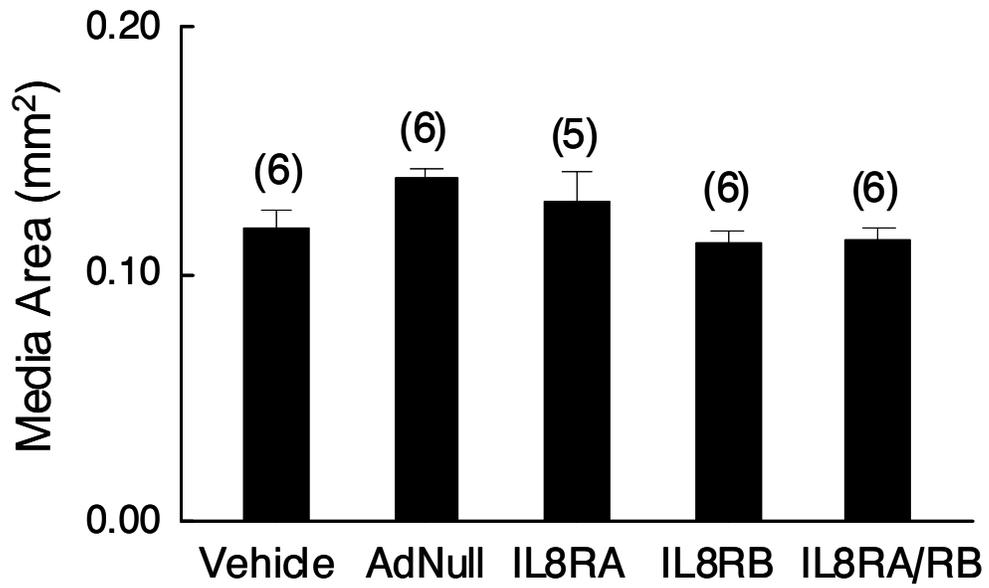
Negative Correlation of Neutrophils and Transfused ECs in Adventitia of Injured Carotid Arteries



Positive Correlation of Monocytes/Macrophages and Neutrophils in Adventitia of Injured Carotid Arteries



Online Supplemental Figure 3: (A) Negative correlation of infiltrated neutrophils (MPO immunohistochemical stained) and transfused ECs (GFP immunohistochemical stained) in adventitia of balloon injured rat carotid arteries 24 hrs post injury. Samples included rats transfused with IL8RA, IL8RB, IL8RA/RB, or AdNull ECs. (B) Positive correlation of infiltrated monocytes/macrophages (ED-1 immunohistochemical stained) and neutrophils (MPO stained) in adventitia of balloon injured rat carotid arteries 24 hrs post injury. Samples included rats transfused with IL8RA, IL8RB, IL8RA/RB, or AdNull ECs.



Online Supplemental Figure 4: Medial area of injured right carotid arteries at 4-wk post balloon injury. Results are means \pm SEM. (n)=number of rats