Endothelial Determinants of Dendritic Cell Adhesion and Migration
New Implications for Vascular Diseases

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Objective—Atherosclerosis is a chronic disease triggered by endothelial injury and sustained by inflammation. Dendritic cells (DCs) are critical for the cell-mediated arm of an immune response and are known to influence inflammatory immunity. A fundamental aspect of DC function is their capacity to adhere and migrate through vascular endothelial cells (ECs). We investigated the role of endothelial activation and dysregulation of the NO pathway on DC adhesion and migration.

Methods and Results—We discovered that DC adhesion and migration are modulated by changes in endothelial function. DC adhesion and transmigration were markedly increased after exposing ECs to hypoxia, oxidized low density lipoprotein, or tumor necrosis factor-α. Specifically, inhibition of endothelial NO synthase increased DC binding and transmigration. L-Arginine or 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibition partially decreased DC-EC interaction.

Conclusions—The results of this study suggest that the adhesion and migration of DCs are increased by stimuli known to accelerate atherogenesis. Vice versa, augmentation of endothelial NO synthase activity prevents DC adhesion. These findings may provide insight into the inflammatory processes occurring in atherosclerosis. Because DCs control immunity, regulating DC-EC interaction may be relevant to inflammation and atherogenesis. (Arterioscler Thromb Vasc Biol. 2002;22:1817-1823.)

Key Words: atherosclerosis ■ 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors ■ inflammation ■ nitric oxide

Atherosclerosis begins as an inflammatory immunological disease.1 Atherosclerotic lesions are the result of a series of highly specific cellular and molecular responses to various endogenous risk factors and antigenic stimuli. The inflammatory response is mediated by functional changes in endothelial cells (ECs), T lymphocytes, monocyte-derived macrophages, and smooth muscle cells.2 Activation of these cells leads to the elaboration of a wide spectrum of inflammatory hydrolases, cytokines, chemokines, adhesion molecules, and growth factors, together with lipid accumulation and proliferation of smooth muscle cells and fibroblasts.1,2 Immuno-histochemical studies have revealed mononuclear cell infiltrations and accumulation of activated T cells in early and late atherosclerotic lesions.3 The mechanisms of T-cell activation and antigen acquisition in atherogenesis are incompletely characterized. Recently, T-cell activation has been attributed to dendritic cells (DCs).

DCs are derived from monocyte precursors and are believed to play a central role in controlling immunity by activating T lymphocytes.4 DCs are involved in the initiation and progression of various diseases, including tumors, viral and bacterial infections, autoimmune diseases, and transplant rejection.4 A fundamental aspect of DC function is the capacity of DCs to migrate. They perform ongoing surveillance for incoming antigens, which induce them to stimulate T cells in secondary lymphoid organs.5 To exit from the bloodstream, DCs first need to tether to the ECs. E- and P-selectins mediate this initial process of endothelial DC rolling.6 The initial contact is followed by the development of a more firm adhesion between DCs and ECs, which is postulated to be required for subsequent transmigration. Mediators involved in DC adhesion and endothelial transmigration include CC-chemokines, lymphocyte function–associated antigen [LFA]-1 and CD11b, intercellular adhesion molecule (ICAM)-2, and its ligand the DC-specific C-type lectin DC-SIGN.7–9 Previous studies investigating vascular DCs have demonstrated their occurrence in atherosclerotic lesions as well as in arterial regions that are known to be
Endothelium-derived NO inhibits endothelium-leukocyte interaction. NO decreases the expression of adhesion molecules such as CD11/CD18, P-selectin, vascular cell adhesion molecule (VCAM)-1, and ICAM-1. Moreover, endothelial NO is a survival factor, protecting the vasculature from apoptosis and oxidative damage. Endothelial activation and dysfunction, as well as increased vascular inflammatory stimulation, precede the development of atherosclerosis and transplant vasculopathy. Although the general principles of leukocyte trafficking are similar, it is becoming increasingly apparent that the underlying cellular and molecular mechanisms are highly dependent on the inflammatory stimulus, the specific leukocyte population, and the tissue involved. We hypothesized that DC adhesion and migration are modulated by endothelial activation. In the present study, we characterize the capacity of blood DCs to adhere to unstimulated and activated/apoptotic macrovascular and microvascular endothelium. We further characterized DC interaction (adhesion and transmigration) with ECs after modulation of the endothelial NO synthase (NOS) pathway. Finally, we describe potential molecular regulators of DC adhesion to activated ECs.

