# The Role of Tissue-Resident Macrophages in Transplant Immunity

# THE ROLE OF TISSUE-RESIDENT MACROPHAGES IN TRANSPLANT IMMUNITY

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## Abstract

Successful engraftment of Vascular Composite Allotransplants (VCA), including face transplants, provides functional and quality of life benefits to the recipient. There is significant interest in developing predictive biomarkers that will identify rejection episodes at the earliest possible moment thereby enabling clinicians to preemptively treat rejection episodes, safely guide maintenance immunosuppression minimization or withdrawal, and improve rejection surveillance for investigational therapies.

Tissue-resident macrophages (TRMs) are a population of sentinel phagocytes that are present in virtually all tissues, particularly at barrier sites such as skin that are substantial components of VCA, and arise from yolk-sac not bone marrow derived precursors. We are testing whether a powerful immunoregulatory CD169+ population of TRMs that was previously identified in mice is present in humans. We hypothesize that maintenance of the immunoregulatory phenotype of CD169+ TRMs will correlate with a healthy vascular composite allograft whereas the loss of CD169+ TRMs or their immunoregulatory properties will coincide with incipient and ongoing allograft rejection. Face transplant skin samples from 4 patients were stained for tissue resident macrophage markers and the anticipated immunoregulatory phenotype. We demonstrated the existence of potentially immunoregulatory population of dermal resident macrophages in

normal skin from healthy individuals and quiescent skin in face transplant patients, their disappearance in inflamed conditions and ambiguous behavior upon rejection episodes.

While our data is preliminary we suspect that through evolution a class of TRMs that are cytoprotective in the quiescent state die or lose immunosuppressive properties in response to danger signals. The results suggest further and more detailed examination of undescribed subsets of tissue resident macrophage population. These studies have the potential to provide a powerful new biomarker that will guide clinical care by accurately measuring allograft health and may offer new mechanistic insights into the earliest events that precipitate VCA rejection thereby identifying novel TRM-related therapeutic targets for the establishment of drug-free VCA transplant tolerance.

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# Chapter I: Background

## 1.1 Background

Macrophages are the major population of mononuclear phagocytic system with the main function of preserving homeostasis in tissues. Circulating monocytes and macrophages are derived from bone marrow residing common monocyte-macrophage DC progenitor (MDP) which further gives rise in mice to Ly6C$^{hi}$ monocytes[1]. Upon differentiation monocytes exit to the blood and further migrate and infiltrate tissues under inflammatory conditions[2]. Ly6C$^{hi}$ monocytes express high levels of CC-chemokine receptor 2 (CCR2) which binds to its ligand CC-chemokine ligand 2 (CCL2). High circulating levels of CCL2 in serum and within inflamed tissues are induced by many infections. Macrophage colony stimulating factor (M-CSF, CSF-1), growth factor produced by stromal cells within the blood and in tissues[3], has dual effects on macrophages: it promotes differentiation of myeloid progenitors into monocytes in bone marrow; and regulates migration, proliferation and function of macrophages in peripheral tissues[4]. Other growth factors that stimulate macrophage colony formation include GM-CSF, IFN-γ and IL-3 [5,6,7].

When monocytes enter inflamed tissues, they differentiate into macrophages with the acquisition of a functional phenotype that depends on micro-environmental signals. Macrophages are categorized into two subsets: M1 being aggressive/ inflammatory, playing the major role in central host defense against bacterial and viral infections[8], and M2 being healing/anti-inflammatory, associated with tissue remodeling, fibrosis and tumor progression[9]. Activation of macrophages into the M1 phenotype is mediated in response to bacterial and viral-derived products such as LPS and IFN-γ and M1 function is directed toward killing of microbial pathogens[10]. Macrophages have germline-encoded pattern recognition receptors on their surface that make them efficient at phagocytosis and induce production of inflammatory mediators[11]. In addition to

ingesting pathogens, macrophages also phagocytose dead host cells before the release of their contents and induction of inflammatory responses. Macrophages also serve as antigen presenting cells, displaying antigens and activating T-cells. In contrast, the M2 phenotype, also named alternatively activated phenotype, is induced by the T helper 2 (Th2) cytokines interleukin-4 (IL-4) and IL-13 and promotes tissue repair and remodeling[12]. Upon depletion of Ly6C$^{hi}$ macrophages, the pool is renewed by monocytes derived from hematopoietic stem cells in bone marrow.

However, the existence of macrophages independent of bone marrow derived monocytes implies a distinct origin. Recent fate-mapping studies revealed a Ly6C$^{lo}$ population of macrophages arising from yolk-sac progenitors[13]. Early works by Takahashi et al. reported that fetal macrophages first appear in blood islands of mouse yolk sac at day 9 of gestation and in fetal liver at embryonic day 10; they actively proliferate and colonize embryonic tissues and organs via blood stream circulation and thereafter do not circulate in blood[14]. Modern fate-mapping experiments in mice identify erythro-myeloid progenitors, generated through Tie2+ (Tek) cellular pathway, as a common origin of tissue resident macrophages[15]. Yolk sac macrophage progenitors develop independently of Myb4, a transcription factor required for hematopoietic stem cells, and fetal macrophages were shown to persist in adult tissues[16]. These macrophages undergo homeostatic proliferation which can be driven by IL-34, the second ligand for the CSF-1R[17]. Moreover, IL-34 specifically directs differentiation of myeloid cells towards tissue macrophages in skin and central nervous system[18]. During acute inflammation, tissue resident macrophages undergo a proliferative burst to repopulate the inflamed tissue[19].

As noted, two distinct populations of macrophages can be distinguished by differences in their progenitors, development, Ly6C expression, and mechanism of maintenance. Ly6C$^{hi}$ macrophages differentiate from bone marrow precursors and are released into the circulation,

seeding tissues throughout the body within a few days and replenished by circulating precursors, are referred as monocyte- derived or tissue-infiltrating macrophages. Ly6C$^{lo}$ tissue resident macrophages (TRMs) originate from yolk-sac-derived erythro-myeloid progenitors, reside in tissue and repopulate independently of hematopoietic stem cells[15]. Fully differentiated tissue resident macrophages can re-enter the cell cycle and maintain their numbers by local proliferation.

TRMs strategically seed peripheral tissues during early development and are divided into subpopulations according to their anatomical sites and functionality. Some of specialized TRMs include: alveolar macrophages in lungs, which eliminate dust, allergens and microorganisms; Kupffer cells in liver, which clear pathogens and toxins from circulation; intestinal macrophages, which recognize and remove enteric pathogens and regulate tolerance to food and microbiota. Distinct population of TRMs are also present in secondary lymphoid organs, such as marginal zone macrophages in the spleen and subcapsular sinus macrophages of lymph nodes (LNs), and the main functions of these TRMs include aiding local homeostasis by suppressing innate and adaptive immunity[20].

One of the most essential barrier sites is skin, composed of epidermis and dermis with subcutaneous fatty region, and populated by a variety of cells to maintain protective functions[21]. TRMs and their role in skin are not thoroughly investigated and it is not clear if the resident subpopulation correlates with monocyte-derived macrophages, or if their functions are antagonistic. Some authors characterize Langerhans cells, primarily located in the epidermis, as macrophages because of their common yolk-sac progenitors and the dependence on M-CSF[22], while Langerhans cells are classically described as migratory professional antigen presenting cells

of the skin[23]. TRMs, presumably populating dermis, respond to danger signals and initiate inflammatory response, which later leads to wound healing[24].

Macrophages are orchestrated by variety of cell surface molecules, which reflect their maturation, differentiation, adhesion, phagocytosis, apoptosis, etc. One of the cell receptors, specific for TRMs is Sialoadhesin (Sn), also called CD169, Siglec-1, a member of the sialic acid binding, immunoglobulin-like lectins (Siglec) family[25]. Studies indicate that the predicted protein sequences of human and mouse Sialoadhesin are about 72% identical[26]. Using a CD169-diphtheria toxin receptor (DTR) transgenic mouse line, in which CD169+ macrophages are specifically ablated following application of diphtheria toxin (DT), Gupta et al. reported that CD169+ macrophages can control parasite propagation and restrain inflammation [27].

TIM-4 (T cell Immunoglobulin & Mucin domain containing protein 4) is exclusively expressed on professional antigen presenting cells such as macrophages and conventional dendritic cells[28]. TIM-4 acts as a phosphatidylserine receptor binding the surface of apoptotic cells mediating their engulfment, and as a costimulatory signal. Efficient clearance of dying cells is a vital prerequisite for maintenance of tissue homeostasis, tolerance and prevention of autoimmunity and inflammation[29]. Compared to other TIM family members, the cytoplasmic tail of TIM-4 lacks putative signaling motifs, consequently it cannot mediate direct inward signaling[30]. TIM-4 is not expressed on T-cells, but it is the ligand for TIM-1, and TIM-1:TIM4 costimulatory interaction induces the proliferation and expansion of T-cells[31]. In segmental hepatic warm ischemia mice models TIM-4 blockade inhibited T cell differentiation and facilitated CD4+ CD25+ Foxp3+ T-regulatory cell expansion through the inhibition of IL-4/Stat6 signaling[32]. Blockade of TIM-4 on activated dendritic cells in vivo promotes differentiation of naïve CD4+ T-cells into FoxP3+ T-regulatory cells and induces prolonged skin allograft survival[36].

Thornley et al. investigated CD169+TIM-4+ TRMs, their phenotype and role in transplant immunity[34].The authors demonstrated that the CD169+TIM-4+, not CD169+TIM-4– subset of CD45+Cd11b+F4/80+Ly6C– skin TRMs migrate to secondary lymphoid organs following oxidative stress. Phenotype analysis of TRMs revealed that CD169+TIM-4+ TRMs had lower expression of antigen presentation (MHCII) and costimulatory (CD80, CD86) molecules compared to CD169– TRMs. But CD169+TIM-4+ macrophages express high levels of CD39 and CD73 and galectin-9, with galectin-9 being an identified ligand for TIM-3 and inhibiting T helper 1 (Th1) responses[35] . CD39/CD73 system of catabolic enzymes cleaves ATP to ADP and converts ADP into adenosine, and adenosine possesses potent anti-inflammatory and immunosuppressive activities[36]. In short, hypo-expression of costimulatory CD80/86 proteins, and antigen presenting MHC molecules as well as expression of immunoregulatory proteins may determine anti-inflammatory function of CD169+TIM-4+ TRMs. In the mixed lymphocyte reaction (MLR) these cells induce 2.5-fold increase in T-regulatory cells differentiation and proliferation compared to CD169− macrophages[37]. Moreover, MLR experiments showed lower expression of TNF-a, IL-6, IL-5 and IL-4, increased levels of FoxP3 and poor overall T-cell survival in cultures stimulated with of CD169+TIM-4+ TRMs compared to co-cultivation with CD169− macrophages. Furthermore, Thornley et al. used TIM-4 knockout mouse model which supposes the survival of TIM-4+ cells by preventing their phosphatidylserine pocket binding to apoptotic cells and, therefore, enabling the survival of these cells by obviating their attraction to the sites of inflammation. Investigation of TIM-4-/- mice determined that TIM-4-/- CD169+ TRMs are increased in number and frequency in the draining lymph nodes after oxidative stress and these cells are also resistant to stress induced apoptosis. TIM-4 -/- heart transplants in mice also induced prolonged graft survival in a T-regulatory cell dependent way. Another interesting study,

consistent with the theory of immunoregulatory subset of TRMs, was performed in lung tissue of naive, un-manipulated mice and indicated that CD11c+ F4/80+ lung resident macrophages generated Foxp3+ regulatory T cells and promoted airway tolerance[38].

