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Antibody-Functionalized Peptidic Membranes for Neutralization of Allogeneic Skin Antigen-Presenting Cells

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Abstract

We report herein application of an in situ material strategy to attenuate allograft T cell responses in a skin transplant mouse model. Functionalized peptidic membranes were used to impede trafficking of donor antigen-presenting cells (dAPCs) from skin allografts in recipient mice. Membranes formed by self-assembling peptides (SAPs) presenting antibodies were found to remain underneath grafted skins for up to 6 days. At the host-graft interface, dAPCs were targeted by using a monoclonal antibody that binds to a class II MHC molecule (IAd) expressed exclusively by donor cells. Using a novel cell labeling near-infrared nanoemulsion, we found more dAPCs remained in allografts treated with membranes loaded with aI-Ad than without. In vitro, dAPCs released from skin explants were found adsorbed preferentially on aI-Ad membranes. Recipient T cells from these mice produced lower concentrations of interferon-gamma cultured ex vivo with donor cells. Taken together, the data indicate that the strategy has the potential to alter the natural course of rejection immune mechanisms in stringent allogeneic models.

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Keywords

Skin allograft; self-assembling peptides; EAK16-II; His-tags; protein formulation; injectable membranes

1. Introduction

The performance of an *in situ* forming fibrillar membrane in attenuating T cell responses toward allogeneic skin grafts was investigated. Human skin allografts are important biological dressings for temporary wound closure [1]. Patients with partial and full-thickness burns benefit from intact epidermis and dermis; together these structures serve as a protective barrier to minimize desiccation of the underlying exposed tissues, limit water evaporation, reduce bacterial contamination, relieve pain, and promote wound healing by accelerating re-epithelialization [1]. However, the heightened antigenicity of skin allografts drives powerful allospecific T cell responses in recipients [2].

Calcineurin inhibitors are the mainstay in managing allograft rejection [3–5]. These agents exert their immunosuppressive effects mainly by dampening the activation, proliferation and survival of all T cells through down-regulation of interleukin-2. Patients exposed to these drugs have increased risk of developing opportunistic infections because skin flora may contain antibiotic-resistant *Staphylococcus aureus* [6, 7]. Herein we propose to generate selective immunosuppression by exploiting a fundamental molecular difference between donor and recipient cells: class II MHC (MHC-II) molecules expressed by donor antigen-presenting cells (dAPCs).

Acute rejection is driven by mismatched class I and II MHC molecules expressed by donors and recipients whereby the latter mount potent T cell responses against skin allografts [8]. Allograft survival correlates with the density of resident dAPCs. Within hours following allogeneic skin transplantation, dAPCs residing within allografts begin migrating to recipient draining lymph nodes [9–11]. More than three-quarters of the resident dAPCs would egress from skins within three days [12]. Once in lymph nodes, dAPCs activate allospecific T cells via MHC and costimulatory molecules [2, 8]. Inside lymph nodes host CD4 helper T cells recognizing mismatched MHC-II molecules are activated to drive CD8 T cell differentiation into cytotoxic T cells (CTLs) (Fig. 1). Presentation of MHC-II antigens is critical because generation of CTLs is compromised without CD4 T cell participation. With visceral allografts, activation of CD4 and CD8 T cells is correlated with the frequency of dAPCs in lymph nodes [13–15]. Activated allospecific CD8 CTLs in turn migrate to the transplant site and damage graft parenchyma via recognition of MHC class I molecules [8]. The magnitude of the rejection depends on the qualitative and quantitative encounter between dAPCs and recipient T cells shortly after grafting [12].

Because acute rejection is a function of dAPCs accumulating in recipient lymph nodes, preclinical modalities have been devised to deplete dAPCs prior to transplantation. Typically the methods require systemic infusion of anti-leukocyte antibodies into donor animals before organ harvest [16–18]. While such preemptive strategies can be effective in delaying rejection of allografts in animal models, translation to humans is complicated by

potential harms that can be done to the donors. Recognizing the unmet need, we envisaged a new strategy by which dAPCs trafficking can be impeded selectively after transplantation.