**Methods**

**Generation of DCs**

**Production of Autologous MCM**

We adopted techniques from Thurner et al to produce monocyte-conditioned medium (MCM). IgG-coated bacteriological plates (85 mm, Falcon) were prepared not later than 30 minutes before use. The plates were coated with D-PBS (10 mL, BioWhittaker) supplemented with IgG for 30 minutes at room temperature. After the plates were coated, they were rinsed twice with D-PBS. Peripheral blood mononuclear cells (PBMCs, 8 × 10⁷) were plated in RPMI 1640 (BioWhittaker) containing 1% autologous serum for 24 hours (37°C, 5% CO₂). Supernatant was harvested and then centrifuged (1350g, 5 minutes, 25°C) and sterile filtered (0.22 µm, Millipore) to divide into aliquots and stored at −20°C.

**Isolation of Monocytes**

Peripheral blood was obtained from the Stanford Medical School Blood Center as standard buffy coat preparations from normal donors. The buffy coat (35 mL, mixed at 1:1 with PBS) was layered over Ficoll (15 mL) and centrifuged (30 minutes, 1300g, room temperature). PBMCs were harvested from the interface and washed 3 times with PBS containing 1% autologous serum. PBMCs were depleted of T, B, and natural killer cells by means of an immunomagnetic technique (Dynabeads, No. 113.09, Dynal). The kit contains anti-CD2, -CD7, -CD16 (a and b), -CD56, and -CD19 monoclonal antibodies. Dynabeads were washed 4 times with PBS containing 1% autologous serum. PBMCs (10⁵/mL) were incubated with magnetic beads at 4°C for 30 minutes by using the Dynal mixer. Lymphocytes were then depleted by means of the Dynal magnet. Nonadherent cells were harvested and washed once, and depletion was repeated once. The Dynal kit isolates monocytes with >98% viability and >94% yield and purity. Fluorescence-activated cell sorter scan revealed the starting cell population to be CD14+ (85% of total monocytes), while CD19+ cells were <5%. These cells are referred to below as monocytes.

**Generation of Immature and Mature DCs**

**From Monocytes**

We adopted techniques from Romani et al to generate immature DCs (or precursor DCs, referred to as pDCs; 7 days of culture) and mature DCs (10 days of culture, referred as DCs). Lymphocyte-depleted PBMCs were plated in 6-well culture plates at a density of 2 × 10⁷ cells per well in complete medium (CM; 3 mL). CM was RPMI 1640 containing 1% autologous serum supplemented with granulocyte-macrophage colony–stimulating factor (800 U/mL, Peprotech) and interleukin-4 (1000 U/mL, Peprotech). Cultures were fed every other day (days 2, 4, and 6) by removing 1.5 mL and adding back 1.5 mL fresh CM. On day 7, buoyant cells were harvested, depleted once again, and split to analyze, or they were taken for experiments as pDCs or transferred to new 6-well plates and further cultured. Seven-day pDCs were kept in culture until day 10 in the presence of MCM and CM at a ratio of 1:3 and were finally harvested and analyzed, or they were used for experiments as mature DCs. DCs and pDCs were labeled with 5-chloromethylfluorescein diacetate (CellTracker Green CMFDA, Molecular Probes) and used for adhesion/transmigration assays not later than 1 hour after labeling.

**EC Culture**

Human dermal microvascular ECs (HMVECs) and human aortic ECs (HAECs) were purchased from Clonetics and cultured in EC basal medium (Clonetics) supplemented with Bulletkit (Clonetics), including 10% FBS, human epidermal growth factor (10 ng/mL), bovine brain extract (12 µg/mL), hydrocortisone (1.0 µg/mL), gentamicin (50 µg/mL), and amphotericin B (50 ng/mL). ECs were grown at 37°C in a humidified 5% CO₂ and 95% air atmosphere. HAECs and HMVECs were used between passages 3 and 6. In some experiments, ECs were exposed to 2% O₂ for 24 hours by using hypoxic chambers (Billups-Rothenberg) and a gas analyzer.

