

Review

# The effect of three-dimensional matrix-embedding of endothelial cells on the humoral and cellular immune response

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## Abstract

The endothelium is a unique immunologic target. The first host–donor reaction in any cell, tissue or organ transplant occurs at the blood–tissue interface, the endothelium. When endothelial cells are themselves the primary component of the implant a second set of immunologic reactions arises. Injections of free endothelial cell implants elicit a profound major histocompatibility complex (MHC) II dominated immune response with significant sensitivity, cascade enhancement and immune memory. Endothelial cells embedded within three-dimensional matrices retain all the biosecretory capacity of quiescent endothelial cells. Perivascular implants of such cells are the most potent inhibitor of intimal hyperplasia and thrombosis following controlled vascular injury, but without any immune reactivity. Allo- and even xenogeneic endothelial cells evoke no significant humoral or cellular immune response in immunocompetent hosts when embedded within matrices. Moreover, endothelial implants are immunomodulatory, reducing the extent of the memory response to previous free cell implants. Attenuated immunogenicity results in muted activation of adaptive and innate immune cells.

These findings point toward a pivotal role of matrix–cell–interconnectivity for the cellular immune phenotype and might therefore assist in the design of extracellular matrix components for successful tissue engineering.

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## 1. Introduction

The artery is an intensely sensitive organ, and its trilaminar architecture is an essential determining component of its ability to maintain vascular homeostasis. The intact blood vessel is thromboresistant, balances vascular tone with patent flow and initiates cascades of repair without impinging on the lumen. The vascular endothelium, covering nearly 700 m<sup>2</sup> in the average person, is the luminal lining of the blood vessels in the body and detects minute changes in the local environment [1]. The endothelium not only serves as a simple physical barrier between circulating (immune) cells, metabolites and the underlying tissue but actively regulates vasomotor tone, thrombogenicity, proliferation and interaction with immune cells of adaptive and innate lineage. In the usual quiescent state, turnover rates of endothelial cells are on the order of months to years, but these cells are easily

damaged by mechanical, oxidative, metabolic, and immunologic stressors [1]. Over a century ago, Virchow recognized endothelial dysfunction as one of the triad of elements essential for loss of vascular homeostasis and thrombotic occlusion. Each phase of the vascular response to injury: thrombosis, inflammation, cellular proliferation, and vascular remodeling, is influenced if not controlled by the endothelium [2]. Environmental exposure classically contributed to endothelial injury. More recently therapeutic techniques such as balloon angioplasty, endovascular stent implantation, and vascular surgery all significantly impair the integrity of the endothelium as an intact monolayer [2,3]. Endothelial dysfunction with endothelial cell immune activation, increased leakiness, apoptosis and angiogenesis has been demonstrated for a wide variety of diseases, including rheumatoid arthritis, psoriasis, multiple sclerosis, gut inflammation, chronic lung disease, diabetes, and atherosclerosis [4–8].

## 2. Endothelial cells and immune response

Inflammatory changes of the endothelium favor local recruitment of leukocytes, and transendothelial migration of monocytes

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and T cells into the subintimal space, which is a fundamental step in the response to tissue-invading microbes and antigens. When this phase is overexuberant significant damage can occur. Indeed, leukocyte infiltration is a crucial initiating step in the development of atheroma within the vessel wall. Endothelial immune behavior is tightly regulated. Recruitment and migration of circulating immune cells is mediated by endothelial-expressed and secreted chemokines (e.g., MCP-1, IL-6, IL-8, MIP-1 $\alpha$ ,  $\beta$ , and fractalkine), and adhesion molecules (e.g., P-, L-, and E-selectin, ICAM-1, VCAM-1) [9,10]. Endothelial cells are directly implicated in innate and adaptive immune signaling. Several reports have demonstrated Toll-like receptor (TLR) expression on endothelial cells [11–16] and lipopolysaccharide-mediated cytokine-induced endothelial up-regulation of chemokines and adhesion molecules [17]. Endothelial cells can even act as antigen-presenting cells, with the concomitant expression of costimulatory molecules and presentation of antigens via major histocompatibility complex (MHC) class I and II. This activity has been documented *in vitro*, as to whether it occurs *in vivo* is still debatable. Endothelial cells from different species express various costimulatory and accessory molecules, e.g., CD80, CD86, ICOS-L, programmed death ligand (PD-L) 1 and 2, CD40, CD134L [18]. Endothelial cells also express CD40 that has an important role in increasing the T cell receptor-mediated signal and to drive differentiation of naive