Can the potent effects of TRMs in mouse transplant immunity be investigated in human recipients of composite vascular allografts? Vascularized Composite Allotransplantation (VCA) involves the transplantation of heterogeneous structures such as skin, bone, muscles, blood vessels, nerves and connective tissue. The United Network for Organ Sharing established 9 criteria characterizing VCA as a transplant which: (1) is vascularized and requires blood flow by surgical connection of blood vessels to function after transplantation; (2) contains multiple tissue types; (3) is recovered from a human donor as an anatomical/structural unit; (4) is transplanted into a human recipient as an anatomical/structural unit; (5) is minimally manipulated (i.e., processing that does not alter the original relevant characteristics of the organ relating to the organ's utility for reconstruction, repair, or replacement); (6) is intended for homologous use (the replacement or supplementation of a recipient's organ with an organ that performs the same basic function or functions in the recipient as in the donor); (7) is not combined with another article such as a device; (8) is susceptible to ischemia, and therefore, only stored temporarily and not cryopreserved; (9) is susceptible to allograft rejection, generally requiring immunosuppression that may increase infectious disease risk to the recipient[39]. Among the common types of VCA there are hand (single and double) and face (partial and full) transplantations.

Face transplant surgery is an innovative and promising treatment for patients who suffer from severe facial disfigurement due to traumatic injuries, electrical/chemical burns, congenital defects, etc. Though it is not a life-saving operation, functional and aesthetic outcomes lead to improved quality of life of these patients and a chance to integrate in social life. With world's first

partial face transplant operation on a living recipient performed in 2005 and full face transplant in 2010, these procedures are subject to significant ethical, technical and medical challenges. Ideas or knowledge concerning optimal immunosuppressive regimen, long-term outcomes and patient selection are unknown or in flux[40]. VCA is in transition from experimental reconstructive operation to established clinical treatment.

One of the major limitations in VCA is the necessity in long-term immunosuppressive therapy used currently in the clinic after transplantation to inhibit the immune system and prevent acute rejection[41]. Prolonged immunosuppression can lead to development of life-threatening infections and susceptibility to develop malignancies. Moreover, commonly used calcineurin inhibitors, such as cyclosporine and tacrolimus, may increase risk of hypertension, hyperglycemia, and hyperlipidemia and may generate neurotoxic and post-transplant diabetic complications[42].

The other significant problem is the absence of a unified VCA transplant registry, though there is a voluntary self-reporting "International Registry on Hand and Composite Tissue Transplantation". This registry analyzed short and long- term outcomes in 39 patients who received hand transplants and 15 cases of partial or total face allo-transplantation[43]. Acute skin rejection was documented at least at 85% of all the patients within the first post-operative year. Acute, untreatable rejections can results in a massive loss of allograft, open wounds and even fatal consequences.

The diagnosis of rejection in VCA can be a challenge. There are no unified assays to control VCA graft rejection, unlike solid organ transplantation, i.e. creatinine levels in kidney transplants. Vascular composite transplants consist of different tissues with skin being the most antigenic one, containing immune cells in both layers. Skin-containing allografts, because of the ability of visual assessment of rejection due to its dermatologic manifestations, are easier to be examined at the

bed side, compared to solid organ transplantation. Skin biopsy is one of the least invasive procedures used to evaluate dermatological pathology. Macroscopic features that allow to suspect rejection are skin redness, swelling, induration or mucosal ulceration[44]. Suspicion of rejection based on visual inspection needs to be confirmed by histopathological examination. The Banff Classification is a schema for nomenclature and classification of renal allograft pathology, established in 1991. The first Banff VCA meeting was held in 2007 to classify and characterize stages of rejection. Skin allograft pathology Banff classification was adopted to serve as a standard for VCA rejection[45]. The Banff scale is used for adjudicating the histopathological grade of rejection in which samples are deemed 0, no chance of rejection, if they are free of inflammation or Banff I or II for low-grade abnormalities such as mild perivascular inflammation. Grade III and IV scores indicate severe rejection with features of epidermal involvement and epithelial apoptosis, dyskeratosis or even necrotizing events (Table 1).

**Table 1. BANFF system for grading skin-containing composite tissue allografts.**

| GRADE | HISTOPATHOLOGICAL FEATURES |
|---|---|
| ***grade 0*** | no or rare inflammatory infiltrates |
| ***grade I (mild)*** | mild perivascular infiltration (with no epidermal involvement) |
| ***grade II (moderate)*** | moderate-to-severe perivascular inflammation with or without mild epidermal and/or adnexal involvement |
| ***grade III (severe)*** | dense inflammation and epidermal involvement with epithelial apoptosis, dyskeratosis, and/or keratinolysis |
| ***grade IV (necrotizing acute rejection)*** | frank necrosis of epidermis or other skin structures |

However, it is important to keep in mind that immunogenic skin lesions can develop in response to allergic or inflammatory processes common in immunosuppressed patients. Not all the

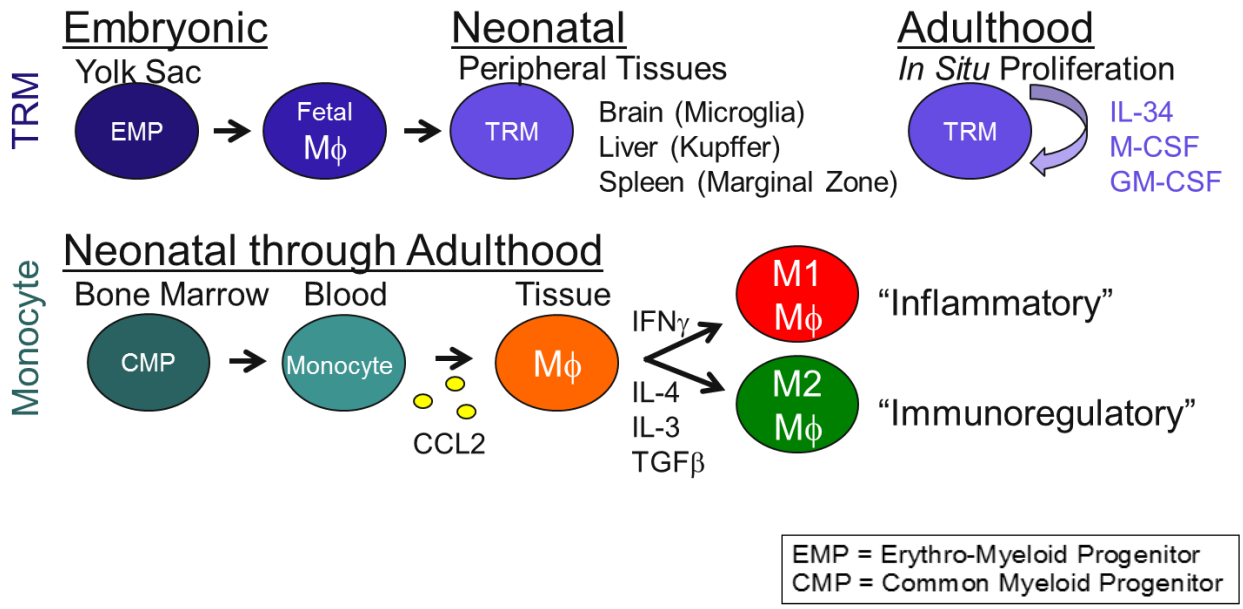
changes in VCA skin samples represent rejection episodes. Therefore, histological assessment is absolutely crucial to determine the grade and severity of rejection or infectious, autoimmune, allergic dermatological components of the pathological reaction. For example, one of the first full face transplant patients in Brigham and Women's Hospital suffered from pathological skin changes which were at first misinterpreted as acute graft rejection, but further analysis revealed a donor history of rosacea. After appropriate treatment for rosacea, the inflammatory episode resolved[46]. This episode is of particular interest, as we further worked with these skin samples. Fischer et al. further conclude that skin is not the only target of rejection in VCA, showing that oral mucosa can also undergo severe rejection processes. The other point is that although skin maybe graded as Banff 0 or I, underlying tissue pathology, like vasculopathy or muscle fibrosis, can represent graft failure. In animal studies, Lee at al. transplanted individual vascularized limb tissues (skin, subcutaneous tissue, muscle, bone, and blood vessels) in rats and showed that inflammatory reactions varied in timing and intensity[47].

Even though acute rejection incidence in VCA is quite high, chronic rejection occurs rarer than in solid organ transplantation. Prompt diagnosis of rejection at early stages reduces the chances of forming large numbers of donor-reactive T-memory cells and thereby decreases chances for chronic rejection. Due to current insufficient data available, the Banff 2007 classification has not included chronic rejection grading in composite tissue grafts yet[48]. The clinical and pathological features that could represent chronic rejection are: vascular narrowing, loss of adnexa, skin and muscle atrophy, fibrosis of deep tissues, myointimal proliferation and nail changes. Moleron et al. evaluated composite tissue allografts by histological assessment, ultrasonography, magnetic resonance imaging and high resolution tomography scan without finding any evidence of chronic rejection[49]. Possible explanations of relative resistance of

composite allografts to chronic rejection can be the different mechanisms involved in this kind of rejection (both cellular and humoral immune reactions like in solid organ transplantation), also shaped by intensive immunosuppressive therapy[39].

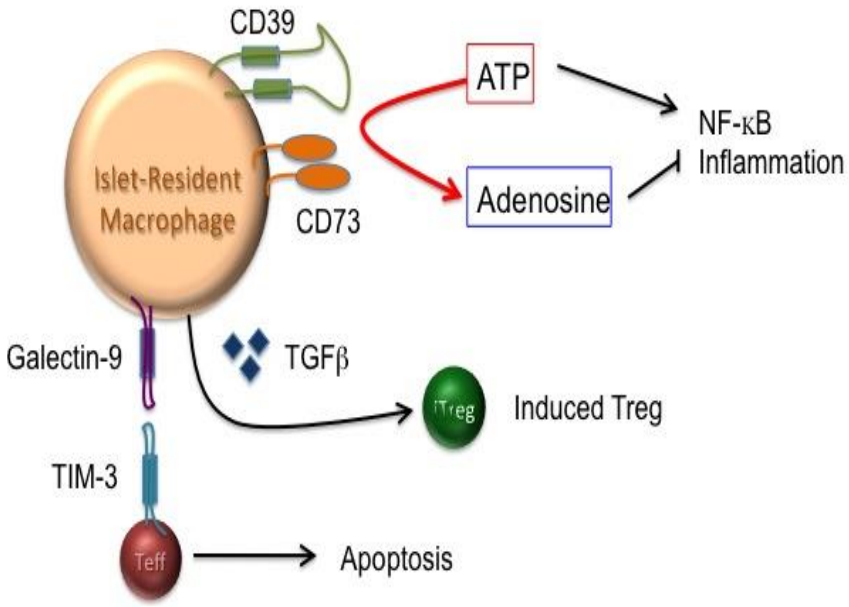
Meanwhile, the best treatment of both acute and chronic rejections is prevention and early diagnosis, with better understanding of immune cell functions involved in both homeostasis and rejection. Previous experiments in mice revealed that preserving the function and viability of CD169+TRMs will serve to prevent rejection for substantial periods of time and tilt immunity toward tolerance[37]. In this study we aim to determine whether a change in the abundance of CD169+TIM-4+ TRMs correlates with healthy VCA transplants in humans and whether TRMs can be a therapeutic target in VCA rejection or tolerance.

## 1.2. Schematic figures




Schematic figure 1. Tissue-resident macrophages are a distinct lineage from monocyte-derived macrophages.

- Tissue-resident macrophages come from embryonic yolk sac derived precursors
- Tissue-resident macrophages seed the tissues and maintain their presence there through homeostatic proliferation which can be driven by IL-34, Macrophage Colony Stimulating Factor And Granulocyte Macrophage Colony Stimulating Factors.