Previously we have reported an injectable platform by which retention of IgG molecules in local tissues can be enhanced using EAK16-II, a self-assembling peptide (SAP) with the sequence AEAEAKAKAEAEAKAK [19]. This and related SAPs are primarily utilized for their environmental responsiveness [20, 21]. These peptides undergo sol-gel phase transition at high ionic strengths (> 20 mM NaCl); in deionized water, EAK16-II can be injected into physiological environment to establish gels *in situ*. A system containing EAK16-II, its histidinylated analogue EAKIIH6, protein linkers (α H6-IgG and pAG) can localize model therapeutic IgG molecules *in vivo* through directional binding [19, 22]. Membranes loaded with antibodies can be established *in vivo* by subcutaneous injection. Such immobilization partially overcomes antibody clearance mechanisms in tumors, as evidenced by prolonged retention of IgG in mouse mammary and melanoma lesions [22]. In both tumor types, localized IgG remained in tumors significantly longer than free IgG.

In the present study, we characterize the system of materials designed to localize anti-MHC-II antibodies specific to dAPCs (Fig. 1). The rationale is that EAK16-II, its histidinylated analogue EAKIIH6, protein linkers, and anti-MHC-II antibodies spontaneously complex non-covalently upon mixing in syringes, with the resultant assembly localizes at the graft-host interface. dAPCs migrating toward draining lymph nodes would interact with the antibody-functionalized membranes, resulting in delayed and/or diminished encounter with T cells. The strategy was examined in the current study using an allogeneic mouse skin graft model. All MHC alleles between the donor and recipient are mismatched, rendering this a stringent model. Although delay in rejection was not expected, the work was aimed to characterize membranes presenting anti-I-A^d IgG (referred hereafter as “ α I-A^d”) *in vitro* and *in vivo* to assess feasibility of the strategy. To this end, we investigated retention of IgG co-administered with the self-assembling components under normal and grafted skins. A nanoemulsion encapsulated with a fluorescent tracer was used to image dAPCs *in vitro* and *in vivo*. The immune responses were determined by analyzing production of IFN- γ from T cells in recipient mice. The data indicate that the platform has the potential to become a useful clinical tool by which skin graft rejection can be mitigated.

2. Methods and Materials

2.1. Materials

Peptides were custom synthesized by American Peptide Company (Sunnyvale, CA) at greater than 95% purity with termini acetylated or amidated. Peptides were reconstituted in sterile MilliQ water (18.2 M Ω at 25°C) at 5 mg/mL (3 mM, EAK16-II, AcNH-AEAEAKAKAEAEAKAK-CONH₂) or 7.5 mg/mL (3 mM, EAKIIH6, AcNH-AEAEAKAKAEAEAKAKHHHHHH-CONH₂). Rabbit anti-His-tag polyclonal antibody (α H6-IgG, 0.2 mg/mL) was obtained from AnaSpec, Inc. (San Jose, CA). Recombinant protein A/G (pAG) was obtained from Pierce Biotechnology (Rockford, IL) and reconstituted to 0.25 mg/mL in sterile deionized water. Anti-mouse I-A^d (MHC-II) antibody (α I-A^d, 0.5 mg/mL; IgG3 κ , clone: 39-10-8) was purchased from Biolegend (San Diego, CA). Allophycocyanin conjugated anti-mouse MHC-II I-A^d antibody (0.2 mg/mL) was

obtained from eBioscience (San Diego, CA). DyLight™ 800 conjugated goat anti-chicken IgG (IgG⁸⁰⁰) was obtained from KPL (Gaithersburg, MD) and reconstituted to 1 mg/mL in sterile deionized water. RPMI-1640 and fetal bovine serum (FBS) were obtained from HyClone (Logan, Utah). ACK lysing buffer and penicillin/streptomycin solutions were purchased from Lonza (Walkersville, MD). Perfluorocarbon (PFC), Perfluoro-15-Crown-5 Ether was purchased from Exflur Research Corporation (Round Rock, TX). Lipophilic carbocyanine DiOC18(7) (“DiR”) was purchased from Life Technologies and used without further purification.