T cells into effector T cells. Binding of endothelial-expressed CD40 with CD40 ligand perpetuates the inflammatory response. The nature of the endothelial cell presents a unique platform by which to examine the immune response to injury, the processes of tissue repair and specific aspects of endothelial biology.

### 3. Three-dimensional matrix-embedding of endothelial cells

It is increasingly evident that organ tissues yield significant control over local immune reactions and cell behavior [19]. As such three-dimensional cell culture systems offer a milieu to study endothelial biosecretory, migratory, and proliferative functionality that is perhaps more physiological and robust than the conventional two-dimensional state of plated cultures [20–24]. The three-dimensional state offers the opportunity to consider external mechanical and cell adhesion stimuli, which dramatically affect integrin ligation, expression, cell contraction and associated intracellular signaling [25,26]. Endothelial cells embedded within three-dimensional collagen scaffolds line the interstices of the collagen-based sponge thereby mimicking a confluent monolayer similar to a physiologic vessel network (Fig. 1) [27,28]. To challenge the notion that one must completely restore tissue structure before function can emerge we created constructs of endothelial cells

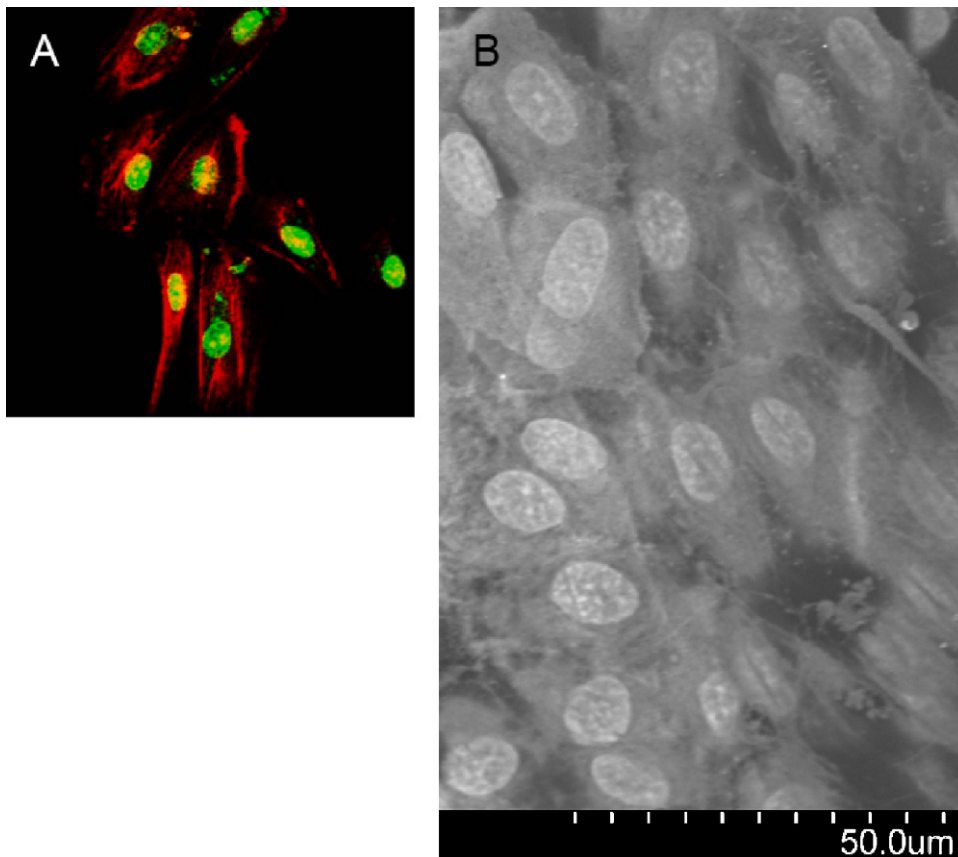


Fig. 1. Endothelial cells line the three-dimensional scaffold as a monolayer mimicking a physiologic vessel network. (A) Human aortic endothelial cells: FITC-YOYO-1 nuclei staining, F-actin staining (63 $\times$  magnification); (B) Human aortic endothelial cells line the interstices of the three-dimensional collagen-based matrix (wet SEM, 800 $\times$  magnification).

embedded within three-dimensional collagen-based scaffolds *in vitro* before perivascular placement around injured vessel segments. Such constructs allow endothelial cells to retain quiescence, secretion of essential regulatory factors and the associated potential for vasoregulatory control, within vehicles that can be stored, manipulated, functionally validated and implanted at will at sites protected from environmental forces [28–31].

