

Endothelial cell-matrix interactions determine maturation of dendritic cells

Heiko Methe¹, Shmuel Hess¹ and Elazer R. Edelman^{1,2}

¹ Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, USA

² Cardiovascular Division, Brigham and Women's Hospital, Harvard Medical School, Boston, USA

The fate of allo- and xenogeneic endothelial cell (EC) implants is regulated by EC-matrix interactions. While free EC are destroyed by a vigorous immune reaction, EC embedded within 3D collagen cells are well tolerated. Given the critical role DC serve in immune reactivity, we hypothesized that EC-driven DC maturation depends on EC-matrix contact. In marked contrast to DC co-cultured with a cytokine cocktail or with allo- and xenogeneic EC grown to confluence on 2D tissue culture plates, DC exposed to 3D matrix-embedded allo- and xenogeneic EC failed to mature, retaining their endocytic activity and exhibiting significantly reduced expression of maturation markers (costimulatory molecules, HLA-DR, CD83; $p < 0.01$). Matrix-embedded EC also limited cytokine-induced maturation and activity of DC. Incubation with matrix-embedded EC inhibited DC induction of allogeneic lymphocyte proliferation ($p < 0.002$) and EC cross-activation (ICAM-1, VCAM-1, HLA-DR, TLR2 and 4; $p < 0.01$). The endothelium in its quiescent state is confluent and substrate adherent. The former ensures secretion of growth inhibitors rather than promoters, and the latter may ensure immune acceptance. We now demonstrate for the first time that interactions of EC with an underlying 3D matrix affect the ability of EC to drive DC maturation.

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Introduction

DC are antigen-presenting cells that play a central role in priming the immune response to foreign antigens. Immature DC constantly screen for and internalize antigens efficiently through endocytosis, but exhibit limited ability to stimulate naive T cells until they mature

[1]. Maturation of DC is triggered by endogenous and/or exogenous mediators including pro-inflammatory cytokines, bacterial or viral components, and interactions with molecules of the TNF receptor family [2]. Up-regulation of MHC and costimulatory molecule expression, together with an extraordinary efficiency in priming naive T lymphocytes, is a hallmark of successful DC maturation [3, 4]. DC maturation is accompanied by down-regulation of endocytic capacity and changes in migratory behavior. Interaction of DC with endothelial cells (EC) is of pivotal pathophysiologic importance in a range of diseases including those governed by atherosclerosis, autoimmunity, and graft rejection [5–7]. The endothelium serves as a site for DC adhesion and diapedesis to sites of inflammation, and when injured and activated, EC on their own can promote attraction and subsequent maturation of DC [8–10]. Diseased

Correspondence: Heiko Methe, Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Bldg 56-322, Cambridge, MA 02139, USA

Fax: +1-617-2532514

e-mail: hmethe@mit.edu

Abbreviations: **EC:** endothelial cell · **HAE:** human aortic endothelial cell · **PAE:** porcine aortic endothelial cell ·

TCPS: tissue culture polystyrene plates

endothelium is characterized by impaired biochemical function, heightened immunogenicity, and alterations in cell-matrix contact [11–13]. Others and we have demonstrated that cellular immunogenicity is partially controlled by substrate adherence in a physiologic 3D environment [14–16].

We hypothesized that maturation of allo- and xenogeneic DC depends on EC substrate adherence to and spatial formation of the subendothelial matrix. In the present study, we compared the maturation state of DC upon exposure to non-adherent and substrate-adherent EC with cytokine-induced maturation. We further characterized induction of T cell proliferation by these DC. Finally, we explored cross-activation of EC.

Our results indicate a relationship between endothelial matrix interconnectivity as a marker for endothelial health and initiation of an immune cascade. Substrate adherence to a three-dimensional matrix limits the ability of allo- and xenogeneic EC to induce maturation of DC.

Results

Non-adherent EC drive maturation of monocyte-derived DC

In line with previous observations [17], monocytes differentiated into immature DC after 5 days of culture in GM-CSF and IL-4. In this state, DC expression of costimulatory molecules was muted, and without additional stimulation maturation was arrested (Fig. 1A; Supporting Information Fig. A). Further stimulation with IL-1 β , TNF- α , and IL-6 for 48 h induced up-regulation of costimulatory (CD40: 2.3-fold increase in MFI compared to immature DC, CD80: 3.3-fold, CD86: 1.6-fold) and HLA-DR molecules (2.1-fold) in concert with increased evidence of DC maturation, as CD83 rose 2.2-fold ($p < 0.005$; Fig. 1A; Supporting Information Fig. A). Exposure to saline suspensions of allo- and xenogeneic EC after growth to confluence in tissue culture polystyrene plates (TCPS) induced full maturation of monocyte-derived DC virtually identically to prolonged treatment with a cytokine cocktail. Human (HAE) or porcine aortic EC (PAE) alone induced DC costimulatory molecule expression with increases in CD40 (HAE: 2.1-fold increase in MFI compared to immature DC, PAE: 2.5-fold), CD80 (3.6-fold, 4.8-fold; $p < 0.05$ vs. cytokine stimulation), CD86 (3.6-fold, 3.4-fold; $p < 0.02$ vs. cytokine stimulation), HLA-DR (2.3-fold, 2.9-fold; $p < 0.05$ vs. HAE, $p < 0.002$ vs. cytokine stimulation), and CD83 (2.6-fold; $p < 0.05$ vs. cytokine stimulation, 3.2-fold; $p < 0.02$ vs. HAE, $p < 0.001$ vs. cytokine stimulation; Fig. 1A; Supporting Information Figs. A, B). Co-culture of DC with confluent HAE still

attached to TCPS for 48 h increased expression of CD40 (61 ± 4 MFI), CD83 (64 ± 3 MFI), CD80 (78 ± 3 MFI), CD86 (90 ± 6 MFI), and HLA-DR (289 ± 22 MFI; Supporting Information Fig. A). Yet, the degree of DC maturation was significantly lower than after co-culture with saline-suspended HAE ($p < 0.05$). Collagen coating of TCPS was without additional influence on the ability of HAE to induce maturation of allogeneic DC (Fig. 1B). Furthermore, supernatants isolated from HAE after growth to confluence on TCPS had a reduced capacity to induce DC maturation ($p < 0.05$ vs. HAE attached to TCPS, $p < 0.002$ vs. saline solutions of HAE; Fig. 1).

mRNA transcript levels tracked protein expression (Fig. 2). Additionally, DC matured exposed to cytokines or non-adherent EC displayed significant up-regulation of IL-12p40 mRNA (immature: 0.03 ± 0.02 relative units (RU), cytokine-stimulated: 0.23 ± 0.03 RU, $p < 0.002$, HAE-stimulated: 0.31 ± 0.05 RU, $p < 0.001$, PAE-stimulated: 0.28 ± 0.03 , $p < 0.002$; Fig. 2). Overall, saline suspensions of HAE and PAE grown to confluence on TCPS and HAE attached to TCPS induced *in vitro* maturation of human DC comparable to a well-established cytokine cocktail.