**Adhesion Assay**

Human ECs exposed to the above conditions and cultured in 8-well chamber slides (LabTekII) were washed with HBSS (Irvine Scientific) containing (in mmol/L) CaCl₂ 2, MgCl₂ 2, and HEPES 20. Fluorescence labeling of DCs was achieved by incubating DCs with 1:2000 dilution (in binding buffer to remove cytokines and growth factors) of CFMFA twice for 30 minutes each, followed by washing to remove free dye. Chamber slides were then placed on a rotating platform, and fluorescently labeled DCs (2 × 10⁵/mL) were incubated with ECs for 4 hours at 37°C under constant flow conditions. Medium was aspirated and replaced with fresh HBSS to remove nonadherent cells. After a second washing, dishes were returned to the rocker platform for an additional 5 minutes. Medium was again aspirated and replaced with HBSS containing 3.7% paraformaldehyde. In some experiments, the cells were incubated with fluorescent annexin V (annexin V, Alexa Fluor 594, Molecular Probes) over 30 minutes before fixation for detection of early apoptosis. After 20 minutes of fixation and washing, adherent cells were labeled by using a confocal microscope. All adhesion experiments were performed with ≥5 different preparations of DCs at least in triplicate. Endothelial adhesion of prestained DCs was analyzed in 6 independent high-power fields for each experiment (see Confocal Microscopy).

**Transmigration Assay**

For transmigration assays, fibronectin-coated transwell inserts (Millipore) were used to culture endothelial monolayers in 24-well plates. Just before the assay, the monolayers were rinsed with serum-free HBSS, and prelabeled DCs were added to the upper chamber. At the same time, 200 µL serum-free medium, supplemented with 100 ng/mL monocyte chemotactic protein (MCP)-1 (R&D Systems), was added to the lower chamber. Transmigration was stopped after 2, 4, and 8 hours, and transmigrated cells were immediately counted in the lower chamber with the use of a fluorescence multimeter (GENios, Tecan). Relative fluorescence intensity has been shown to increase linearly with cell number. The migration index was calculated on the basis of the ratio between the absolute numbers of migrated cells to the DC-EC binding ratio (DC adhesion and transmigration were determined from the same well). All transmigration experiments were performed with ≥5 different preparations of DC-CM at least in triplicate.
were performed with ≥5 different preparations of DCs at least in duplicate.

Confocal Microscopy
Imaging was performed at Stanford’s Cell Sciences Imaging Facility on a MultiProbe 2010 laser confocal microscope (Molecular Dynamics). The MultiProbe uses an argon/krypton mixed gas laser with excitation lines of 488, 568, and 647 nm and is built on a Nikon Diaphot 200 inverted microscope. To collect the dual signal, the sample was excited with 488- and 568-nm lines, and the emitted light was passed through a 488/568/647-nm triple dichroic beam splitter. The emitted light corresponding to FITC was collected with a 515- to 545-nm bandpass filter, and Alexa Fluor 594 emission was collected with a 590-nm long-pass filter. A Nikon ×60 (numerical aperture 1.4, Planapo) objective was used. Images of serial optical sections were recorded every 1.5 μm per vertical step along the z-axis. Five sections in each of the 6 random fields were analyzed offline for each experiment in a blinded fashion, and each experiment was performed at least in triplicate. The average number of DCs adhering to ECs was calculated and expressed as the DC/EC ratio. Interobserver and intraobserver variability was ≤4.5%.

Drugs
The following drugs were used: The substrate for NOS, L-arginine (Sigma), was used to enhance NO synthesis, and the calcium ionophore (A23187, Sigma) was used as a receptor-independent stimulus of NOS. Asymmetric dimethyl L-arginine (ADMA, Sigma), an endogenous NO inhibitor, was used to block endothelial NO activity. Cervinastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitor (statin), was kindly provided by Bayer AG (Leverkusen, Germany). Atorvastatin was a gift from U. Laufs (Homburg, Germany). Oxidized LDL (oxLDL) was prepared by incubation of LDL (100 μg/mL) with CuSO₄ (10 μg/mL) at 37°C for 24 hours and donated by P. Tsao (Stanford University, Stanford, Calif).