These perivascular implanted endothelial cells – not exposed to flow – exhibited the full spectrum of a healthy physiologic endothelium and were able to control vascular repair processes: placement of endothelial implants onto the adventitial surfaces of injured vessels effectively diminished intimal thickening and proliferation of smooth muscle cells after angioplasty and the creation of arteriovenous fistulas [30,31]. These perivascular implants did not evoke a significant immune response in immunocompetent animals [32] even with allo- or xenogeneic endothelial cells, and therapeutic efficacy was observed for more than six months [29]. This prompted us to investigate the effect of three-dimensional matrix environment on endothelial immunogenicity.

#### 4. Influence of matrix composition on endothelial cell phenotype

Matrix environment influences pivotal endothelial cell functions: receptors for the extracellular matrix, in particular the integrins, act not only to provide anchorage for endothelial cells, but also provide information about the local microenvironment that facilitates their decision to proliferate, migrate or die [33] and mediate endothelial permeability [34]. Integrins actively affect intracellular signaling, e.g., via focal adhesion kinase, and signaling via different integrins modulates cellular (immune) phenotypes [35]. Additionally, focal adhesions serve as primary mechanosensors for multiple signaling cascades that transduce hemodynamic forces (e.g., shear force, cyclic stretch) to the endothelium and smooth muscle cells [36]. Reduced blood flow velocity and oscillatory flow patterns exert pro-atherogenic activity via modifications of subendothelial matrix protein composition. In contrast, the atheroprotective effects of high shear, unidirectional laminar blood flow is not associated with changes in subendothelial matrix composition [37,38]. Furthermore, heterogeneity of endothelial cells from different vascular beds is thought to derive from differences in matrix environment and binding modalities of endothelial cells to extracellular matrix proteins as well [39,40].

Extracellular matrix remodeling plays a major role in the pathogenesis of atherosclerosis and restenosis as early stages of atherosclerotic lesions are characterized by deposition of fibronectin and fibrinogen within the extracellular matrix [38]. Integrin expression largely varies with composition of the matrix proteins, microarchitecture, tension, mechanical stimuli, etc. Retention of lipoproteins in the subendothelial matrix, hyperglycemic-mediated protein modifications as well as mechanical stressors modulate the synthesis of almost all major components of the extracellular matrix, including collagen, elastin, proteoglycans, glycosaminoglycans, glycoproteins,

and various soluble proteins, that in turn provoke alterations in the integrin expression pattern [37,38].