Incomplete DC maturation upon incubation with substrate-adherent, matrix-embedded EC

Maturation of DC co-cultured with substrate-adherent HAE or PAE embedded within a 3D matrix was significantly restricted compared to DC in the presence of non-adherent EC or EC attached to TCPS. Control over maturation was from unique molecular interactions rather than simple physical limitation of contact, as immune cells are free to infiltrate the matrices and encounter embedded EC. DC in the presence of matrix-embedded EC displayed only weak up-regulation of CD40, CD86, CD83 ($p < 0.0005$ vs. non-adherent EC), and CD80 ($p < 0.01$), and no induction of HLA-DR expression ($p < 0.001$; Fig. 1; Supporting Information Figs. A, B).

Supernatants isolated from matrix-embedded HAE regulated DC maturation equivalent to matrix-embedded HAE (CD83: 34 ± 2 MFI, HLA-DR: 167 ± 14 MFI, CD86: 65 ± 2 MFI; Fig. 1B), and the degree of maturation was significantly less after incubation of DC with supernatants from TCPS-grown HAE ($p < 0.05$; Fig. 1B). Matrix-embedded HAE limited cytokine-induced DC maturation as well. Co-incubation of immature DC with a cytokine cocktail (IL-1 β , TNF- α , and IL-6 for 48 h) in the presence of matrix-embedded HAE resulted in a 1.3–1.6-fold lower up-regulation of CD83, HLA-DR and CD86 expression than with the cytokine cocktail alone ($p < 0.05$; Fig. 1; Supporting Information Fig. A). Incubation with empty Gelfoam matrices alone had no effect on the maturation of

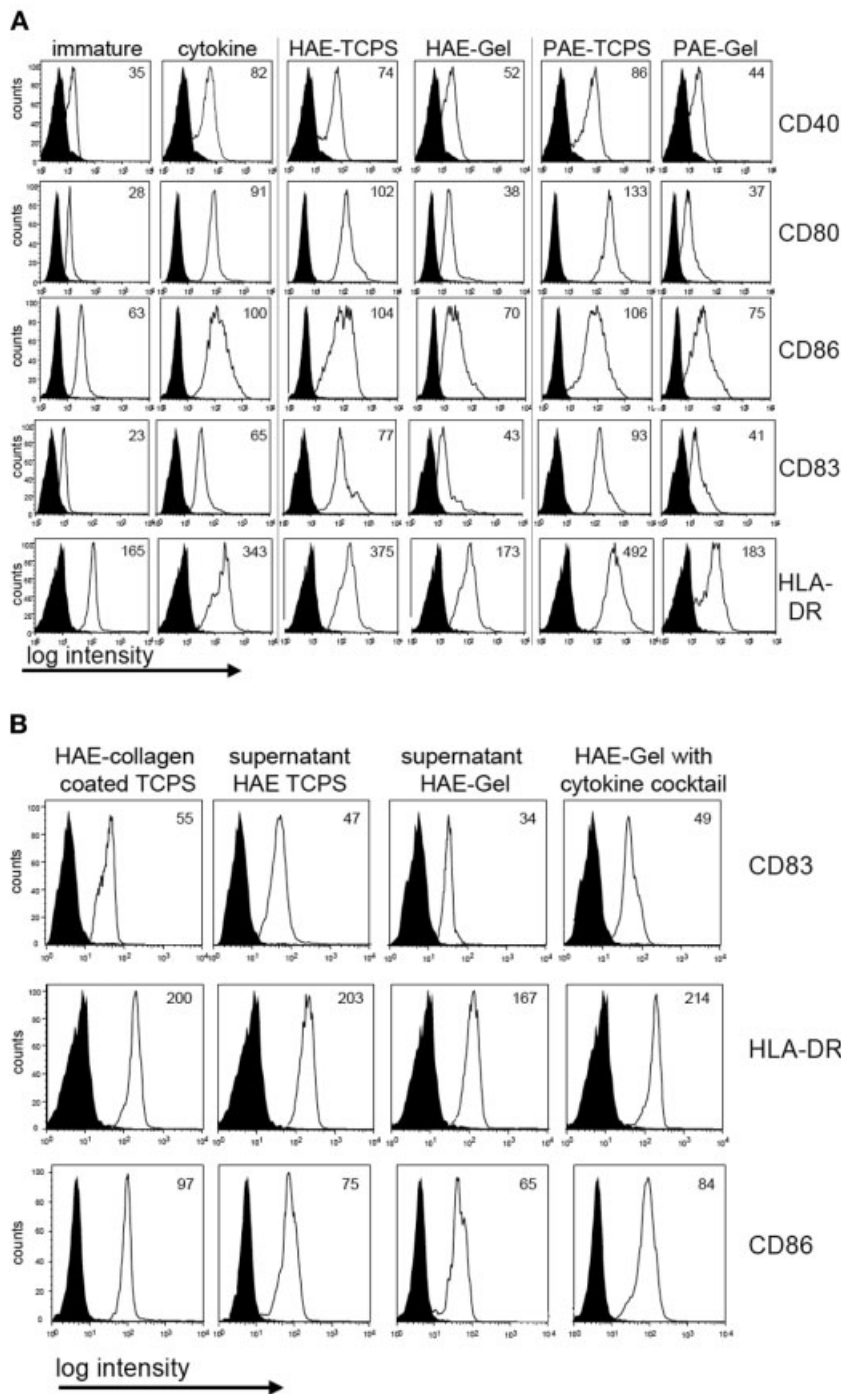


Figure 1. Incubation with substrate-adherent, matrix-embedded HAE and PAE results in incomplete DC maturation. In contrast, stimulation with a cytokine cocktail and incubation with HAE and PAE suspensions grown on TCPS results in enhanced expression of CD40, costimulatory molecules, CD83 and HLA-DR (A). Coating TCPS with collagen has no influence on the maturation ability of HAE. Supernatants of HAE-TCPS and HAE-Gelfoam only partially induce maturation of allogeneic DC; the presence of matrix-embedded HAE limited up-regulation of CD83, HLA-DR, and CD86 expression upon stimulation of immature DC with a cytokine cocktail (B). Adherent cells isolated from peripheral blood were cultured *in vitro* with 20 ng/mL IL-4 and GM-CSF for 5 days (immature DC; CD40: 35 MFI, CD83: 29 MFI, CD80: 28 MFI, CD86: 63 MFI, HLA-DR: 165 MFI). For the last 48 h, 10 ng/mL IL-1 β , 1000 U/mL IL-6, and 20 ng/mL TNF- α (cytokine), or 1.5×10^5 HAE or PAE grown on TCPS or embedded within Gelfoam were added. Histograms show the levels of CD40, CD80, CD86, HLA-DR, and CD83 expression on monocyte-derived DC in relation to isotype control (black) and are representative of four similar experiments. Numbers denote MFI values.