2.2. Skin transplantation

Six-to-eight week old female (certified-virus-free) BALB/c and C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) and housed in the Duquesne University Animal Care Facility. Animals were handled in accordance to protocols approved by the Duquesne University Institutional Animal Care & Use Committee. The transplant procedure was modified from a protocol described by Garrod et al. [23]. Briefly, the recipient C57BL/6 mice were anesthetized using isoflurane (induction: 5%; maintenance: 3%) and subsequently administered with Buprenex at 0.1 mg/kg via the intraperitoneal route. Ear skin explants (dorsal layer) were prepared from BALB/c mice and transplanted onto graft beds (each ~0.5 cm²) prepared in the flank of C57BL/6 mice. Upon completion of the surgery, C57BL/6 mice were bandaged and returned to cage with their activities monitored regularly.

2.3. In vivo stability of IgG membrane in subcutaneous space and under the skin graft

Anti-chicken IgG labeled with a near infrared dye emitting at 800 nm (IgG⁸⁰⁰) was used as a model antibody to investigate the assembling and stability of the system *in vivo*. EAK16-II (100 µg in 20 µL) was mixed with 37.5 µg (in 5 µL) of EAKIIH6. In a second vial, αH6-IgG (2 µg), IgG⁸⁰⁰ (2 µg), and pAG (0.375 µg) were pipet mixed. The peptides and antibodies solution were withdrawn into the same insulin syringe (28 G' needle) and injected into the subcutaneous space in footpad or the posterior flank of BALB/c mice. Fluorescence in the footpad was imaged *ex vivo* at 30 min, 6, 24, 48, and 72 hours. Excised footpads were stretched out on a 15×100 mm dish using scotch tapes for *ex vivo* imaging using an Odyssey Imager (Li-Cor, Inc., Lincoln, NE) and measured at an intensity of 1.0 with focal distances of 3.2 mm and 3.5 mm. The averaged signal of three blank footpads at each focal distance was subtracted as background. Data reported represent intensities averaged from the measurement performed at each focal distance.

Signal at the posterior flank was recorded in real time *in vivo* for up to 4 days. Anesthetization was induced using isoflurane (induction 5%, maintenance 3 %) for real time *in vivo* imaging. Abdominal hairs were removed prior to scanning. Mice were scanned prior to injection and monitored at the indicated times after injection using a Pearl Impulse imager (Li-Cor, Inc.). All images were collected using the same threshold and resolution (170 µm) settings. Oval-shaped regions of interest were defined at the site of injection using the contralateral flank as background. Fluorescence was quantified using the Impulse (2.0) software (Li-Cor, Inc.) with a standard deviation multiplier of 10 and a search limit of 100 pixels. To demonstrate the assembling and stability of the system under the skin graft, the

same amount of peptides and antibodies solutions were mixed and administrated onto the graft bed of C57BL/6 mice before placement of the skin. Signal was monitored in real time using a Li-Cor Pearl Impulse Imager for up to 6 days as in the previous experiment.

2.4. Detection of I-A^d + dAPCs in draining lymph nodes

The capability of anti-I-A^d membrane to impede the trafficking of dAPCs to host draining lymph nodes was studied using flow cytometry. α I-A^d IgG with or without the self-assembling components were administrated onto the graft bed prior to the skin graft. Draining lymph nodes (inguinal, popliteal and axillary) were collected and pooled 1 day, 2 days, or 3 days after transplantation. Single cell suspensions were prepared by mechanical crushing and filtering through 70 μ m nylon cell strainers. APCs (both donor and recipient) were enriched using BD IMag (BD Bioscience, San Jose, CA). APCs were re-suspended in 200 μ L 3% FBS PBS and stained with Allophycocyanin conjugated anti-mouse MHC-II I-A^d antibody (4 μ g/mL). Samples were analyzed using an Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ).