Whereas the physiologic endothelial phenotype has been defined, quiescent, activated dysfunctional endothelial cells display a different integrin expression pattern (e.g., increased expression of integrin  $\alpha_v\beta_3$  and  $\alpha_5\beta_1$ ) [33]. Atherosclerotic lesions are marked by integrin expression patterns that have been associated with an activated endothelial phenotype [37,38,41]. The predominant endothelial phenotype in three-dimensional matrices in contrast to endothelial cells grown on two-dimensional tissue culture plates is quiescent and marked by a physiologic integrin expression pattern (i.e., up-regulated  $\alpha_2\beta_1$ -,  $\alpha_1\beta_1$ -,  $\alpha_6\beta_1$ -,  $\alpha_6\beta_4$ - and down-regulated  $\alpha_v\beta_3$ -,  $\alpha_5\beta_1$ - integrins), and expression of a physiologic matrix protein profile (e.g., up-regulated collagen IV and laminin, down-regulated fibronectin gene expression; unpublished results) [42]. Alterations in cellular immune behavior by the immediate matrix environment have been described previously: fibroblast MHC class II expression is limited in a signal transducers and activators of transcription (STAT)-1 dependent pathway [43], smooth muscle cell response is lessened when cultured in a three-dimensional environment [44] and T cell development in the thymus depends on the spatial tissue formation [45].

#### 5. Immune phenotype of three-dimensional matrix-embedded endothelial cells

As previously demonstrated in other cell systems intracellular cytokine signaling pathways are modulated by the surrounding tissue architecture. We therefore analyzed endothelial expression of adhesion, costimulatory and MHC molecules upon TNF- $\alpha$  and IFN- $\gamma$  stimulation. Endothelial cells were either placed within collagen-based scaffolds (three-dimensional), grown to confluence on two-dimensional polystyrene tissue culture plates or tissue plates coated with collagen. Whereas the matrix environment was without influence on the endothelial biosecretion, we detected significant changes in the respective immune phenotype. Using real-time PCR, flow cytometry, and ELISA we demonstrated muted up-regulation of adhesion molecules (VCAM-1, ICAM-1, L-, E- and P-selectin), chemokines (fractalkine, MCP-1, IL-6, IL-8), costimulatory molecules (CD80, CD86, CD40, ICOS-L) and MHC class II molecules in endothelial cells embedded within three-dimensional collagen-based scaffolds when compared to the two-dimensional growing environments [32,42]. Chemokine and adhesion molecule expression are essential for attraction of immune cells and subsequent diapedesis through the endothelial layer, expression of MHC class II molecules and costimulatory allows endothelial cells to act as antigen-presenting cells, especially important in transplant rejection.

Further analysis revealed profound modifications of intracellular signaling pathway activities in endothelial cells by the underlying matrix [42]: IFN- $\gamma$  dependent JAK/STAT pathways (Fig. 2) and TNF- $\alpha$ -mediated NF- $\kappa$ B activation were significantly inhibited in matrix-embedded endothelial cells. This effect could not be explained simply by the composition of