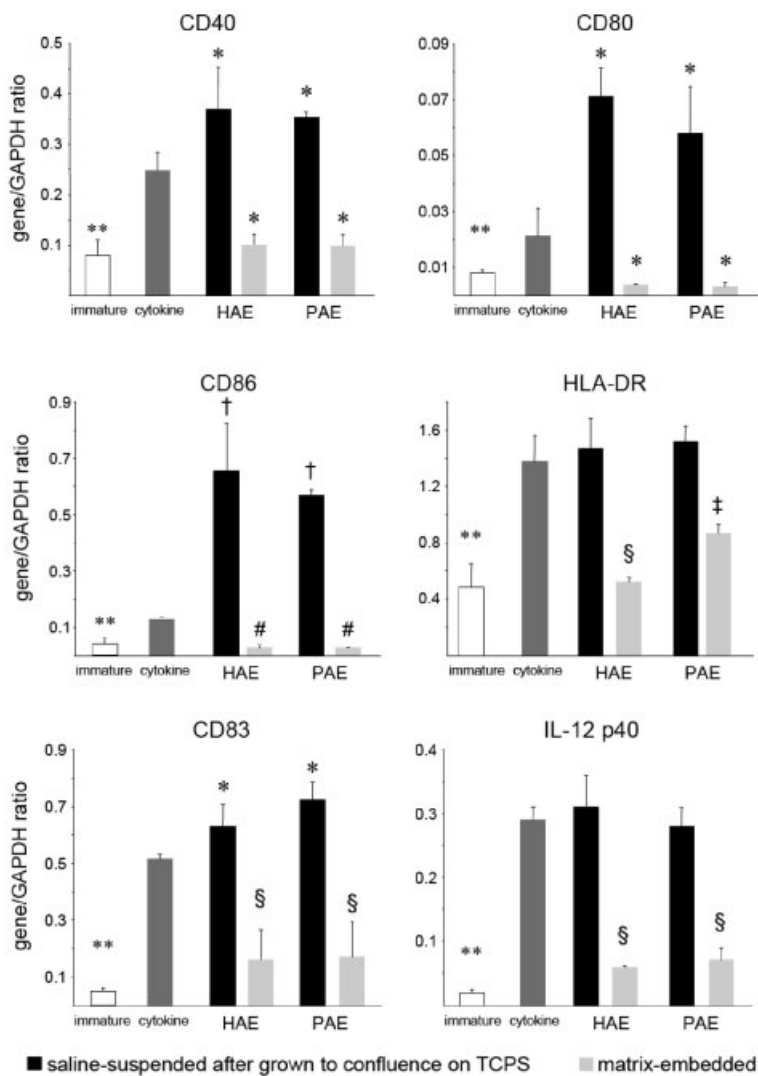


Figure 2. Compared to monocyte-derived DC matured with matrix-embedded surface-adherent EC, mRNA transcript levels for CD40, CD80, CD86, HLA-DR, CD83 and IL-12p40 are all increased when cells were matured with EC suspension after growth to confluence on TCPS. Real-time PCR results were normalized for GAPDH expression levels. Data are expressed as means \pm SD of four independent experiments. * p < 0.05 EC vs. cytokine, † p < 0.01 EC vs. cytokine, ‡ p < 0.05 EC saline suspensions vs. matrix-embedded EC, § p < 0.001 EC saline suspensions vs. matrix-embedded EC, # p < 0.0002 EC saline suspensions vs. matrix-embedded EC, ** p < 0.02 immature vs. cytokine.

monocyte-derived DC (CD40: 38 ± 2 MFI, CD83: 22 ± 1 MFI, CD80: 29 ± 3 MFI, CD86: 59 ± 5 MFI, HLA-DR expression: 179 ± 27 MFI; Supporting Information Fig. B).

Real-time PCR analysis revealed the same pattern of incomplete maturation and reduced IL-12p40 expression (p < 0.001) when DC were exposed to substrate-adherent allo- and xenogeneic EC (Fig. 2).

Maturation control by matrix-embedded EC might be mediated *via* increased TGF- β secretion by matrix-embedded HAE when compared with HAE attached to TCPS (378 ± 52 vs. 61 ± 23 pg/mL; p < 0.002) as TGF- β signaling had currently been proposed to induce an inhibitory regulatory DC phenotype [18]. Recapitulating, matrix-embedded HAE and PAE (in marked

contrast to saline suspensions of EC grown to confluence on TCPS) are significantly limited in their ability to induce *in vitro* maturation of DC. In addition, subsequent maturation by an established cytokine cocktail after first contact to matrix-embedded EC is lessened.

Incubation with substrate-adherent, matrix-embedded EC sustains DC endocytic activity

Immature DC are efficient at antigen capture and exhibit a high level of endocytosis. FITC-conjugated dextran uptake increased when monocytes were cultured for 3 and 5 days in GM-CSF and IL-4 (423.3 ± 121.8 MFI, 239.8 ± 42.8 MFI; p < 0.0001). Whereas dextran uptake decreased with continued cytokine stimulation

(89.7 ± 14.7 MFI) and by co-incubation with non-adherent HAE (92 ± 20.3 MFI) or PAE (82.4 ± 16.5 MFI; all $p < 0.0001$ vs. day 5), DC retained their endocytic activity when EC were presented embedded within a 3D matrix ($p < 0.0001$ vs. cytokine- and free EC-stimulated; Fig. 3; Supporting Information Fig. C).