2.5 Preparation and characterization of near infrared dye-encapsulated PFC nanoemulsions

Nanoemulsions were produced using microfluidization by adapting our earlier described published procedures [24–30]. Briefly, the lipophilic payload, NIR dye (DiR) was dissolved at room temperature in Miglyol® 812N (synthetic hydrocarbon oil). This oil solution was mixed with PFC and surfactant mixtures to form a pre-emulsion which was then processed on a Microfluidizer M110S (Microfluidics Corp, Westwood, MA) to produce the final nanoemulsion. Droplet size and zeta potential were measured using dynamic light scattering on a Zetasizer Nano (Malvern, Westborough, PA). Measurements were taken after diluting the nanoemulsion in deionized water (1:40 v/v, made at 25°C) using 173° scattering angle with respect to the incident beam. The stability of the nanoemulsions was assessed by measuring the hydrodynamic diameter (Z average) and half width of polydispersity index at different time points (days). Zeta potential was measured at the same dilution using specialized zeta cells with electrodes following the manufacturer's instructions. Li-Cor Odyssey was used to confirm labeling of the nanoemulsion with DiR. For the measurement, four dilutions were prepared with 100 μ L of each dilution placed in a 96-well plate. The plates were scanned at 800 nm with intensity setting at 2.5 and the offset 3.0 mm with the resulting images analyzed using Li-Cor software.

2.6. Ex vivo labeling of skin explants with PFC nanoemulsions and imaging of cells in vitro and in vivo

Dorsal ear skin from BALB/c mice was collected and incubated with 4 mL of diluted with PBS PFC NIR nanoemulsion (2 mg/mL) in a 6-well plate for 2 h. Labeled ear skin was rinsed with PBS 3 times before grafting. After 2 days, recipient mice were scanned in Li-Cor Pearl Imager as previously described in the stability studies. For *in vitro* confocal microscopy, donor ear skin was incubated with 4 mL of serum free RPMI diluted PFC NIR nanoemulsion (2 mg/mL) overnight in incubator (2 skin grafts per sample). CCL21 (final concentration 10 ng/mL) and GM-CSF (final concentration 5 ng/mL) were added to each

well to facilitate emigration of dAPCs. On the second day, adherent and suspended cells released from the ear explants were harvested and centrifuged to remove unincorporated nanoemulsion. The cells were then re-suspended in 200 μ L serum free medium and added to the glass bottom culture dish with SAPs and linker proteins with or without α I-A^d stained with Congo red. The membrane-cell mixtures were fixed with 2% PFA for 0.5 h and washed twice with PBS containing 1% fetal bovine serum. Cells were then stained with 0.5 mL of 1 (μ g/mL Hoechst 33342 for 10 min before washing twice with buffer. Anti-fade mounting solution (Prolong® Gold Antifade, Life Technologies) was added to cover slide and placed over the membrane. Images were collected using a Zeiss Axio Observer microscope equipped with the ApoTome 2 imaging system. Nuclear images were obtained with a DAPI filter set (excitation/emission: 335–383 nm/420–470 nm). α I-A^d or control membranes were imaged with a Cy3 filter set (excitation/emission: 538–562 nm/570–640 nm). Z-stack was used to generate three-dimensional (3D) images.

2.7. Allogeneic APC-T cells reaction

To demonstrate that systemic T cell responses were attenuated, splenic T cells from recipient C57BL/6 were cultured with naïve BALB/c APCs. Six days after transplantation, the spleen was collected from BALB/c mice to prepare naïve APCs. Single cell suspensions were prepared by dish crushing, hypotonic solution (ACK lysing buffer to remove erythrocytes) treatment and filtering through 70 μ m nylon mesh strainers. Cells were cultured in 1 mL complete RPMI medium with 5 ng/mL GM-CSF at a density of 1×10^6 cells/mL overnight. Culture medium was gently pipetted to remove non-adherent cells. Recipient C57BL/6 mice were euthanized and single cell suspensions were prepared from spleen adjusted to 5×10^5 cells/mL. C57BL/6 splenic T cells (1×10^6 cells in 2 mL medium) were added into previously obtained naïve BALB/c APCs, and cultured for 48 h. Cell culture medium was centrifuged to remove debris. ELISA was used to quantify concentrations of IFN- γ (DuoSet, R&D Systems, Minneapolis, MN) produced by recipient C57BL/6 splenic T cells.

2.6. Statistical analysis

Statistical analysis was performed in GraphPad Prism 5.0. Data were analyzed using one way ANOVA and Tukey-Kramer multiple comparison ($\alpha=0.05$) or one-tailed Student's t-test ($\alpha=0.05$) as indicated. Data shown represent mean and standard deviation.