Blocking Monoclonal Antibodies
Endothelial dysfunction may be associated with enhanced expression of the adhesion molecules ICAM-1, VCAM-1, and platelet and endothelial cell adhesion molecule (PECAM)-1.18 Therefore, we were interested in describing their roles as potential regulators of DC interaction with activated ECs by the use of blocking antibodies. Anti-human PECAM-1 (HEC7, 20 μg/mL), VCAM-1 (1G11, 50 μg/mL), and LFA-1α (CD11a, TS1/22, 50 μg/mL) were obtained from Endogen, and anti-ICAM-1 (11C91, 50 μg/mL) was purchased from R&D Systems. All monoclonal antibodies have been previously characterized as specific blocking antibodies in ECs.19,20

Statistical Analysis
Data are expressed as mean±SEM. Comparisons of multiple mean values were by ANOVA, followed by the Student-Newman-Keuls post hoc test for statistical significance. A value of P<0.05 was accepted as statistically significant.

Results
Characterization of Cultured DCs
HLA-DR+, CD14+, CD11c+, and lineage-negative DC precursors were cultured for 7 days in serum-free media supplemented with granulocyte-macrophage colony-stimulating factor and interleukin-4 to give rise to immature and mature DCs. Immature DCs were characterized as CD80+/−, CD1a+, and CD11c+. Further cultivation with MCM until day 11 yielded mature DCs with upregulated CD80, CD83, and CD86 (please see online Figure 1, available at http://atvb.ahajournals.org).
sion molecules. Anti–ICAM-1 antibody partially inhibited DC adhesion to normoxic ECs and hypoxic ECs. Anti–VCAM-1 and anti-PECAM antibodies did not affect DC binding to unstimulated ECs, whereas anti–PECAM-1 antibody partially inhibited DC adhesion to hypoxic ECs. Preincubation of DCs with anti–LFA-1 (DC counterreceptor to ICAM-1) inhibited DC adhesion on hypoxic ECs. Combined blockade of LFA-1 and ICAM-1 inhibited DC adhesion between 62% and 84%. Results in Table 2 are shown for HMVECs and could be confirmed by using HAECs (data not shown).

Figure 1. DC adhesion on activated human microvascular ECs. Fluorescence-stained unstimulated DCs (white) were incubated on HMVECs (gray) activated by TNF-α (100 ng/mL), oxLDL (10 μg/mL), or hypoxia (2% O2). DC adhesion was significantly increased on activated endothelium compared with nonactivated endothelium (P<0.01 and **P<0.001 vs control). The NOS agonist A23187 partially suppressed DC adhesion on activated endothelium (§P<0.01 vs hypoxia+oxLDL). Representative images of 5 experiments (5 different preparations of DCs were analyzed in triplicate) are depicted at the top.

Figure 2. Role of NO in DC adhesion on ECs. Fluorescence-stained unstimulated DCs (white) were incubated on HMVECs (gray) pre-treated with ADMA (1 to 10 μmol/L), ADMA (10 μmol/L)+L-arginine (1 mmol/L), and cerivastatin (CEV, 0.01 μmol/L) or atorvastatin (ATV, 0.01 μmol/L) for 24 hours. ADMA concentration-dependently increased DC adhesion, which was partially inhibited by L-arginine. In contrast, statin treatment downregulated DC adhesion. *P<0.0001 and **P<0.01 vs control; §P<0.01 vs ADMA (10 μmol/L).
Transmigration of DCs Through HMVECs

Endothelial migration of DCs is a crucial step after endothelial adhesion. To uncover factors relevant for DC migration, a migration index was calculated on the basis of the ratio between the absolute numbers of migrated cells to the DC/EC binding ratio. EC activation with TNF-α (100 ng/mL), oxLDL (10 μg/mL), or hypoxia enhanced DC migration by 23±7%, 48±13%, and 19±10%, respectively (P<0.01 versus unstimulated ECs).