Schematic figure 2. CD169+TIM-4+ tissue-resident macrophages are migratory and have an immunoregulatory phenotype.

- ***Migratory:***

✓ Migrate to the dLN in response to oxidative stress

- ***Immunoregulatory phenotype:***

✓ CD39/CD73: Cleave ATP and generate adenosine collectively
✓ TGF β: induces iTregs
✓ Galectin-9: induces apoptosis in TIM-3+ Th1/ Th17 effector T cells
✓ Low levels of MHCII/ antigen presentation & costimulatory CD80,CD86, CD40 molecules

## Chapter II: DATA AND METHODS

### 2.1. Short introduction

Successful engraftment of Vascular Composite Allotransplantation (VCA), including face and limb transplants, provide functional and quality of life benefits to the recipient. However, VCA transplants are life-improving but not life-saving and require life-long immunosuppression. As such, the benefits of the VCA transplants must be carefully considered along with the risks of chronic immunosuppression. Thus, there is significant interest in developing predictive biomarkers that will identify rejection episodes at the earliest possible moment thereby enabling clinicians to preemptively treat rejection episodes, safely guide maintenance immunosuppression minimization or withdrawal, and improve rejection surveillance for investigational therapies.

Tissue-resident macrophages (TRMs) are a heterogeneous population of sentinel phagocytes residing in virtually all tissues, particularly at barrier sites such as skin that are substantial components of VCA transplants, and arise from yolk-sac, not bone marrow derived precursors[13]. In a mouse cardiac allograft transplantation, maintenance of CD169+TIM-4+ TRMs with immunoregulatory phenotype and function forecasts allograft survival whereas the loss of this protective population and its immunoregulatory phenotype is a harbinger of allograft rejection[34]. Moreover, efforts to preserve the immunoregulatory function of TRMs powerfully promote prolonged engraftment and lower the barrier to transplant tolerance induction. We aim to test whether a powerful immunoregulatory CD169+ population of tissue resident macrophages that we have previously identified in mice is present in humans. Moreover, we are testing the hypothesis that the presence of these immunoregulatory macrophages within the transplant will be associated with freedom from rejection while recipients with transplant biopsies that fail to bear these cells will experience incipient or ongoing rejection.

## 2.2. Materials and methods

*Tissue samples*

Samples of normal skin, lymph nodes, tonsils, inflamed skin (hypersensitivity Type IV, contact dermatitis) and other tissues for positive and negative controls were obtained from our collaborators at Brigham and Women's Hospital (BWH, Boston, MA, USA).

*Patients*

Serial skin biopsy samples from a total of four patients receiving face transplant at BWH between 2009 and 2013 were evaluated (Table 2).

**Table 2. Characteristics of four full face transplant recipients**

| | **PATIENT 1** | | | **PATIENT 2** | | **PATIENT 3** | | **PATIENT 4** | |
|---|---|---|---|---|---|---|---|---|---|
| ***Donor age*** | 31 | | | 42 | | 56 | | 51 | |
| ***Recipient age*** | 30 | | | 57 | | 44 | | 38 | |
| ***Biopsy sites*** | Lateral face/neck | | | Lateral face/neck | | Lateral face/neck | | Lateral face | |
| ***Time after transplantation*** | 4 mo | 24 mo | 3 yr | 2.5 yr | 3 yr | 7 mo | 1.5 yr | 9 mo | 11 mo |
| ***Stage (Banff criteria)*** | I/early II | II/minimal III | non-rejection | early stage III rejection | Non-rejection | non-rejection | I/early II | Rejection III | Non-rejection |

The paraffin-embedded blocks were taken from the repository of our collaborators at BWH and cut into 5-mm-thick slides at their core facility. The patients were on immunosuppressive

therapy to maintain tolerance and prevent rejection according to standards and protocols[46]. We analyzed both surveillance and diagnostic biopsies. The clinical features that led the clinicians to suspect rejection were redness, swelling, induration or mucosal ulceration.

*Immunohistochemistry and Immunofluorescence*

All specimens were evaluated by conventional immunohistochemistry. 5-mm-thick sections were prepared from formalin-fixed, paraffin-embedded tissues, taken from tissue repository. After deparaffinization, the Pascal pressure cooker was used for antigen unmasking. Primary antibodies utilized are: anti-CD68 (ab955, mouse polyclonal antibody, Abcam, Cambridge, MA, USA), anti- CD11c (ab52632, rabbit polyclonal antibody, Abcam), anti-CD169 (ab183356, rabbit polyclonal antibody, Abcam), anti-TIM-4(NBP1-87569, rabbit polyclonal antibody, Novus Biologicals, CO, USA), anti-TIM-4 (AF2929, goat polyclonal antibody, R&D systems, MN, USA), anti-ENTPD1 (CD39) (rabbit polyclonal antibody, Sigma Aldrich, MO, USA), anti-IL-10 (BS-0698R, rabbit polyclonal antibody, Bioss, MA, USA) (Table 3). For substrate staining we used peroxidase substrate kit (Vector laboratory, Burlingame, CA, USA), and counterstained sections with hematoxylin. After mounting the sections, samples were evaluated in light microscope (Nikon Eclipse 80i), and the pictures were captured using software NIS-elements freeware 2.10. Positive and negative tissue controls and isotype-specific irrelevant antibody controls were used to ensure specificity.

**Table 3. Antibodies used in immunohistochemistry.**

| **Common name** | ***Gene*** | **Functions** |
|---|---|---|
| CD68 | *Cd68* | Plays a role in phagocytic activities of tissue macrophages, both in intracellular lysosomal metabolism and extracellular cell-cell and cell-pathogen interactions. Binds to tissue- and organ-specific lectins or selectins, allowing homing of macrophage subsets to particular sites. |
| CD11c | *Itgax* | Expressed on monocyte-derived cells, including macrophages. Mediates cell-cell interaction during inflammatory responses. Especially important in monocyte adhesion and chemotaxis. |
| CD169 | *Siglec-1* | Expressed only by tissue resident macrophages and is involved in mediating cell-cell interactions |
| TIM-4 | *TIMD4* | Phosphatidylserine receptor that enhances the engulfment of apoptotic cells. Also binds to TIM-1 and mediates a costimulatory activation signal |
| CD39 | *ENTPD1* | Hydrolyzes extracellular ATP and ADP to AMP |
| IL-10 | *IL-10* | Immunoregulatory cytokine, produced primarily by monocytes |

Dual-labeling immunofluorescence was performed to identify co-distribution of different antigens in the same sample. Following overnight incubation of the tissues with a mix of two primary antibodies from different species (rabbit and mouse), samples further incubated with Alexa Fluor 594-conjugated anti-mouse IgG and Alexa Fluor 488-conjugated anti-rabbit IgG (Invitrogen). The sections were cover slipped with ProLong Gold anti-fade with DAPI (Invitrogen). The covered samples were evaluated with a BX51/BX52 microscope (Olympus America, Melville, NY, USA), and images were captured using the CytoVision 3.6 software (Applied Imaging, San Jose, CA, USA).

## 2.3. Results

To test our technique, we stained lymphoid tissue- non-stimulated tonsils with anti-CD68, anti-CD11c, anti-CD169 and anti-TIM-4 antibodies before testing the valuable skin biopsy specimens. Anti-CD68 and anti-CD11c antibodies bind to epitopes in the B-cell zone follicle of the tonsil, marginal zone and T-cell zone that surrounds it (Figure 1, A-B). While anti-CD11c and anti-CD68 antibodies stain the same population of dendritic cells and macrophages, anti-CD169 stains macrophages in T-cell zone that surrounds B-cell zone; and anti- TIM-4 (AF2929) lightly stains many CD169 positive cells in the T-cell zone (Figure 1, C-D). We can conclude that CD169+ cells are a distinct population of macrophages from monocyte-derived macrophages, at least in non-stimulated tonsils.

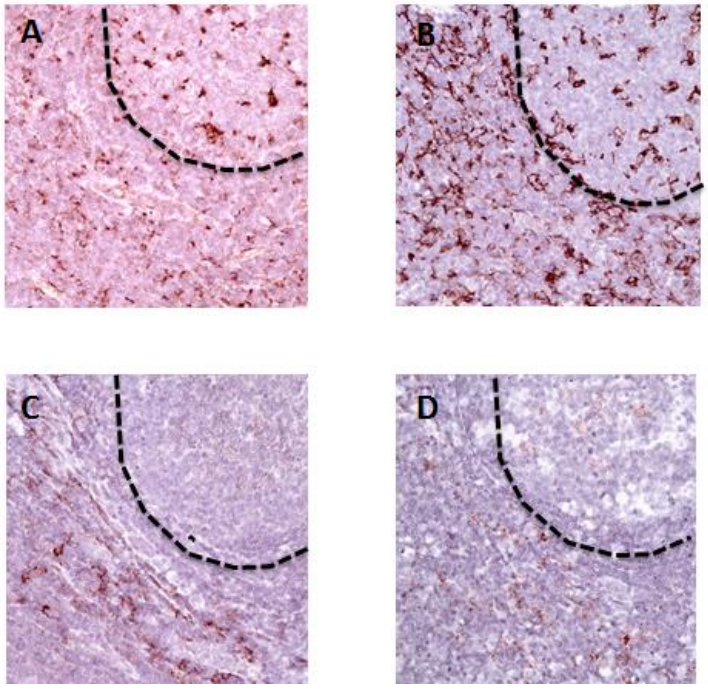



Figure 1. Immunohistochemistry (Formalin/PFA-fixed paraffin embedded tonsil).**A.** Anti-CD68 antibody**.** **B**. Anti-11c antibody. **C**. Anti-CD169 antibody. **D.** Anti-TIM-4 antibody

Next we stained normal skin with anti-CD11c, anti-CD68 and anti-CD169 antibodies which allow us to discriminate CD169+ tissue resident macrophages from CD169− monocyte-derived macrophages (Figure 2). In normal skin anti-CD11c and anti-CD68 antibodies similarly stain perivascular macrophages and monocytes around superficial vessels, while anti-CD169 stains perivascular component as well as interstitial cells, logically proving their tissue resident status. Interestingly, a closer look at CD169+ macrophages shows that these cells have considerably long dendrites (Figure 3).