Non-adherent but not matrix-embedded EC induce TLR expression by DC

Immature DC weakly expressed TLR2 (73 ± 4 MFI) and almost no TLR4 (39 ± 2 MFI). Saline suspensions of HAE up-regulated DC TLR2 and TLR4 expression equivalent to or greater than (1.5- and 2.5-fold increase in MFI, respectively) cytokine stimulation (TLR2: 111 ± 8 MFI, TLR4: 61 ± 4 MFI). This effect was even more pronounced after co-incubation of DC with non-adherent xenogeneic PAE (TLR2: 2.4-fold; $p < 0.05$ vs. cytokine- and HAE-stimulated, TLR4: 3-fold; $p < 0.05$ vs. HAE, $p < 0.001$ vs. cytokine stimulation; Fig. 4A; Supporting Information Fig. D). In contrast, DC co-cultured with matrix-embedded EC displayed only weak up-regulation of TLR4 ($p < 0.0005$ vs. non-adherent EC) and no induction of TLR2 expression ($p < 0.001$; Fig. 4A; Supporting Information Fig. D). mRNA transcript levels tracked protein expression (Fig. 4B). There was no induction of TLR expression on immature DC left untreated (data not shown).

DC co-cultured with matrix-embedded EC secrete increased levels of IL-10 and TGF-β

ELISA analysis revealed significantly higher secretion of IL-10 and TGF-β by DC co-cultured with matrix-embedded EC, compared with cytokine-treated DC or DC co-cultured with non-adherent EC. IL-10 secretion was 60-fold (TGF-β 1.8-fold) higher than by cytokine-induced DC and 22-fold (TGF-β 1.9-fold) higher than by DC co-cultured with non-adherent EC (Fig. 5). DC

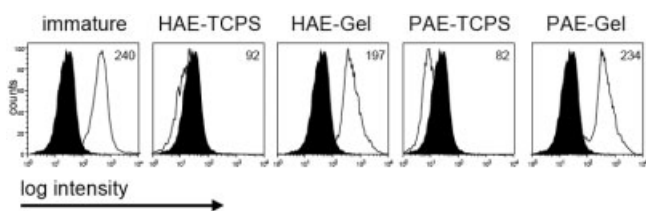


Figure 3. Incubation with substrate-adherent, matrix-embedded EC sustains DC endocytic activity. Histograms show FITC-dextran uptake by immature DC and by DC after incubation with suspensions of HAE and PAE after growth on TCPS or with EC embedded in Gelfoam matrices in relation to uptake by cytokine-matured DC (black). Histograms are representative of six similar experiments. Numbers denote MFI values.

matured with supernatants of matrix-embedded EC also displayed increased IL-10 (512 ± 32 pg/mL) and TGF-β secretion (1042 ± 32 pg/mL; $p < 0.05$).

DC co-cultured with matrix-embedded EC express TLR2 and TLR4 to a significantly lesser extent than cytokine-induced DC or DC co-cultured with saline suspensions of EC. In addition, the former are characterized by significantly greater secretion of IL-10 and TGF-β.

DC exhibit reduced T cell proliferation activity after cultivation with substrate-adherent EC

The ability to promote T cell differentiation into effector and memory cells is an important functional marker for the maturation grade of DC. Whereas cytokine-treated and non-adherent EC-exposed DC induced T cell proliferation over the full spectrum of DC/T cell ratios tested (74 789 ± 1777, HAE: 97 522 ± 1630, and PAE:

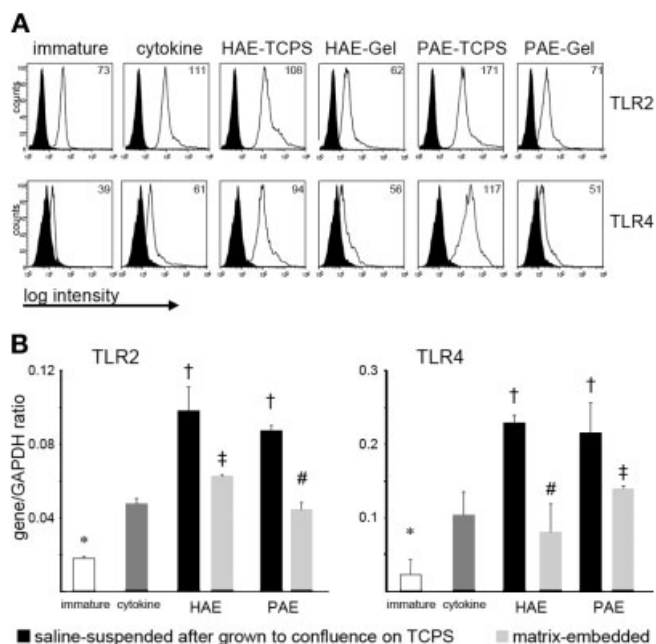


Figure 4. Suspensions of EC after growth to confluence on TCPS induce higher expression of TLR2 and TLR4 on monocyte-derived DC than a cytokine cocktail. Matrix embedding nearly completely inhibits up-regulation of TLR2 and TLR4 on DC. Monocyte-derived DC were differentiated *in vitro* with a cytokine cocktail, HAE and PAE cultured on TCPS or embedded within Gelfoam matrices for 48 h. (A) Histograms show the levels of TLR2 and TLR4 expression on DC in relation to isotype control (black) and are representative of four similar experiments. Numbers denote MFI values. (B) Compared to DC matured with matrix-embedded EC, mRNA transcript levels for TLR2 and 4 are increased when cells were matured with EC suspension. Real-time PCR results were normalized for GAPDH expression levels. Data are expressed as means ± SD of four independent experiments. * $p < 0.05$ immature vs. cytokine, † $p < 0.01$ EC vs. cytokine, ‡ $p < 0.05$ saline suspensions vs. matrix-embedded, # $p < 0.01$ saline suspensions vs. matrix-embedded.

101 616 ± 4302 cpm), this ability was significantly muted in DC after incubation with matrix-embedded HAE (18 320 ± 1000 cpm; $p < 0.002$) and PAE (20 080 ± 683 cpm; $p < 0.0001$; Fig. 6).