DC migration was unaffected by preincubation with blocking ICAM-1 and VCAM-1 antibodies and was slightly decreased by LFA-1 blockade. In contrast, inhibition of PECAM-1 reduced DC migration by 52% (Figure 3a). The combined use of ICAM-1 and PECAM-1 antibodies had no further inhibitory effect on DC migration (Figure 3a). However, the absolute number of transmigrated DCs (net effect of adhesion and migration) was significantly reduced by the combined use of ICAM-1 and PECAM-1 antibodies (−72±14% versus −45±9% in ECs treated with anti–ICAM-1 and −24±4% in ECs treated with anti–ICAM-1, P<0.001).

ADMA (10 μmol/L) time-dependently enhanced DC migration, whereas cerivastatin and atorvastatin significantly blocked DC migration. The latter effect was inhibited in part by 10 μmol/L ADMA (38±20% inhibition, P<0.05). EC preincubation with calcium ionophore (1 μmol/L) significantly suppressed DC migration (Figure 3b). We could partially abrogate the A23187 effects on DC migration by use of coincubation with 100 μmol/L Nω-monomethyl-L-arginine (−39±7%, P<0.01) or 10 μmol/L ADMA (−48±15%, P<0.001). Symmetric dimethylarginine, which is not an inhibitor of NOS, had no effect on A23187-mediated blockade of DC migration.

Discussion

The salient findings of this investigation are as follows: (1) endothelial adhesion and migration of DCs is increased by stimuli known to accelerate atherosclerosis, (2) enhanced endothelial NO activity inhibits DC-EC interaction, and (3) HMG-CoA reductase inhibition reduces endothelial DC transmigration.

Inflammation and Atherosclerosis

T lymphocytes and macrophages are present in atherosclerotic plaques as components of the inflammatory process.
oxLDL, TNF-α, or hypoxia are much more vulnerable to DC attachment and transmigration than are nonactivated ECs. Of interest, EC apoptosis markedly enhanced DC adhesion. These results support the concept that endothelial activation accelerates DC-mediated immune activation, finally leading to enhanced vascular inflammation. In this regard, it has been shown that accumulation of DCs occurs in atherosclerotic areas and in vascular regions predisposed to atherosclerosis, i.e., those regions exposed to high cyclic strain and low shear stress. These vascular regions are also sites of endothelial dysfunction.

Evidence suggests that NO is an endogenous antiatherogenic molecule. Accordingly, we wished to characterize its role in EC-DC interaction. We demonstrated that DC interaction (adhesion and transmigration) with ECs is strongly enhanced after blocking endothelial NOS activity by using the endogenous NOS inhibitor ADMA. Moreover, the precursor of NOS, L-arginine, partially abolished the increased DC adhesion to ECs exposed to ADMA or oxLDL. Calcium ionophore as a receptor-independent stimulus of NOS strongly diminished DC adhesion and transmigration. These results extend previous investigations that revealed that endogenous NO can decrease endothelial monocyte adhesion. Specific mechanisms by which NO interferes with DC transmigration might include alterations in adhesion molecule expression, changes in endothelial permeability, and modification of gap junction function. Moreover, data from one of our earlier reports suggest that NO activity may regulate monocyte migration by affecting EC oxidative stress. Future studies should focus on the role of oxidative stress and nuclear factor-κB-regulated transcriptional pathways on DC-EC interaction.

HMG-CoA Reductase Inhibition and DC-EC Interaction
Statins have been shown to protect endothelial function, and they are powerful drugs for the prevention of cardiovascular diseases. The beneficial effects of statins may be partially independent of their lipid-lowering potential. Statins increase endothelial NO bioactivity, decrease endothelial apoptosis, and inhibit smooth muscle cell proliferation. In the present study, we demonstrate for the first time that physiological concentrations of statins decrease DC adhesion and transmigration. Statin-mediated effects were at least in part mediated by NO, inasmuch as coinubcation with the NOS inhibitor ADMA diminished the effect of statins. Thus, by enhancing NO synthesis, statins may diminish inflammatory activity (e.g., DC invasion and T-cell activation) in the phase of early atherosclerosis.