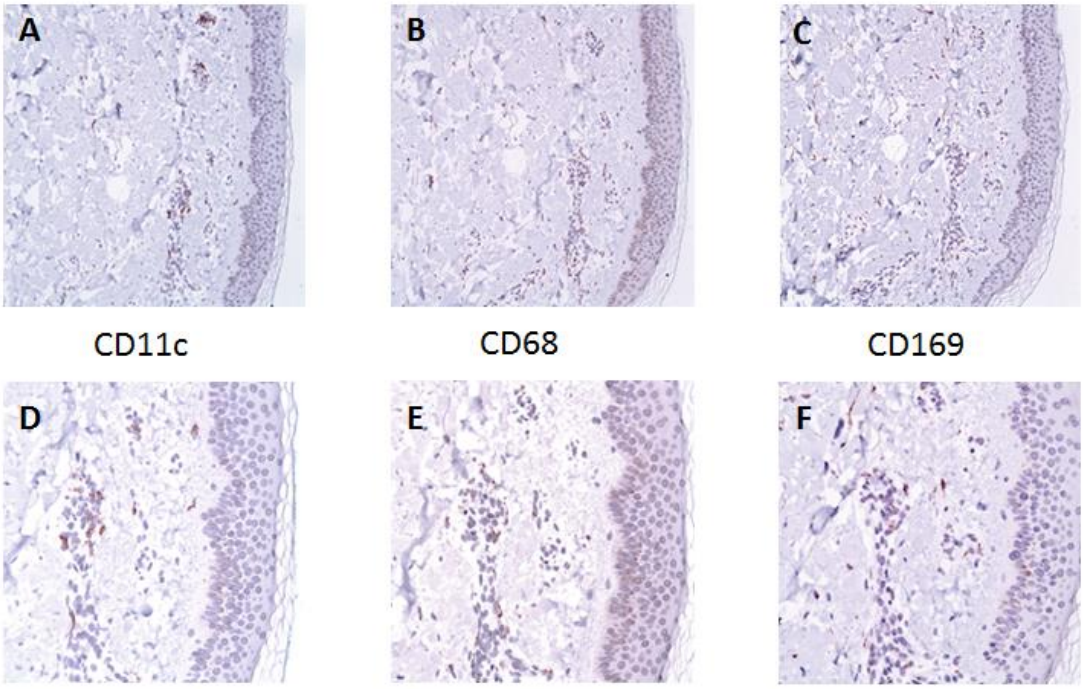



Figure 2. Immunohistochemistry (Formalin/PFA-fixed paraffin embedded sections). Normal skin.
**A, B, C**- magnification × 20; **D, E, F**- mag.x40

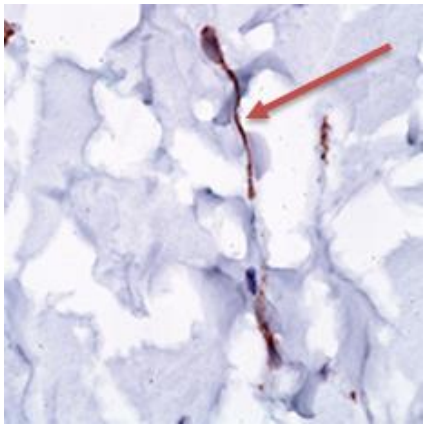


Figure 3. Immunohistochemistry (Formalin/PFA-fixed paraffin embedded sections). Normal skin. Anti-CD169 antibody. mag.x100

Further, we assessed the characteristics of these cells during inflammation in biopsies from spongiotic dermatitis, which is a cell-mediated, type IV hypersensitivity reaction. The results show that CD11c+ and CD68+ cells expand during hypersensitivity type IV reaction, whereas CD169+ cells significantly reduce in numbers in the inflammatory environment (Figure 4). CD11c+ and CD68+ cells are abundant in perivascular area and the epidermis; and the CD169+ staining perivascular component disappears upon inflammation and we can only see a few CD169+ interstitial cells.

Double staining of normal and inflamed samples with anti-CD68 and anti-CD169 antibodies was consistent with previous results (Figure 5). Skin sample from patients diagnosed with contact dermatitis contains CD68, a pan macrophage marker, cells but these CD68+ cells do not express the CD169, marker of TRMs, at the site of inflammation.

Following these preliminary studies we were ready to evaluate valuable samples from patients who received face transplants. Using single staining IHC we determined that CD169+ macrophages were present during the period of quiescence and disappeared upon rejection II/minimal III in Patient 1 (Figure 6). Though rejection samples may seem to have some positively

stained cells, a closer image (Figure 6 C-D x100*)* showed difference in color and melanin granules stained within the cytoplasm in the rejected sample, compared to membrane staining in quiescent skin sample.

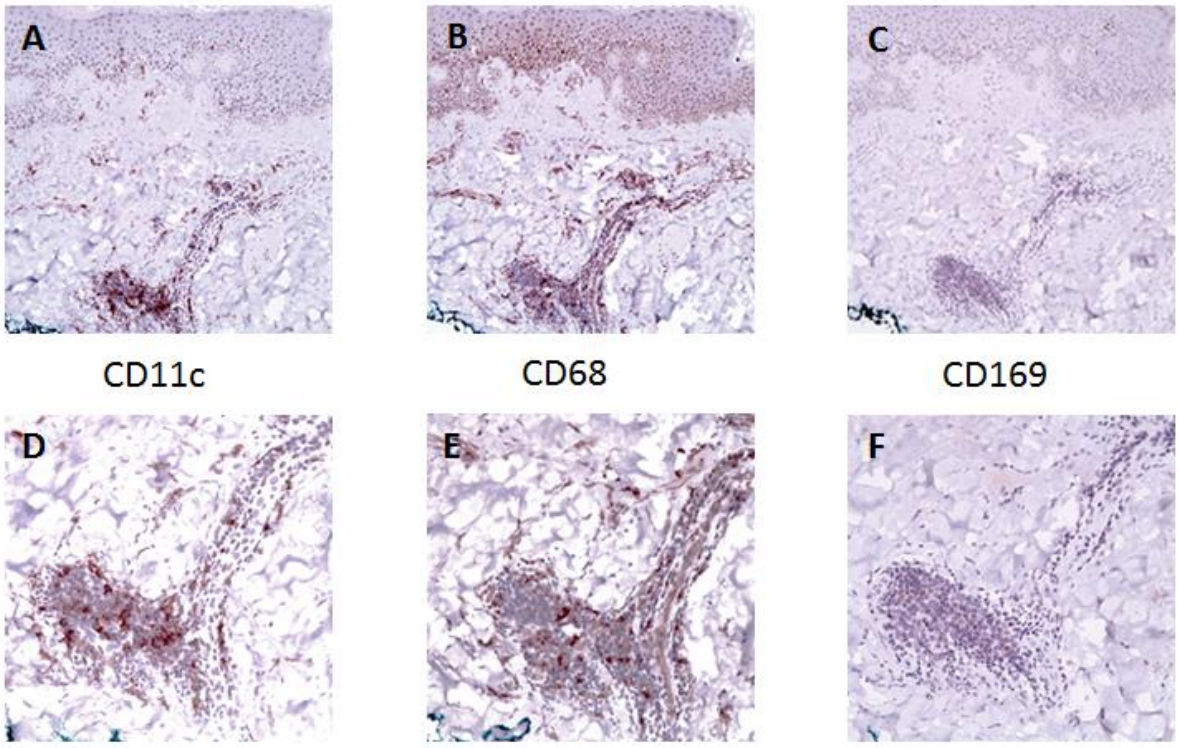



Figure 4. Immunohistochemistry (Formalin/PFA-fixed paraffin embedded sections). Spongiotic dermatitis.
**A, B, C**- magnification x20; **D, E, F**- mag.x40

To prove that we performed double staining of face transplant skin samples with anti-CD169 and anti-CD68 antibodies in the same Patient 1 and the results determined population of CD68+ macrophages that co-express CD169 in samples graded as Banff 0 (Figure 7). In contrast, in Banff grade III or II/III samples we identify numerous CD68+ macrophages but fail to see CD68+ CD169+ double positive macrophages. Circulating CD68+ macrophages must seed the inflamed tissue.

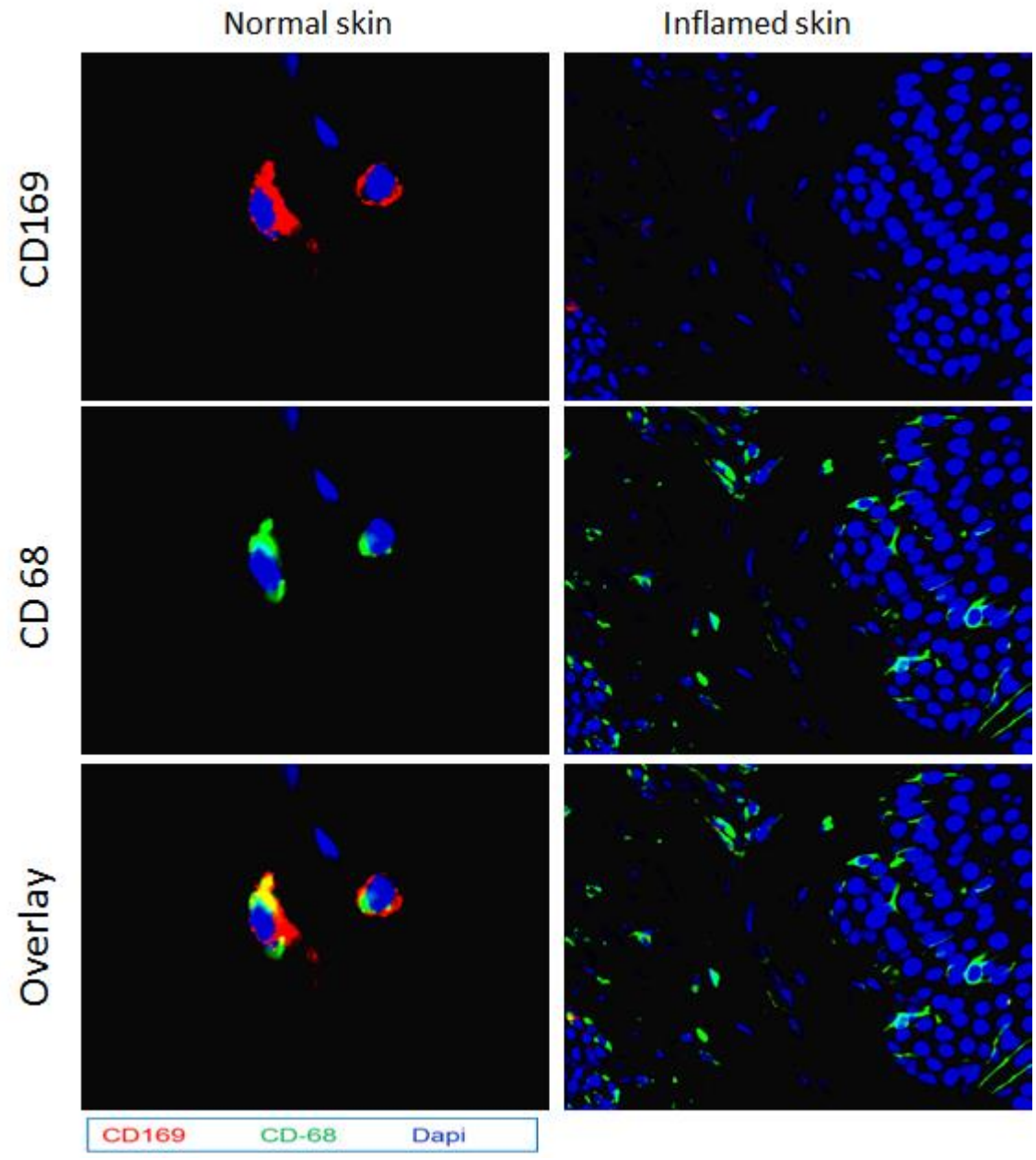



Figure 5. Immunofluorescence (Formalin/PFA-fixed paraffin embedded sections) - Anti-CD169 antibody (red), anti-CD68 antibody (green) in normal skin and inflamed skin biopsies (spongiotic dermatitis).