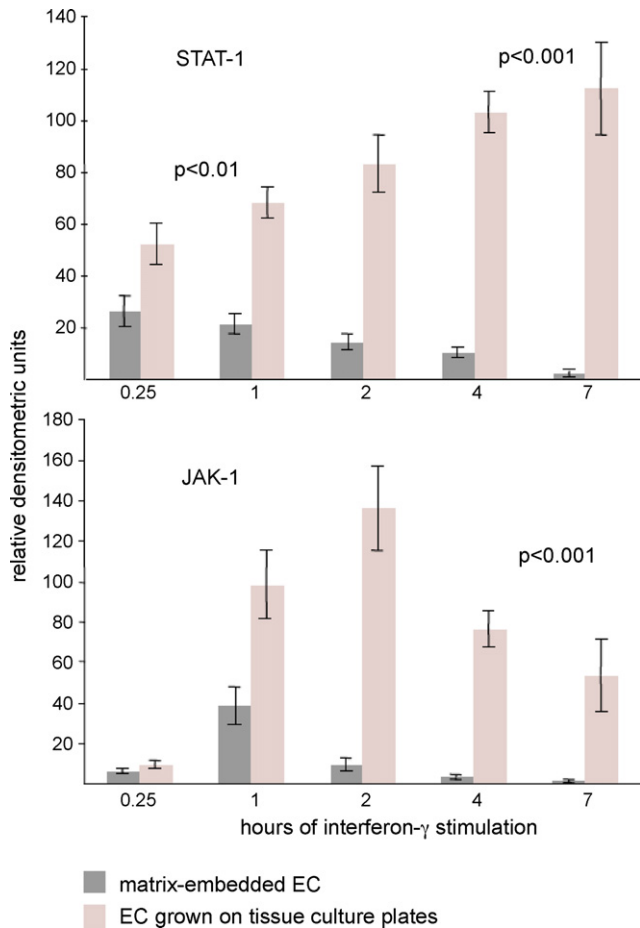


Fig. 2. Densitometric analysis of Western blots demonstrated attenuated phosphorylation of STAT-1 and JAK-1 after IFN- $\gamma$  stimulation for indicated time periods in matrix-embedded human aortic EC when compared to human aortic EC grown to confluence on tissue culture polystyrene plates. Data are expressed as mean values  $\pm$  S.D.

the scaffold, i.e., gelatin as coating of tissue culture plates with gelatin was without effect on intracellular signaling pathways [32,42].

## 6. Interaction with immune cells

Progressive infiltration of engrafted tissues by host mononuclear cells is characteristic of acute rejection. Surprisingly, we could not detect a significant humoral or cellular rejection after implantation of matrix-embedded allo- and even xenogeneic endothelial cells in immunocompetent hosts [32]. In marked contrast to endothelial cells injected as saline suspensions, matrix-embedded endothelial cells did not evoke formation of endothelial-specific antibodies (Fig. 3) or host immune cell infiltration to the site of implantation. Furthermore, matrix-embedded endothelial cells limit T cell activation, their subsequent differentiation and proliferation, and reduced differentiation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells into CD62L<sup>low</sup>CD44<sup>high</sup> effector T cells [32,42,46]. Interestingly these effects were also seen in hosts with preformed anti-endothelial cell immunity: mice repeatedly exposed to saline-suspended endothelial cells demonstrated a vigorous humoral and cellular immune

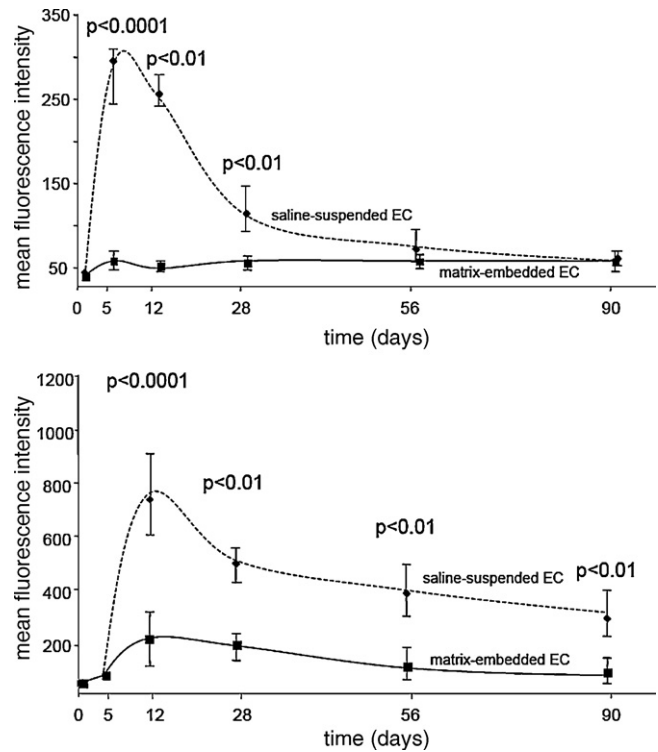


Fig. 3. Circulating EC-specific IgM (upper panel) and IgG<sub>2a</sub> (lower panel) in immunocompetent mice after s.c. injection of saline-suspended porcine aortic EC after grown to confluence on tissue culture plates or s.c. implantation of three-dimensional collagen-based matrix with embedded porcine aortic EC. Flow cytometry revealed a statistically significant difference between animals that had received matrix-embedded and saline-suspended EC. Data are expressed as mean values  $\pm$  S.D.

response to another injection of saline-suspended endothelial cells whereas we observed a decline over time in endothelial-specific antibodies and host immune cell reactivity in mice that received matrix-embedded endothelial cells [46].