3. Results

Mouse ear skin grafting is a robust and stringent method for measuring the magnitude of allogeneic T cell responses [31]. Excision of epidermal and dermal layers results in exposed underlying tissues that mimic debrided burnt skins [32]. The targets of the current strategy, dAPCs, are interspersed within skin layers of mouse and human [8]. Skin dAPCs include donor Langerhans' cells (dLCs) in the epidermis and dermal dendritic cells (dDCs). dLCs and dDCs migrate in waves, with the trafficking complete within 4–5 days in rodent models [12]. LCs are immature DCs but promptly mature upon encountering alloantigens while migrating to secondary lymphoid tissues. The density of these cells per unit area is correlated with immunogenicity. For example, mouse tail skin, which contains few LCs, is

significantly less antigenic than ear skins [12]. We postulated that SAPs could form stable structures at the host-graft interface and that anti-graft T cell responses could be attenuated by establishing membranes that capture dAPCs.

3.1. In vivo retention of IgG membranes in subcutaneous space

To determine the extent to which the system was able to localize antibodies in subcutaneous space *in vivo*, the fate of a NIR ($\lambda=800\text{nm}$) labeled IgG (hereafter “IgG⁸⁰⁰”) was tracked in BALB/c mice (Fig. 2 and 3). Retention was demonstrated by injecting the proteins and peptides into the footpads of BALB/c mice subcutaneously (Fig. 2). Fluorescence could be detected for at least 72 h when IgG⁸⁰⁰ was injected with the assembling components (“System+IgG⁸⁰⁰”), while IgG⁸⁰⁰ in saline (“free IgG”) was significantly lower at 6 h ($p < 0.001$) and near background after 24 h ($p < 0.001$). Without pAG, retention of IgG⁸⁰⁰ was significantly lower at 6 h ($p < 0.01$), 24 h ($p < 0.001$), 48 h ($p < 0.001$) and 72 h ($p < 0.001$) compared to mice received the antibody-membrane treatment. This confirmed previous results showing that the bioaffinity interactions involve pAG [22]. Further evidence of localization was supported by real-time optical measurements. When injected into the mouse flank, solutions containing IgG⁸⁰⁰ with the self-assembling components showed remarkable localization (Fig. 3). Strong fluorescence was detected at the injection site for at least 96 h, while relative low fluorescence was observed with IgG⁸⁰⁰ delivered in saline after 24 h. These *in vivo* results show that the injectable formulation was effective in retaining IgG locally.

3.2. In vivo stability of IgG membranes under skin grafts

Having established that the self-assembly and bioaffinity interactions could take place *in vivo* in normal subcutaneous space, the ability of the system to localize at the site of allograft implantation was examined. Membrane components mixed with IgG⁸⁰⁰ were established in the graft bed immediately prior to placement of allogeneic skins. Fluorescence underneath the graft was monitored in live mice for 6 days (Fig. 4). IgG⁸⁰⁰ delivered with the assembling components showed superior localization; as early as 5 minutes after injection, local fluorescence registered at least two-fold higher in intensity than in mice received IgG⁸⁰⁰ without the components. Superior fluorescence was observed at all time-points monitored; fluorescence in mice that received the antibody in membrane were significantly higher on day 3 ($p < 0.01$) and day 6 ($p < 0.01$) when compared to those in mice that received IgG⁸⁰⁰ in saline. These results indicate that the assembling components provided a mechanism by which IgG were retained at the graft-host interface.

3.3. Detecting dAPCs in lymph nodes

To determine the extent to which dAPCs trafficking was impeded by membranes loaded with anti-I-A^d IgG (hereafter referred to as “ $\alpha\text{I-A}^{\text{d}}$ ”), draining lymph nodes (inguinal, axillary, and popliteal) of recipient mice were analyzed for the presence of I-A^d+ cells (Fig. 5). $\alpha\text{I-A}^{\text{d}}$ was placed on graft beds with or without the self-assembling components prior to placement of ear skins. At predetermined time points after transplantation, draining lymph nodes were excised from the euthanized mice. Lymph nodes from each mouse were pooled and stained for I-A^d+ cells. Flow cytometric analyses indicated that on day 2 $\alpha\text{I-A}^{\text{d}}$