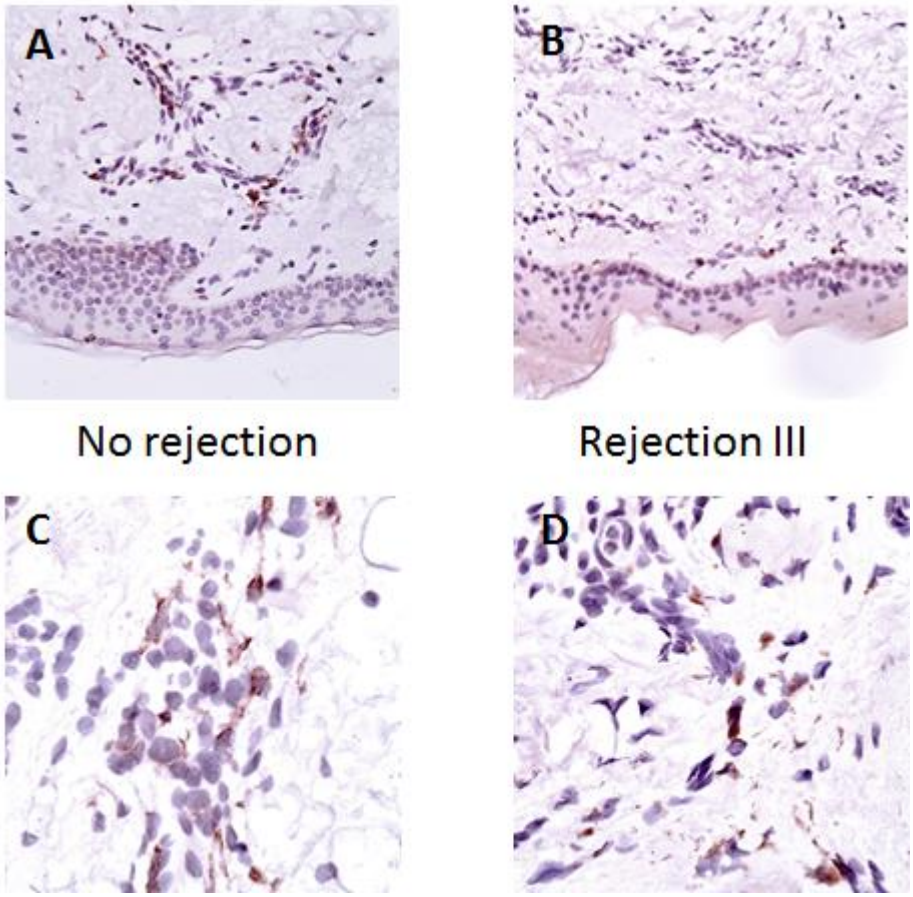



Figure 6. Immunohistochemistry (Formalin/PFA-fixed paraffin embedded sections) - Anti-CD169 antibody. Banff score in Patient 1: A, C - non-rejection (0), B, D- early stage II/ minimal III rejection.
**A, B**- magnification x20; **C, D**- mag.x100.

Further analysis of skin samples from Patients 2-4 were quite contradictive (Figures 8-10). While skin biopsy from Patient 2 revealed the same previously noted pattern of CD169+ macrophages present in quiescence and disappearing upon rejection (Figure 8), the pattern found in face transplant samples from Patient 3 and Patient 4 was unexpected. CD169+CD68+ macrophages were viable during rejection episodes in skin samples from these patients (Figures 9-10).

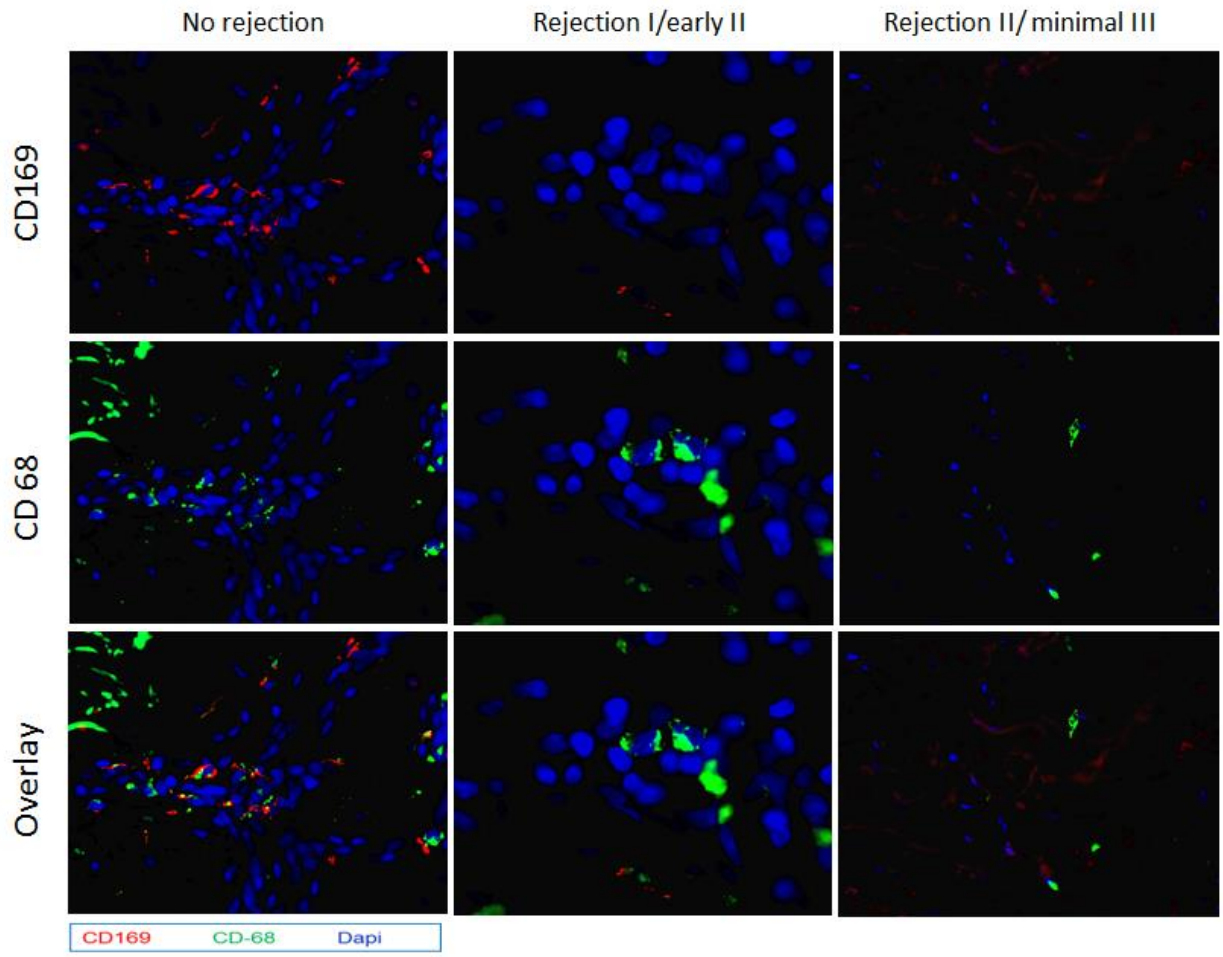



Figure 7. Immunofluorescence (Formalin/PFA-fixed paraffin embedded sections) - Anti-CD169 antibody (red), anti-CD68 antibody (green). Banff score in Patient 1: non-rejection (0), stage I/early II, early stage II/ minimal III rejection.

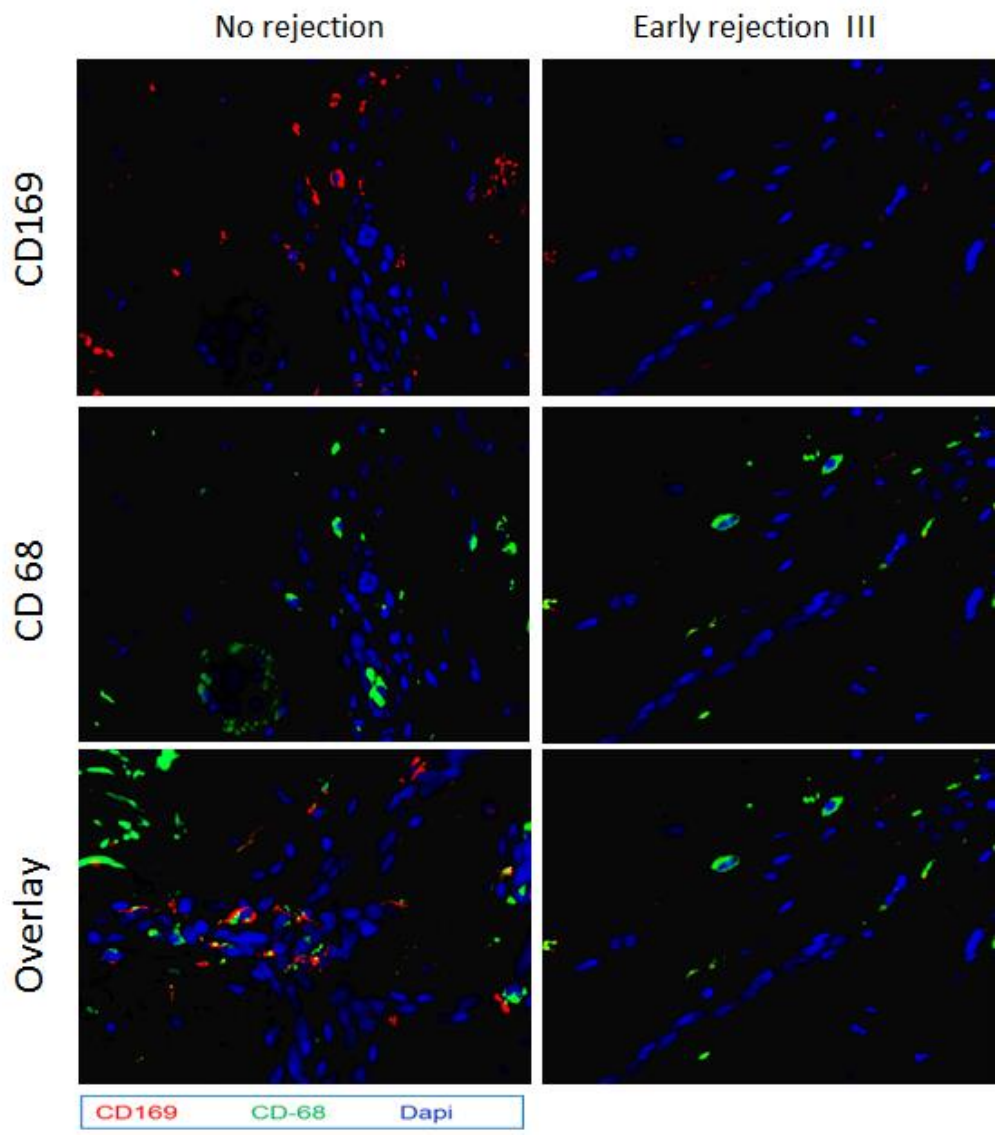



Figure 8. Immunofluorescence (Formalin/PFA-fixed paraffin embedded sections) - Anti-CD169 antibody (red), anti-CD68 antibody (green). Banff score in Patient 2: non-rejection (0), early rejection stage III.