Reduced activation of matrix-embedded EC when co-cultured with DC

Real-time PCR, flow cytometry and Western blot analysis revealed reduced activation of matrix-embedded HAE and PAE after co-culture for 2 days with DC. After magnetic-bead-based isolation of DC, the remaining cells were greater than 95% pure for the EC-specific marker CD31. mRNA expression levels for adhesion molecules, CD58, HLA-DR and TLR molecules on matrix-embedded HAE were significantly reduced when compared to their non-adherent counterparts (real-time PCR; Fig. 7A). Reduced mRNA expression levels associated with reduced surface and intracellular expression, with 3.6-fold lower expression of ICAM-1 on matrix-embedded when compared to non-adherent HAE (1.3-fold decrease for PAE), 4.9-fold decrease of VCAM-1 on HAE (PAE: 2.7-fold), and 16-fold decrease of HLA-DR (PAE: 23-fold decrease; Fig. 7B). TLR2 (HAE: 2.1-fold increase, PAE: 2.4-fold increase; $p < 0.02$) and

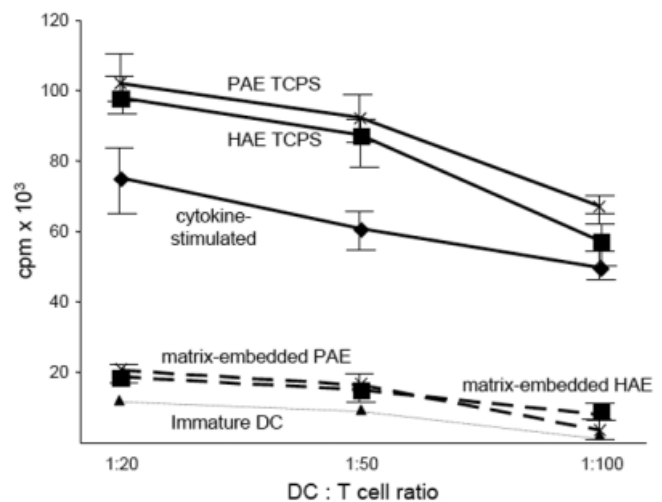


Figure 6. DC exhibit reduced T cell proliferation activity after cultivation with substrate-adherent EC. Purified cytokine- or EC-matured DC were γ -irradiated and added to purified CD3⁺ T cells at the indicated DC/T cell ratio and incubated for an additional 5 days before addition of [³H]thymidine to assess T cell proliferation. Data shown represent one of three experiments that gave similar results. Error bars represent the SD of triplicate determinations.

TLR4 expression (HAE: 1.9-fold increase; $p < 0.05$, PAE: 4.2-fold increase; $p < 0.01$) were significantly increased in non-adherent EC when compared to matrix-embedded EC after co-incubation with DC for 48 h (Fig. 7C). Saline-suspended and matrix-embedded EC not incubated with DC did not express significant mRNA transcript levels for adhesion molecules, HLA-DR, and TLR molecules (Fig. 7A, B). We therefore conclude that matrix-embedding limits *in vitro* cross-activation of EC by allo- and xenogeneic DC.

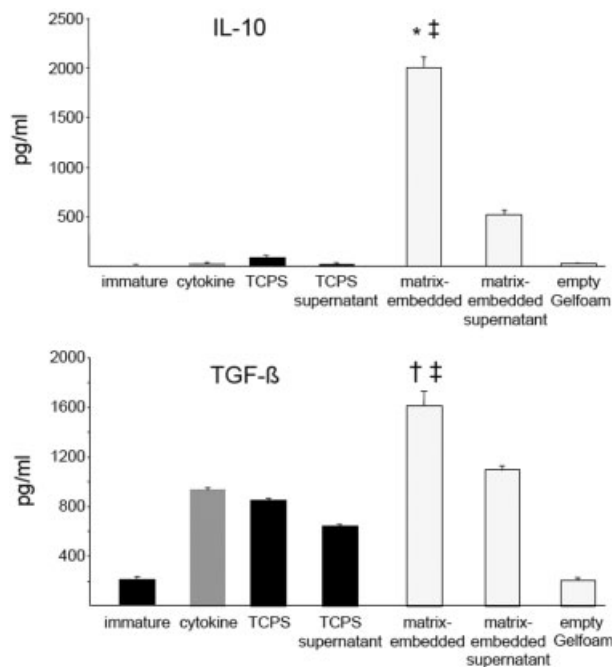


Figure 5. DC co-cultured with matrix-embedded EC secrete increased levels of IL-10 and TGF- β . ELISA analysis of IL-10 and TGF- β secretion by DC matured with a cytokine cocktail, suspension of HAE after growth to confluence on TCPS (TCPS), supernatants of HAE grown on TCPS, Gelfoam-embedded HAE, supernatants of matrix-embedded HAE, or with empty Gelfoam. * $p < 0.001$, † $p < 0.02$, ‡ $p < 0.05$ vs. supernatants of matrix-embedded HAE.

Discussion

The endothelium is a highly specialized, metabolically active interface between blood and the underlying tissue, maintaining vascular tone, thrombo-resistance and a selective permeability to cells and proteins. It is also a critical transducer of inflammatory signals and pivotally important to attraction of circulating immune cells to sites of local injury. We now show that endothelial structure and function come together as part of the immunoregulatory capacity of EC. EC are normally matrix adherent within a 3D microenvironment. Circulating and/or partially detached EC, as well as the anti-EC antibodies they induce, have been demonstrated in atherosclerosis, vasculitis, and graft rejection [13, 19–24]. It has also been demonstrated that metabolic diseases such as hyperlipidemia and diabetes mellitus are associated with microscopic changes in