DC Invasion and Adhesion Molecules
To determine which adhesion molecules might be mediating enhanced DC adhesion on activated ECs, functional binding studies were performed. DC adhesion to hypoxic ECs was partially mediated by ICAM-1, whereas monocyte binding was primarily mediated by VCAM-1. Notably, stimulation of NO by the calcium ionophore reduced ICAM-1 expression on ECs (data not shown).

Unlike adhesion, migration of DCs is not blocked by anti–ICAM-1 antibodies. Our investigation that DC migration is reduced by PECAM-1 antibodies extends previous findings by D’Amico et al obtained with human umbilical vein ECs to primary microvascular ECs. Moreover, we demonstrate for the first time that the combined use of anti–ICAM-1 and anti–PECAM-1 antibodies inhibits the absolute number of transmigrated DCs (as a net effect of adhesion and transmigration) by 70%. The scheme in Figure 4 illustrates the role of adhesion molecules ICAM-1, PECAM-1, and VCAM-1 in EC-DC interaction. Other potential adhesion molecules that might be responsible for endothelial adhesion or transmigration of DCs are CD18 and ICAM-2.

In summary, DC adhesion and transmigration is enhanced by TNF-α, oxLDL cholesterol, and hypoxia, effects that are partially mediated by the adhesion molecules ICAM-1 (adhesion) and PECAM-1 (transmigration). Stimulation of the endothelium to release NO inhibits adhesion and transmigration of DCs, whereas inhibition of NO synthesis increases DC interaction with the endothelium. Statins decrease DC adhesion and transmigration, an effect mediated in part by NO.

In conclusion, we have characterized important determinants of DC adhesion to ECs. The present study indicates that alterations in endothelial function have a significant impact on DC adherence to the endothelium. Endothelial activation and DC recruitment may play an important role in atherosclerosis and transplant vasculopathy. Delineation of the determinants of DC-EC interaction may lead to new therapeutic avenues. Inhibition of DC-EC interaction may have an application in reducing the progression of cardiovascular disease. Conversely, enhancing DC-EC interaction may be a novel strategy for immunotherapy by initiating T-cell responses against cancer and viral and bacterial infections.

Acknowledgments
This study was supported in part by grants from the National Institutes of Health (grant CA09151-26 to Drs Schlichting and Engleman) and the National Heart, Lung, and Blood Institute (grant R01 HL-58638 to Dr Cooke). Dr Schlichting was a fellow of the Stanford University Tumor Biology Training Program. Dr Weis was supported by the German Society of Cardiology, Germany. Dr Cooke is an Established Investigator of the American Heart Association.

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Arterioscler Thromb Vasc Biol. 2002;22:1817-1823; originally published online September 5, 2002;
doi: 10.1161/01.ATV.000036418.04998.D5
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/22/11/1817

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Figure I
Flow cytometric evaluation of DC progenitors (pDC), immature DCs and mature DCs

Figure Ia
The typical phenotype of DC progenitors (pDC) generated from PBMC is shown. All cells in Box A1 (SSC/FSC scan) are HLA-DR positive. As further outlined these HLA-DR positive pDC are CD3-, CD19-, CD56-, CD1a-, CD80-, CD83-, Fas-L+/-, CD86+/- and CD14+ and CD11c+. HLA-DR+, CD14+, CD11c+ and lineage negative pDC have been processed further and were cultured for 7 days in serum free media supplemented with GM-CSF and IL-4 to give rise to immature and mature DC.

Figure Ib
After 7 day of culture with serum free media supplemented with GM-CSF and IL-4 pDC became immature DCs as indicated by their lin- and CD80- phenotype. These cells are CD80+/-, CD1a+ and CD11c+ (panel A and B). Further cultivation with monocyte conditioned media till day 11 yields mature DC with upregulated CD80, CD83 and CD86 (panel C).
Fig. Ib

- **A**
  - Lin⁻/HLA-DR
  - HLA-DR/CD1a
  - HLA-DR/CD11c

- **B**
  - HLA-DR/CD80
  - HLA-DR/CD83
  - HLA-DR/CD86

- **C**
  - HLA-DR/CD80
  - HLA-DR/CD83
  - HLA-DR/CD86