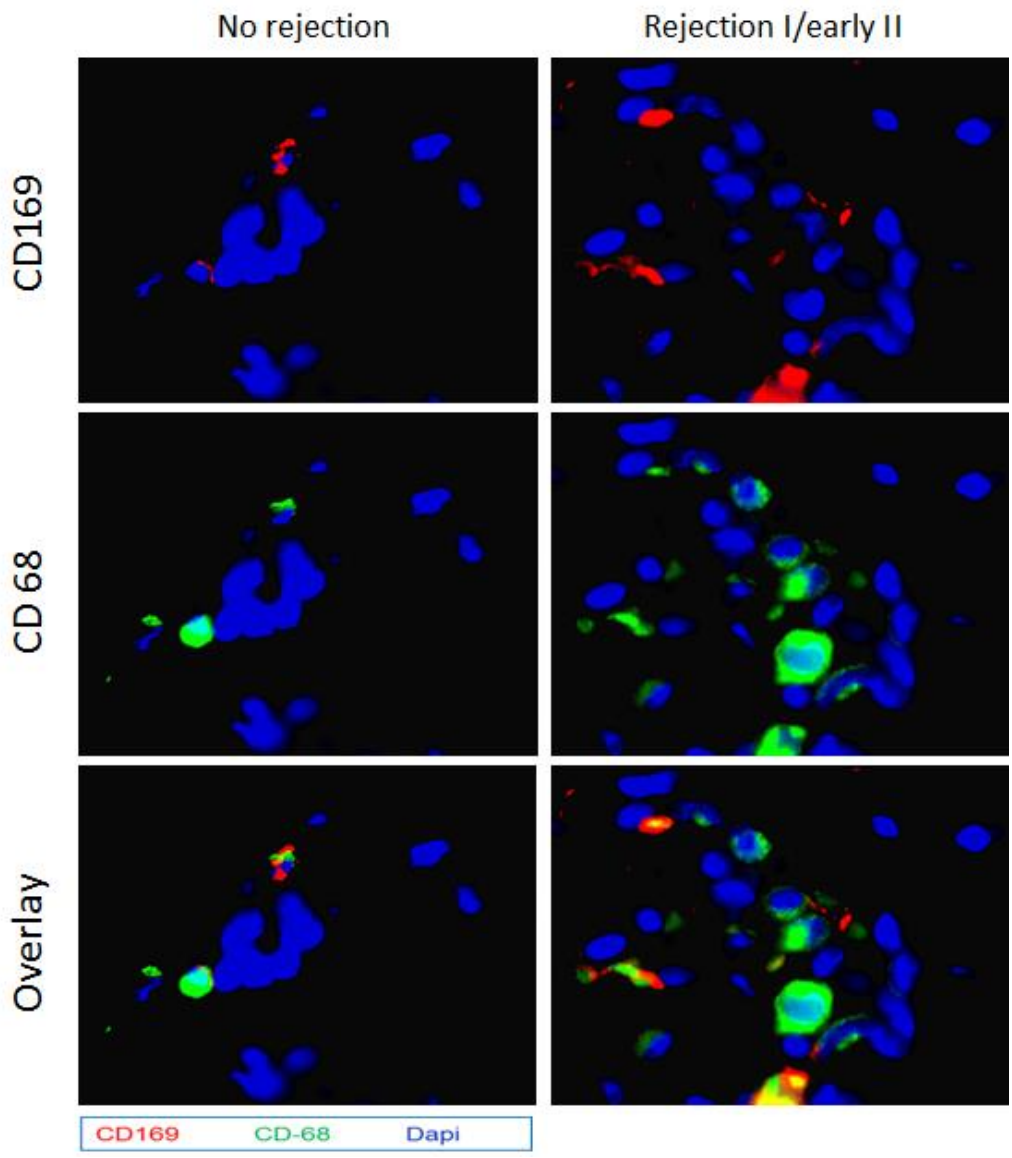



Figure 9. Immunofluorescence (Formalin/PFA-fixed paraffin embedded sections) - Anti-CD169 antibody (red), anti-CD68 antibody (green). Banff score in Patient 3: non-rejection (0), rejection I/early II.

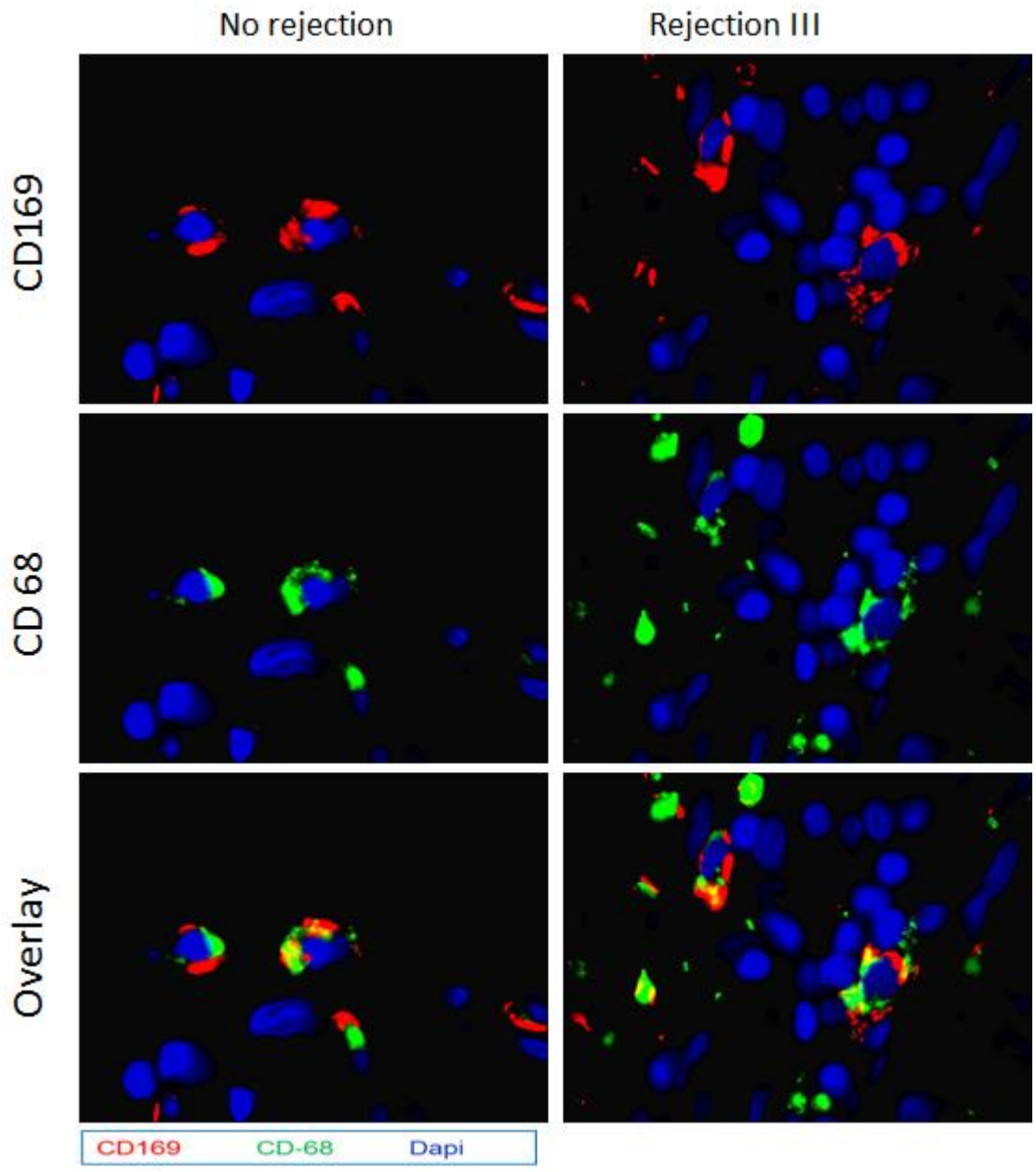



Figure 10. Figure 8. Immunofluorescence (Formalin/PFA-fixed paraffin embedded sections) - Anti-CD169 antibody (red), anti-CD68 antibody (green). Banff score in Patient 4: non-rejection (0), rejection III.

The CD68+CD169+ cells exert immunoregulatory properties in mice, and most of these cells also express TIM-4, a protein expressed not only by macrophages but also other APCs, particularly, mature dendritic cells. Studies to date indicate that CD68+ TIM4+ cells can be identified in skin but have not yet determined whether these cells co-express CD169. IHC with anti-TIM-4 antibody showed that TIM4+ cells are present in both quiescent and rejecting samples (Figure 11) but we do not yet know whether these cells are macrophages or DCs or both. Therefore, we cannot identify correlation between Tim-4 and CD169 staining in non-rejecting and rejecting face transplant samples.

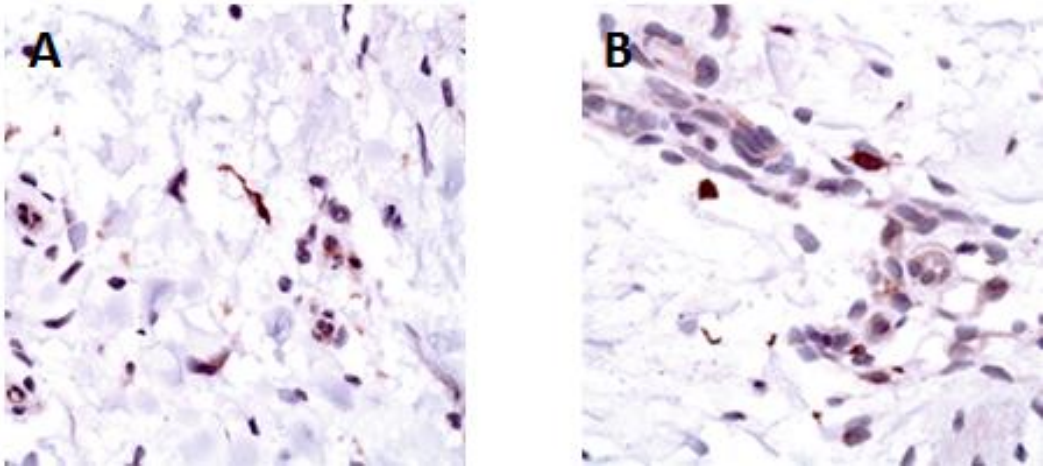



Figure 11. Immunohistochemistry (Formalin/PFA-fixed paraffin embedded sections) - Anti-TIM-4 antibody. Banff score in Patient 2: **A**- non-rejection (0), **B**-early stage III rejection.

Immunofluorescent analysis of TIM-4 and CD68 co-expression in samples from Patients 3 and 4 (Figures 12-13) identified that TIM-4 is expressed on CD68+ positive cells as well as other cell not expressing CD68. We suppose that CD68–TIM-4+ cells might be other antigen presenting cells such as dendritic cells.