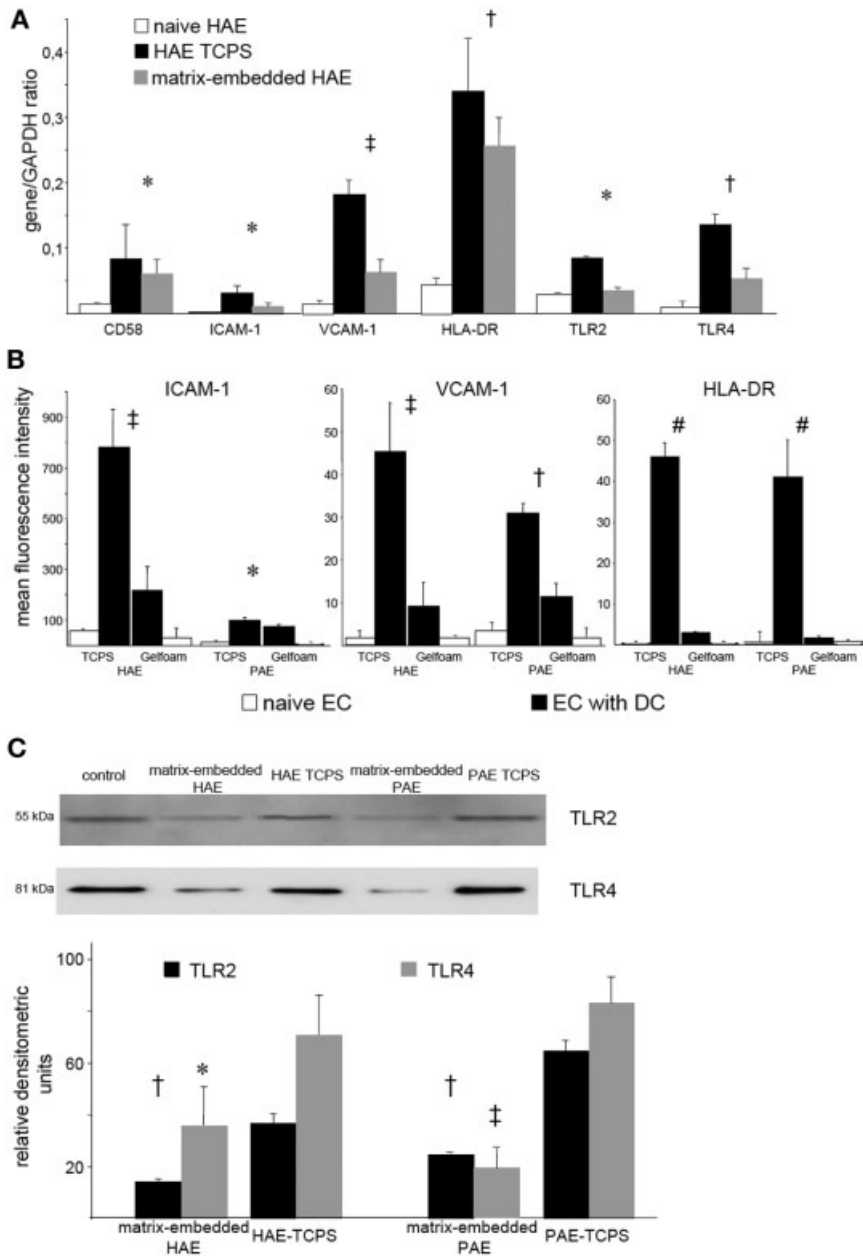


Figure 7. Substrate-adherent EC display significantly lower expression levels of immune and adhesion molecules after cocubation with monocyte-derived DC for 2 days. EC were purified from DC before real-time PCR (A), flow cytometry (B) and Western blot analysis (C). (A) Real-time PCR reveals lower expression of CD58, ICAM-1, VCAM-1, HLA-DR, TLR2 and TLR4 on matrix-embedded surface-adherent HAE when compared to HAE grown on TCPS. Real-time PCR results were normalized for GAPDH expression levels. Data are expressed as means \pm SD of four independent experiments. * p < 0.05 TCPS vs. matrix-embedded, † p < 0.01 TCPS vs. matrix-embedded, ‡ p < 0.002 TCPS vs. matrix-embedded. (B) Increased surface expression of ICAM-1, VCAM-1, and HLA-DR on EC grown on TCPS compared to matrix-embedded EC. Data are expressed as MFI \pm SD of four independent flow cytometry experiments. * p < 0.05 TCPS vs. matrix-embedded, † p < 0.01 TCPS vs. matrix-embedded, ‡ p < 0.002 TCPS vs. matrix-embedded, # p < 0.001 TCPS vs. matrix-embedded. (C) Western blots and densitometric analysis demonstrate increased expression of TLR2 and TLR4 in EC grown on TCPS compared to surface-adherent EC after co-culture with monocyte-derived DC for 48 h. Data are expressed as means \pm SD of four independent experiments with similar results. * p < 0.05 TLR expression TCPS vs. matrix-embedded, † p < 0.02 TLR expression TCPS vs. matrix-embedded, ‡ p < 0.01 TLR expression TCPS vs. matrix-embedded.

subendothelial matrix composition promoting changes in EC-matrix interconnectivity and spatial formation [11, 25, 26]. The importance of cell-matrix contact and spatial formation for immune regulation is increasingly appreciated [14–16]. Others and we have demonstrated that substrate adherence to 3D matrices reduces immunogenicity of fibroblasts and EC upon cytokine activation *via* reduced expression of MHC class II and costimulatory molecules [14, 16]. As the immunogenic capacity of EC is directly proportional to the extent of MHC class II expression on the cell surface [27], we hypothesized that substrate adherence would also affect EC-induced DC maturation. In our experiments, EC that are embedded within 3D matrices behave entirely differently than those that are freed from anchorage after growth to confluence on TCPS or still attached to 2D TCPS.

DC maturation is pivotal for induction of immune responses for which EC might serve as initiators, be it responses against self or other cells and tissues. Dysfunctional syngeneic EC promote DC maturation *in vivo* and *in vitro* [7, 28], and non-activated xenogeneic EC induce maturation of DC *in vitro* [29]. In addition, DC and EC amplify their respective effects in the setting of heightened immune activation. Our results extend these data by delineating the importance of micro-architecture and cell-substrate adherence for EC-induced DC maturation. Whereas non-adherent, free EC induced every aspect of monocyte-derived DC maturation functionally indistinct from a cytokine cocktail, co-incubation with matrix-embedded EC did not. EC substrate adherent to a 3D environment induced only minor DC up-regulation of mRNA transcript and protein levels of adhesion, costimulatory, and HLA-DR molecules. DC co-incubated with substrate-adherent EC also failed to demonstrate up-regulation of the direct maturation markers IL-12 mRNA and CD83 or to mature to the point of loss in phagocytic function.

Studies in murine transplant models have shown that the functional properties of DC depend on their maturational state [30, 31]. Whereas mature DC, which strongly express MHC class II and costimulatory molecules, induce a robust allostimulatory response [30–32], immature DC are characterized by low expression levels of surface MHC, T cell costimulatory and adhesion molecules, low levels of pro-inflammatory cytokines and absence of bioactive IL-12 synthesis [33, 34]. Accordingly, we now present that DC exposed to non-adherent EC display enhanced T cell stimulatory activity in mixed lymphocyte reactions, whereas T cell proliferation after exposure to matrix-embedded EC-matured DC was significantly weaker. Of note, Zhang *et al.* recently identified regulatory DC that inhibited T cell proliferation. They identified TGF- β expressed and secreted by EC-like splenic stroma cells to drive differentiation of

immature DC into this regulatory phenotype [18]. Interestingly, surface-adherent EC cultured within a 3D environment secrete significantly more TGF- β than their non-adherent counterparts.

Furthermore, Lan *et al.* reported “alternatively activated” DC that are not fully matured and preferentially secrete IL-10, and expand regulatory T cells, thereby inducing long-term allograft survival [34]. In line with these results, we now demonstrate that co-culture with matrix-embedded EC and its supernatants induce DC secretion of an anti-inflammatory cytokine milieu (IL-10, TGF- β).