Besides crosstalk with adaptive immune cells endothelial cells directly interact with innate immune cells [47–49]. It is increasingly appreciated that maturation of circulating DC is driven by contact with and/or transmigration through activated endothelium. Evidenced by increased expression of CD83, HLA-DR, and costimulatory molecules allo- and xenogeneic endothelial cells drive *in vitro* maturation of DC to the same extent as an established cytokine cocktail. Yet, embedding endothelial cells in three-dimensional collagen-based matrices interferes with the endothelial-mediated maturation of allo- and xenogeneic DC. Allo- and xenogeneic DC cocultured with matrix-embedded endothelial cells retained their phagocytic ability [50]. Further analysis revealed that matrix-embedded endothelial cells instead induced a dendritic cell phenotype that had recently been characterized as immunoinhibiting/modulating (lacking maturation markers, increased secretion of interleukin-10 and transforming growth factor (TGF)- $\beta$ ) [51,52].

Matrix-embedding is far more than passively protective; it enables endothelial cells to actively modulate immune responses—based on significantly increased secretion of

the immune-regulative cytokine transforming growth factor (TGF)- $\beta$  together with sustained expression of negative costimulatory molecules PD-L1 and PD-L2 matrix-embedded allogeneic endothelial cells induce far greater amounts of CD4<sup>+</sup>CD25<sup>+</sup> Foxp3 expressing T regulatory cells than endothelial cells grown on two-dimensional tissue culture plates [53]. Endothelial cells implants were immunomodulatory for an extended time beyond their residence *in vivo*. Prior implantation of matrix-embedded allogeneic endothelial cells allowed for subsequent injection of saline-suspended endothelial cells with a markedly muted immune response, suggesting that matrix-embedded endothelial cells might induce a certain level of tolerance for subsequent tissue transplantation [53].

## 7. Discussion

Our results corroborate others' results that composition and spatial formation of extracellular matrix components affect cellular behavior. Yet, to the best of our knowledge our group is the first to demonstrate effects of matrix–endothelial cell connectivity for endothelial cell immunogenicity. Immune responses against cellular and non-cellular components of tissue engineered devices are becoming increasingly appreciated as potential limitations to successful therapeutic approaches. As the endothelial lining of all vascularized organs and conduits acts as mediator and target of host immune responses, knowledge of the regulation of endothelial immunogenicity could help in the design of biocompatible devices including vascularized grafts. Our findings might help understand how endothelial cells become immunogenic in arterial disease states. Matrix architecture is critical for modulation of endothelial immunogenicity. As three-dimensional matrix-embedding mimics basal anchorage of endothelial cells these findings might offer novel insights to our understanding of various endothelial-mediated diseases, highlighting the importance of spatial formation and endothelial cell–matrix interactions in maintaining endothelial cell (immune)phenotype and vascular homeostasis. Earlier findings have revealed a multi step well regulated process consisting of signaling via integrins, intracellular signaling cascades, down- and up-regulation of specific genes and finally expression and secretion of a variety of pro-inflammatory mediators [54,55]. Tissue engineering with three-dimensional matrices offers a tool to increase our understanding of determinants for endothelial health and of the pathophysiology of diseased endothelium. Further work is needed to develop physiologic three-dimensional matrix structures that might also allow for examination of more realistic milieus (e.g., application of flow).

Overall, research on the influence of spatial matrix formation in addition to the composition of extracellular matrix on endothelial cells (immune)phenotype will further elucidate and improve the therapeutic effects of tissue engineered devices. Modulation of matrix structure might help design suitable vascular conduits for different vascular beds, as endothelial cells reveal a significant vascular bed-specificity and extracellular matrix defined heterogeneity [40].

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