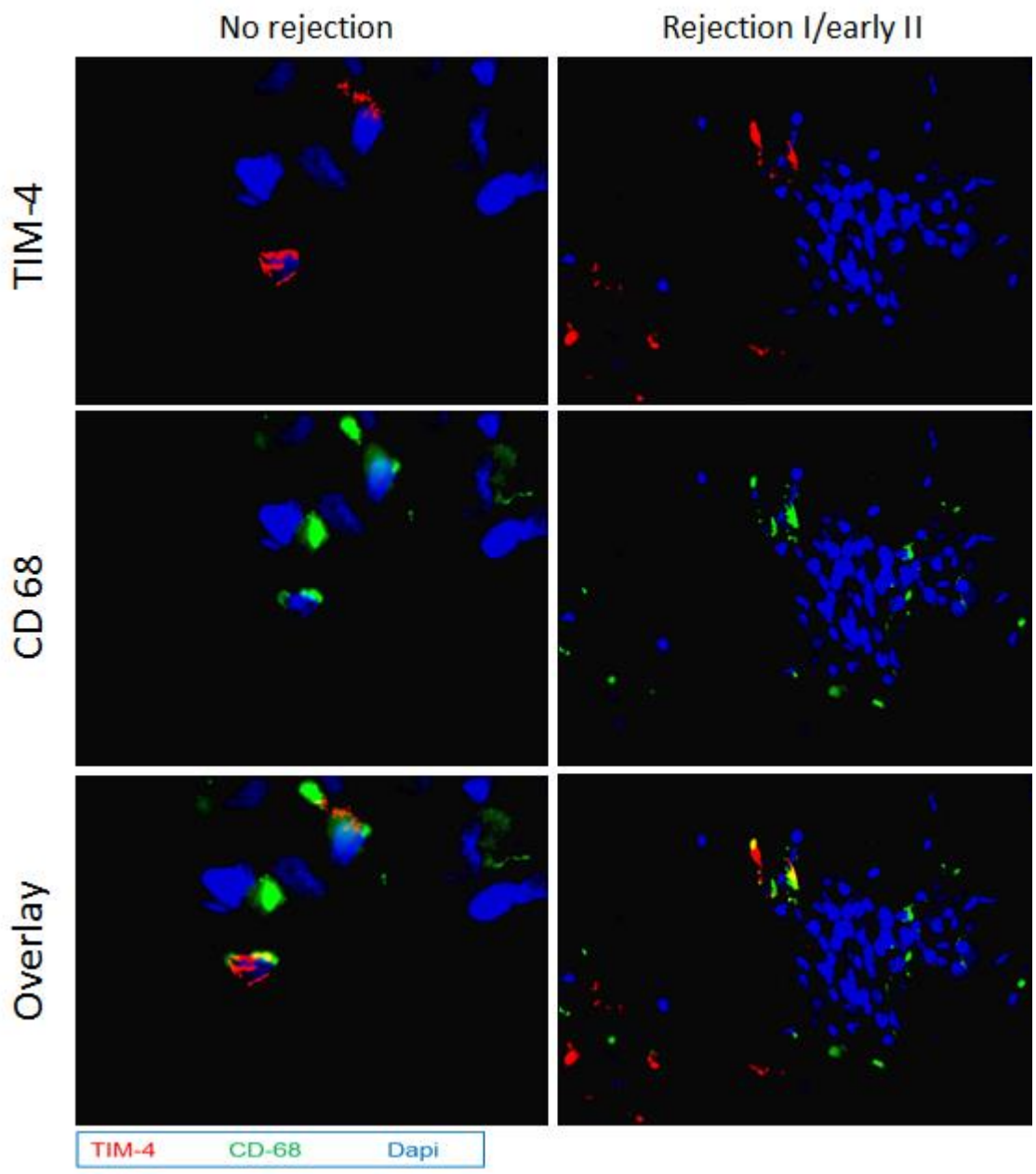



Figure 12. Immunofluorescence (Formalin/PFA-fixed paraffin embedded sections) - Anti-TIM-4 antibody (red), anti-CD68 antibody (green). Patient 3: non-rejection (0), rejection I/early II.

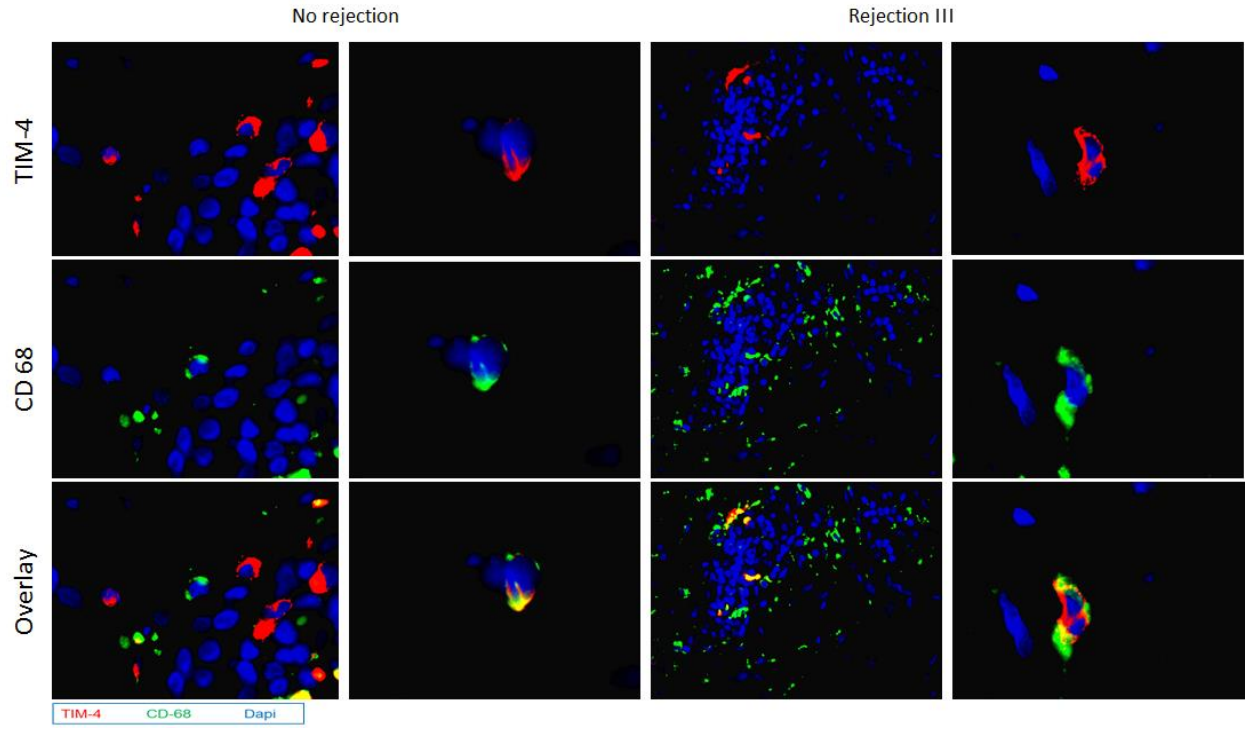



Figure 13. Immunofluorescence (Formalin/PFA-fixed paraffin embedded sections) - Anti-TIM-4 antibody (red), anti-CD68 antibody (green). Banff score in Patient 4: non-rejection (0), rejection III.

Since we have identified that CD169+ tissue resident macrophages are present in healthy skin, quiescent samples from face transplant patients and even some rejecting samples from the same patients, we were interested in immunoregulatory functions it can provide. We used IL-10, one of powerful immunoregulatory molecules, and tried to stain the samples with anti-IL-10 antibody. Though using immunohistochemistry to identify cytokine expression is a quite arguable method due to potential false-positive results, such as staining in keratinocytes, we can see tremendous amount of IL-10 in endothelial cells (Figure 14). One of the cells though can be CD169 positive macrophage. IL-10 secretion was not identified in normal skin *(*Figure 14 A*)*, which can be explained by its unnecessity in homeostasis. In face transplant samples, IL-10 is present in quiescent stage and upregulated in rejection in Patient 4, and IL-10 is absent in rejecting sample in Patient 1, which is interestingly correlated with absence or presence of CD169+ macrophages.

Those samples which were previously positively stained with anti-CD169 antibody are also positive in IL-10 secretion (Figure 14, C- D). In opposite, we didn't identify IL-10 in CD169 negative rejecting tissue from Patient 1 (Figure 14, B). Maybe, inflammation during rejection kills CD169+ cells and/or changes their phenotype so that they are no longer immunoregulatory.

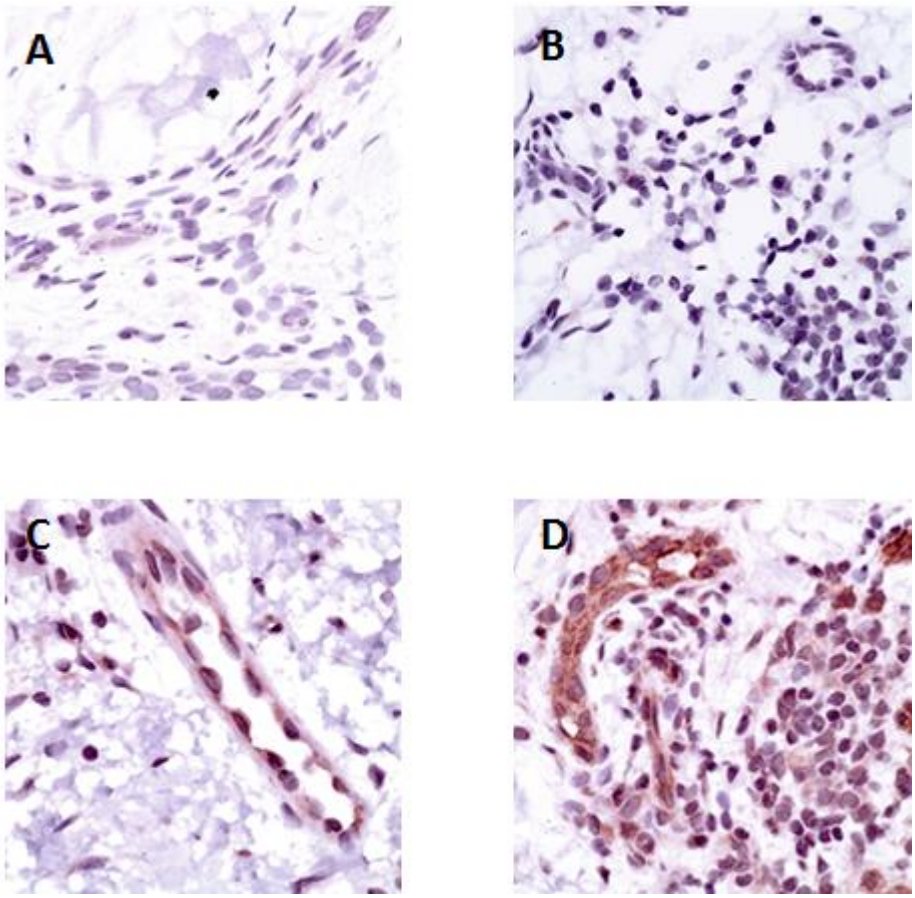



Figure 14. Immunohistochemistry (Formalin/PFA-fixed paraffin embedded sections) - Anti-IL-10 antibody. **A**-normal skin; **B**- Patient 1,stage I/early II, **C**- non-rejection (0), Patient 4, **D**- rejection III, Patient 4.

We also tested the other potent anti-inflammatory molecule CD39 (Figure 15), which collectively with CD73 cleaves ATP into adenosine and inhibits NF-kB pathway. The stain bound to elastic fibers and is very hard to be interpreted. There are also large numbers of dendritic cells in dermis stained by anti-CD39 antibody, including Langerhans cells as well as

endothelial cells and fibroblasts. Therefore, the staining is unspecific and cannot discriminate the subset of tissue resident macrophages.

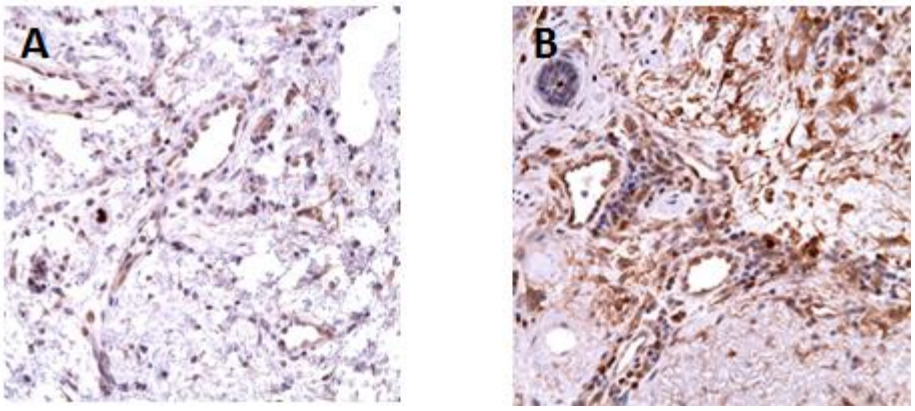



Figure 15. Immunohistochemistry (Formalin/PFA-fixed paraffin embedded sections) - Anti-CD39 antibody. Patient 4: **A**- non-rejection (0), **B**- rejection III.