Interestingly, EC still attached to 2D TCPS displayed 'semi-efficiency' in promoting maturation of DC. Coating TCPS with collagen was without effect on their maturation efficiency, further indicating the predominant importance of 3D *versus* 2D environments for immune behavior of EC. Equally fascinating, supernatants of TCPS-grown as well as of matrix-embedded EC had a reduced capacity to induce DC maturation when compared to the respective direct EC-DC contact. As one explanation, Herrera *et al.* demonstrated that maturation of DC depends predominantly on direct MHC transfer between grafted cells and DC [28]. Yet, supernatants of matrix-embedded EC displayed a significant lower ability to promote DC maturation than supernatants from EC grown to confluence on TCPS, again indicating the presence of a secreted inhibitory factor by matrix-embedded EC (e.g. TGF- β) [18]. Additionally, in the presence of matrix-embedded EC, immature DC lost the ability to undergo maturation upon stimulation with the strong DC maturation cytokine cocktail.

Our results are of special importance for transplantation biology where the non-syngeneic endothelium of donor organs is the first target for host immune responses. We therefore analyzed cross-activation of EC after co-culture with DC, and we are now able to demonstrate that activation of EC while incubated with DC was also significantly reduced when EC were substrate adherent within a 3D matrix. Whereas non-attached EC expressed high levels of adhesion and HLA-DR molecules, we could not detect differences from baseline levels in matrix-embedded EC. These findings further emphasize the importance of cell-matrix contact and matrix microarchitecture in regulating endothelial immunogenicity. Up-regulation of MHC class II expression on non-adherent EC by cross-talk with DC might turn EC into potent antigen-presenting cells that can function in a DC-like manner, further perpetuating endothelial-directed immunity. In addition, TLR2 and TLR4 expression was not up-regulated on matrix-embedded EC after co-culture with DC, in marked contrast to TLR expression on non-adherent EC. Therefore, our results may be of particular importance for

graft rejection, as surgical injury and ischemia-reperfusion damage during the transplantation procedure may disrupt the delicate microarchitecture, activating the grafted endothelium and subsequently providing the maturation signals that host DC require to become immunogenic [35–37]. Enhanced immunogenicity of EC partially or completely detached from the underlying basement membrane has recently been demonstrated [20, 21, 38]. Furthermore, up-regulation of TLR2 and TLR4 on DC exposed to non-adherent allo- and even stronger to xenogeneic EC might indicate involvement of innate immune receptor signaling in recognition of non-self EC [29, 39]. Yet, lack of TLR expression on DC exposed to substrate-adherent EC might serve as an additional indicator for the reduced immunogenicity of EC when in tight contact with an underlying 3D collagen-based matrix.

Overall, EC surface adherent and grown 3D might mimic the physiologic configuration of healthy endothelium that is not prone to damage or recognition by immune mechanisms. Just as intimate contact and interaction of EC with matrix seems to confer immune acceptance, the immune system appears to respond vigorously to dislodgement of EC from their underlying matrix and disruption of matrix architecture. It is in this way that one might envision immune amplification of responses such as reperfusion injury, surgical trauma, ischemia or metabolic derangements (hyperglycemia, hyperlipidemia). The interaction between DC and EC offers an even higher level of immunomodulation by matrix. The immature state of DC after exposure to EC adherent to a 3D matrix might imitate the physiologic role of DC as tolerogenic/regulatory cells to induce and maintain self tolerance in the steady state [40, 41].

In conclusion, EC-DC crosstalk and mutual activation depends on substrate adherence of EC in a 3D environment. Our results have broad implications for our understanding of how endothelial immunogenicity might be controlled and how DC reactivity to EC might be regulated. Further characterization of the impact that continuous cell-matrix contact and matrix architecture has for the endothelial ability to drive DC maturation and the underlying molecular pathways might lead to novel therapeutic schemes in prevention of allorejection (and possibly xenorejection) as well as of endothelial-associated vascular diseases in general.

Materials and methods

Culture and embedding of EC

PAE isolated from LargeWhite swine aortae were grown in DMEM supplemented with 2 mM L-glutamine, 10% FBS (HyClone, UT), 100 U/mL penicillin G, and 100 µg/mL streptomycin (Life Technologies, Grand Island, NY). HAE

were grown in optimized EC growth medium-2 (EGM; both Cambrex, Walkersville, MD) supplemented with 5% FBS. EC were either grown to confluence on TCPS or seeded on Gelfoam blocks (Pharmacia & Upjohn, Kalamazoo, MI) [16]. For some experiments, TCPS were coated with collagen type I (35 µg/mL; Sigma Chemicals, St. Louis, MO) before EC seeding. Initial seeding density and Gelfoam block size were chosen based on the anticipated final cell numbers needed for subsequent co-culture experiments. EC surface attachment and confluence on Gelfoam matrices were demonstrated by confocal microscopy. The number of EC attached to the Gelfoam was counted on a Neubauer's counting chamber after at least two blocks from the same charge were washed with HBSS (Life Technologies) and digested with collagenase (1 mg/mL, type I; Worthington Biochemical Corp). Detachment of EC from TCPS was achieved by brief trypsin treatment (0.5% trypsin in normal saline and 5.3 mM EDTA), which was interrupted by the addition of optimized EGM. Neither trypsin nor collagenase affects the endothelial expression of immunoregulatory molecules [16].

Cell viability was checked by Trypan blue exclusion as well as a LIVE/DEAD viability/cytotoxicity kit (Molecular Probes, Eugene, OR) in which live and dead cells within the matrices were visualized by confocal laser scanning microscopy (BioRad MRC 600). There was no significant difference in live and dead EC between the different culture conditions (data not shown).

Preparation, culture and maturation of DC

Peripheral blood was collected from healthy volunteers and fractionated over Ficoll-Paque (Sigma) using standard procedures. The local ethical committee approved the study and informed consent of all participating subjects was obtained. To derive DC, total PBMC were cultured at 2×10^6 cells/mL in complete medium [RPMI 1640, 10% heat-inactivated FCS, 0.1 mM sodium pyruvate (Life Technologies)] for 1.5 h in tissue culture flasks. Following incubation, non-adherent cells were removed by extensive washing with HBSS (Life Technologies). The remaining adherent cells were then cultured in complete medium containing 20 ng/mL IL-4 and 20 ng/mL GM-CSF (PeproTech, Rocky Hill, NJ) for 5 days in a CO₂ incubator at 37°C. The resulting cells were semi- to non-adherent and MHC class II^{low}/CD14^{-/low}/CD83⁻ (data not shown).

For further maturation, adherent and non-adherent DC were harvested, extensively washed, counted, and 5×10^5 DC were stimulated with a cytokine cocktail (10 ng/mL IL-1β, 1000 U/mL IL-6, 20 ng/mL IL-4, GM-CSF, and TNF-α; all Peprotech), 1.5×10^5 HAE or 1.5×10^5 PAE for 48 h, or respective EC supernatants. EC were either presented as a monolayer or as suspensions after growth to confluence on TCPS or surface adherent embedded within Gelfoam matrices. For negative controls, harvested and washed DC were cultured without cytokine activation or EC for 48 h. Every assay was repeated at least four times. After maturation, DC were isolated from any contaminating EC with magnetic bead-labeled anti-CD1a antibodies (Miltenyi, Bergisch-Gladbach, Germany). Flow cytometry analysis revealed 98% purity of the isolated DC (data not shown). Various co-culture conditions

resulted in different DC morphologies (Supporting Information Fig. E).

Real-time PCR

Total RNA was extracted from isolated DC and the remaining EC using the RNeasy Mini Kit (Qiagen, Valencia, CA). As controls, RNA was extracted from unstimulated DC and EC after growth to confluence on TCPS or embedded within Gelfoam matrices without co-culture with DC. Complementary DNA was synthesized using TaqMan reverse transcription reagents from Applied Biosystems (Foster City, CA). Real-time PCR analysis was performed with an Opticon Real Time PCR Machine (MJ Research, Waltham, MA) using SYBR Green PCR Master Mix (Applied Biosystems) and selected primers (Supporting Information Table 1). Data from the reaction were collected and analyzed by the complementary Opticon computer software. Relative quantification of gene expression was calculated with standard curves and normalized to GAPDH.

Flow cytometry

DC or EC suspensions were washed and 3×10^5 cells were resuspended in FACS buffer (PBS containing 0.1% BSA and 0.1% sodium azide; Sigma). Standard flow cytometric analysis assessed surface expression of various markers. The following mAb directly conjugated with PE or FITC were used in single-color flow cytometric analysis: PE-CD1a (clone HI149, IgG₁), FITC-CD3 (clone UCHT1, IgG₁), PE-CD14 (clone TÜK4, IgG_{2a}), PE-CD31 (clone WM59, IgG₁), FITC-CD40 (clone 5C3, IgG₁), FITC-CD54 (clone 15.2, IgG₁), FITC-CD80 (clone BB1, IgM), FITC-CD83 (clone HB15e, IgG₁), FITC-CD86 (clone 2331, IgG₁), FITC-CD106 (clone 51–10C9, IgG₁), FITC-HLA-DP,DQ,DR (clone CR3/43, IgG₁), FITC-TLR2 (clone TL2.3, IgG_{2a}), and FITC-TLR4 (clone HTA125, IgG_{2a}). Appropriate isotype control antibodies (mouse PE-IgG₁, PE-IgG_{2a}, FITC-IgG₁, FITC-IgG_{2a}, FITC-IgM) were used, respectively. Antibodies were purchased from DakoCytomation (Carpinteria, CA), Serotec (Raleigh, NC) or PharMingen (San Diego, CA). After staining, cells were washed and fixed in 1% paraformaldehyde before analysis on a FACSCalibur instrument and with CellQuest software (Becton Dickinson, Mountain View, CA).

Endocytic activity

Endocytic activity of DC was measured by uptake of FITC-conjugated dextran (MW 40 000; Molecular Probes) as previously described [42]. Briefly, DC at various states of maturation were incubated in complete media with 1 mg/mL FITC-conjugated dextran for 1 h at 37°C to measure specific uptake, or at 4°C to measure nonspecific binding. Cells were then washed extensively and analyzed by flow cytometry as described above.

ELISA

DC isolated after co-culture were left untreated for 24 h and supernatants were assayed for concentrations of IL-10 (Bender

Medsystems, Vienna, Austria) and TGF- β (R&D Systems, Minneapolis, MN) by ELISA. Baseline TGF- β secretion by matrix-embedded HAE and HAE grown to confluence on TCPS was also determined by ELISA. Supernatants were stored at -70°C and measured at the same time by the same ELISA to avoid variations of assay conditions.

Mixed lymphocyte reaction assay

CD3⁺ T cells from an unrelated donor were isolated from total PBMC by negative selection using antibody depletion and magnetic beads according to the manufacturer's instruction (DynaL Biotech, Lake Success, NY). The nonmagnetic fraction contained greater than 95% CD3⁺ T cells, as assessed by flow cytometry. Purified cytokine- or EC-matured DC were γ -irradiated (3000 rad from a ¹³⁷Cs source) and added at 1×10^4 , 4×10^3 , or 2×10^3 cells/well to 2×10^5 CD3⁺ T cells to give final ratios of 1 : 20, 1 : 50, or 1 : 100. On day 5, 1 μ Ci of [³H]thymidine (Perkin-Elmer, Boston, MA) was added to each well. Cells were harvested 18 h later and [³H]thymidine uptake was quantified using a Packard TopCount γ -counter (GMI, Ramsey, MI).

Western blot

After separation from DC, EC were washed in PBS buffer and cell lysates were prepared by incubation with lysis buffer (20 mM Tris, 150 mM NaCl, pH 7.5, 1% Triton X-100, 1% deoxycholate, 0.1% SDS and protease inhibitor; Roche, Indianapolis, IN). Respective protein concentrations were determined with a Qubit fluorometer following the manufacturer's instructions (Invitrogen, Carlsbad, CA). Samples were boiled for 5 min with Laemmli sample buffer and equal amounts of protein were separated on 4–12% Bis-Tris-Acetate Nupage gels (Invitrogen). Jurkat (TLR2) or HL-60 whole cell lysates (TLR4; both Santa Cruz Biotechnologies, Santa Cruz, CA) served as controls. Proteins were then transferred onto nitrocellulose membrane iBlot stacks (Invitrogen). Membranes were blocked and incubated with rabbit anti-human TLR2 (dilution 1 : 250 in blocking buffer) or TLR4 antibodies (dilution 1 : 200; both Santa Cruz Biotechnologies). After three washing steps (PBS with 0.05% Tween 20), membranes were incubated with a goat anti-rabbit mAb conjugated to horseradish peroxidase (Santa Cruz Biotechnology) at a 1 : 1000 dilution in blocking buffer for 2 h at room temperature, followed by washing in five changes of wash buffer. For detection of TLR bands, the blot was incubated with the Western Supersignal Femto kit (Pierce, Rockford, IL), followed by exposure and analysis on a FluorChem SP (Alpha Innotech, San Leandro, CA).

Statistical analysis

All statistical analyses were performed with JMP software (SAS Institute Inc., USA, 2002). Data were normally distributed and expressed as means \pm SD. Comparisons between two groups were analyzed by Student's *t*-test, and comparisons between more than two groups were analyzed by ANOVA. A value of *p* < 0.05 was considered statistically significant.

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