### 2.4. Brief Discussion

In a mouse cardiac allograft transplantation, the continuing presence of CD169+TIM-4+ TRMs with immunoregulatory phenotype and function forecasts allograft survival whereas the loss of this protective population and its immunoregulatory phenotype is a harbinger of allograft rejection[34]. An interesting facet of their immunoregulatory phenotype is their similarity with T-regulatory cells. Both cell types produce immunoregulatory molecules including IL-10, TGF-B, and CD39/CD73 system cleaving ATP and ultimately generating adenosine, which has anti-inflammatory properties. At the same time these cells are not very potent antigen presenting cells because of hypo-expression of MHC-II and costimulatory molecules such as CD80 and CD86 as compared to tissue-infiltrating macrophages. As noted previously, the hypo-stimulatory and pro-immunoregulatory properties of TRMs are evident in MLR, where they induce 2.5-fold increase

in T-regulatory cells differentiation and proliferation as compared to conventional antigen presenting cells[37].

Considering that the majority of our knowledge on TRMs is derived from experiments using rodent models, it seems important to analyze the presence, phenotype and function of TRMs in humans. Hence we have probed for the presence of CD169+ TRMs and their probable immunoregulatory phenotype in human material beginning with an assessment of their role in immunological processes in face transplant patients.

Human CD169+ macrophages are a distinct population from circulating tissue-infiltrating macrophages. They are viable in healthy skin, reside in dermis and possess, as in rodents, characteristically long dendrites. In keeping with results in mouse models, CD169+ macrophages presence in normal skin but disappearance with inflammation was demonstrated in biopsies of spongiotic dermatitis.

Through analysis of VCA samples from patients with face transplantation, we can conclude that CD169+ TRMs are present in skin during the periods of quiescence. In Patients 1 and 2, the abundance of CD169+ cells diminished during rejection, while in Patients 3 and 4 CD169+ macrophages were present in rejecting samples. Is the presence or absence of CD169+ TRMs in transplanted skin a biomarker for rejection? Using the BANFF criteria for rejection, the differences between normal skin and grade I rejection are subtle and difficult to discern. There was a disparity in CD169+ cells abundance in Patient 1 and Patient 3 both having histopathological grading rejection stage I. Perhaps the loss of CD169+ TRMs in skin in VCA transplants can be used as a criteria that distinguishes early face transplant rejection from normal skin and enables diagnosis at an early easily treatable phase of rejection. Another possibility is that CD169+ cells return to skin as a correlate of successful anti-rejection treatment. Patients 3 and 4 whose skin possessed CD169

positive cells may have been on their way to recovery. However, this hypothesis needs to be further tested.

We can also speculate that these TRMs which are immunoregulatory in the steady state can either be destroyed or may change their phenotype from an anti-inflammatory to pro-inflammatory upon rejection. We tried to identify immunoregulatory phenotype of CD169+ TRMs. IL-10 expression has been first evaluated by IHC. Though we were able to detect secreted IL-10, the limitations of cytokine staining by IHC suggest future investigation of IL-10 secretion by TRMs using more precise methods such as in situ hybridization. Unfortunately, another tested molecule CD39 was not able to discriminate between the abundance of dendritic cells and monocytes, therefore, cannot be successfully implemented in describing phenotype of TRMs, at least in immunohistochemistry.

Overall, we were able to obtain some provocative data regarding unexplored subset of macrophages- TRMs in exotic samples from face transplant patients.

# Chapter III: DISCUSSION AND PROSPECTIVES

## 3.1. Limitations

Face transplantation is a promising though evolving non-standardized treatment with only 35 surgeries successfully performed across the world for patients with traumatic injuries, burns and congenital pathologies[50]. We were able to obtain serial samples of skin from 4 face transplant recipients at Brigham and Women's Hospital for a preliminary inquiry into the presence, phenotype and clinical correlates of tissue resident macrophages in the transplant.

The research using immunohistochemical staining is limited by availability of antibodies, with most of them being polyclonal rabbit antibodies. Consequently, we are restricted in antibody combinations for double or triple staining experiments. We haven't been able to co-localize CD169+ cells and TIM-4+ cells due to the fact that successfully working antibodies for the both markers were from the same species. The possible way to overcome antibody specificity limitations is to use new multiplex IHC techniques, which allow to stain with 20-40 epitopes at once instead of standard technique analyzing 3-4 markers. This would allow us to test different antibodies not regarding to their species of origin, and investigate co-localization of different epitopes, but this technique requires specific equipment.

As an alternative for inquiry of possible immunoregulatory functions of CD169+ TRMs, we assessed their expression of IL-10. There are several problems inherent in IHC staining of intracellular soluble cytokines. This method is not quantitative and lacks sensitivity for secreted proteins. If it's critically important to document that these cells express IL-10, we need to do it either by extracting the cells from the tissue, and assessing them by single cell transcriptional or proteomic analysis or applying in situ hybridization, which will allow us to define the type and localization of cytokine-secreting cells.

### 3.2. Future Research

The intriguing question in transplant immunity is how we can manipulate the immune system to regulate and diminish the likelihood of rejection. One of the ways to induce tolerance is upregulating the presence and potency of graft homing or dwelling immunosuppressive cells, such as tissue resident macrophages. In skin transplant rejection samples cytotoxic T-cells target keratinocytes and keratinocyte stem cells, and macrophages phagocytose apoptotic cells[44]. These maybe necessary ingredients for either induction of tolerance that will downregulate immune response or signal that will positively promote immune response.

Based on previous findings, survival of CD169+TIM-4+ macrophages and their immunoregulatory phenotype is affected by inflammatory environment[34]. But what exactly happens with TRMs upon inflammation is still to be studied. Do they completely disappear from the site of inflammation or are they able to alter their phenotype and become pro-immunogenic cells? In order to be able to discriminate between subsets of TRMs we need to further investigate whether these cells secrete pro-inflammatory cytokines and chemokines such as CCL1, CCL2, TNF-$\alpha$, IL-1$\beta$, IL-6, IL-12. We can also test immunoregulatory molecules such as CD39, CD73, galectin-9 expressed by tissue macrophages to specify anti-inflammatory M2-like phenotype of TRMs using flow cytometry or PCR. Moreover, TRMs supposedly secrete IL-10, anti-inflammatory cytokine, which can be assessed by in situ hybridization.

We believe that TIM-4 is a molecule that attracts TRMs to inflammatory site because of its phosphatidylserine binding pocket. Though in current work we couldn't show the role of TIM-4 in transplant tolerance, our previous experience allows us to think of TIM-4 as a characteristic molecule to distinguish immunoregulatory subset of TRMs [34]. In skin transplant model in mice,

TIM-4 blockade was shown to lead to threefold prolongation in graft survival, elimination of T-effector cells and induction of T-regulator cells from naïve CD4+ T-cells[33]. In murine arthritis model, anti-TIM-4 treatment before or even after the onset of disease clearly regressed the development of arthritis by inhibiting production of pro-inflammatory cytokines in the joints[51]. TIM-4 expression on tumor-associated macrophages drives phagocytosis of dying tumor cells, reduces cytotoxic activity of T-cells and decreases antigen presentation[52]. We need to conduct further experiments which will allow us to correlate TIM-4 with non-rejecting samples and samples with different grades of rejection. We don't know if loss of CD169 in rejection involves loss of TIM-4 as well, but we have identified TIM-4 cells that are CD169 negative. But if the cell is CD169+TIM-4+, will it lose TIM-4 at the same time as CD169?

The next question can be: how can one protect CD169+ cells? What induces CD169 to form in the beginning and what molecular pathway is responsible for loss of CD169+ macrophages. When normal cells mature, they frequently differentiate, become post-mitotic, and eventually evolve towards apoptosis. For instance, resting Langerhans cell in epidermis are in immunosuppressive state, and stimulation with antigen drives their transition from immunosuppressive to immunostimulatory state, migration to secondary lymphoid organs to present antigen to T-cells[53]. This type of maturation implies one way. When keratinocyte matures, it goes from basal cell which is mitotically active and relatively undifferentiated, into keratin-producing cell that becomes post-mitotic and eventually can't divide anymore, and undergoes apoptosis[54]. So, is CD169+macrophage maturation a one-way process or can it be reversed during micro-environmental changes is still to be determined. It is possible that stimulus for CD169 loss goes through maturation of the cell. It might go from cell that confers immune privilege to a cell that is stimulatory to immune response as it matures like conventional immature macrophages that

are known to lose their immunosuppressive phenotype upon maturation[55]. So, if there is a hypothetical way of enhancing persistence of immature resident macrophages, blocking their maturation, that would ultimately downregulate rejection. To investigate whether TRMs die, their phenotype is terminal or they can re-acquire immunoregulatory phenotype in certain micro-environment, tracking of these cells and fate-mapping studies would be necessary.

The experimental results where CD169 + cells were present in rejection didn't fulfill with our expectations. If CD169+ macrophages we see in transplant samples are tolerogenic, then loss of CD169 deals with progression, and regression of disease can be characterized by re-acquisition of Sialoadhesin. That could explain the paradox of TRMs present in grade II rejection. But we need to keep in mind that whatever stimuli is driving the cell to mature or acquire certain phenotype, it is suppressed by immunotherapy. As well as being in induction immunosuppressive therapy after vascular composite allo-transplantation[56], these patients receive intense anti-rejection treatment[51]. The transplanted tissues, even in quiescence and with normal functionality, can be similar to healthy tissues but not completely identical due to immune and non-immune processes. So, when we compare skin from face transplantation, does it make more sense to compare samples from rejection 0 and rejection III, or to compare quiescent samples to normal skin from face or other areas?

As skin is a principal component of VCA, we need to better understand the nature of skin TRMs through studying their normal physiology. It may be done through obtaining fresh samples of normal skin, digesting them in order to pull out the cells and describing the phenotype of TRMs using flow cytometry, Cytoff, single cell transcriptional or proteasome arrays. Stimulating the skin with LPS and characterizing the behavior of TRMs under oxidative stress can help us to distinguish

inflammatory and anti-inflammatory subsets of TRMs. Previous *in vitro* experiments showed that CD169+ cells are very sensitive to oxidative stress and phenotype changes with TLR stimulation[34].

Further we can investigate subsets of TRMs in samples from specific diseases including various types of dermatitis like atopic dermatitis, allergic contact dermatitis, seborrheic dermatitis and etc. Different clinical conditions with distinct underlying pathological mechanisms may induce proliferation or depletion of subsets of TRMs. In pancreatic islets in NOD mice as they develop peri-isletic inflammation, TRMs decreased in numbers, and the ratio of DCs to macrophages changed dramatically[37].

The other important point is to determine the relationship of TRMs with other regulatory lymphocytes, such as T-regulatory and B-regulatory cells. Previous experiments with mixed lymphocytic reactions indicated stimulation of T-regulatory cells by TRMs in ABM TCR transgenic model system of alloreactivity[34], and it might be worth conducting the similar experiment with TRMs obtained from fresh skin samples. Though TRMs were shown to attract anti-inflammatory B-cells to the peritoneum[57], the impact of TRMs on immunoregulatory B-cell development remains unclear.

Overall, we demonstrated the existence of potentially immunoregulatory population of dermal resident macrophages in normal skin and quiescent skin in face transplant patients, their disappearance in inflamed conditions and ambiguous behavior upon rejection episodes. While our data is preliminary we suspect that through evolution a class of TRMs that are cytoprotective in the quiescent state die or lose immunosuppressive properties in response to TLR stimulation or other danger signals. In this circumstance TRMs can both promote tissue homeostasis and not subvert anti-microbial defense. These results suggest further and more detailed examination of undescribed subsets of tissue resident macrophage population.

Vascular composite transplants provide significant functional and quality of life benefits but require potentially harmful, long-term immunosuppression. These studies have the potential to provide a powerful new biomarker that will guide clinical care by accurately measuring allograft health and may offer new mechanistic insights into the earliest events that precipitate VCA rejection thereby identifying novel TRM-related therapeutic targets for the establishment of drug-free VCA transplant tolerance.

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