membrane-treated C57BL/6 mice had 2.0% (± 0.18) of dAPCs in draining lymph nodes, significantly lower ($p < 0.05$) than that of C57BL/6 mice received α I-A^d delivered in saline ($3.5\% \pm 0.52$; Fig. 5c). α I-A^d membrane-treated C57BL/6 mice showed lower (but not significantly different) frequency of I-A^d+ cells on day 3 (Fig. 5d). The two groups showed comparable frequencies of I-A^d+ cells on day 1 (Fig. 5b). There may be at least two reasons for observing reduced dAPCs in lymph nodes only on day 2. First, due to limited hydration and salt content, there might be a lag-time (though not evident *in vitro*) for complete assembly of the components in graft beds. Thus, upon placement of skin graft, dAPCs immediately migrated out (within the first hour) might have escaped the membrane barrier. The effect seen on day 2 might result from impeded trafficking from a second wave of emigrated dAPCs. Because egressed dAPCs likely express high densities of foreign MHC molecules, these cells would be killed by host allospecific T cells in lymph nodes.

3.4 Tracing dAPCs with near infrared nanoemulsion

Given the uncertainties in tracking dAPCs in lymph nodes, we used a novel phagocytic cell-specific PFC nanoemulsion to image these cells at the graft-host interface. Similar formulations of the emulsion have been shown to label macrophages and dendritic cells with PFC droplets encapsulating near infrared dyes [25]. The data show that dAPCs in skin grafts can be labeled *ex vivo*; fluorescence in labeled ear explants correlated with the dose of the nanoemulsion used (Fig. S2). Upon grafting of the labeled skins, strong fluorescence was found in graft bed fortified with α I-A^d membrane (Fig. 6), suggesting retention of dAPCs in graft beds. Higher intensity of fluorescence was observed in graft beds with membranes loaded with α I-A^d than without ($p < 0.001$). In a separate *in vitro* experiment, dAPCs egressed from skin explants were found on membranes loaded with α I-A^d (Fig. 7a) but not without (Fig. 7b). These observations suggest that the α I-A^d membrane retained skin-resident dAPCs.

3.4. Suppression of T cell responses in recipient mice

We next hypothesized that reduced dAPCs trafficking would manifest in altered systemic T cell responses toward the allograft. To test this, T cells of recipient mice were analyzed *ex vivo* to evaluate immune reactivity toward donor antigens. Lymphocytes isolated from spleens harvested from transplanted animals were stimulated *in vitro* by culturing with fresh leukocytes from naïve donor mice (Fig. 8a). After 48 h, culture supernatants were analyzed using ELISA to determine the concentrations of IFN- γ , a characteristic cytokine produced by activated T cells in cell-mediated rejection. The results indicate that mice received α I-A^d membrane yielded T cells that were hypo-responsive. Splenic T cells recovered from mice received α I-A^d membrane produced 4.4 ng/ml (± 1.7) IFN- γ , in contrast to 15.1 ng/ml (± 3.4) and 22.1 ng/ml (± 5.1) from mice received only α I-A^d in saline (" α I-A^d IgG, local"; $p < 0.01$) or SAPs ("EAK16-II/EAKIIIH6"; $p < 0.001$), respectively. T cells from transplanted mice administered with the SAPs without α I-A^d produced high levels of IFN- γ . Low levels of IFN- γ were released from T cells from mice transplanted with isografts (C57BL/6 ear $\rightarrow$ C57BL/6 host) in which no rejection was expected (Fig. 8b). As expected, the treated transplanted skins were rejected comparably (scored based on gross necrosis) by day 9 (see supplemental data Fig. S3). This was not unexpected given the extent of MHC disparities.

Overall, the differential IFN- γ produced by recipient T cells support the notion that T cell responses were attenuated specifically with local deposition of α I-A^d membranes.

4. Discussion

The two mouse strains tested here express distinct sets of class II MHC molecules: I-A^d and I-E^d (BALB/c), and I-A^b (C57BL/6). These antigenic mismatches drive a robust rejection. In C57BL/6 recipients, T cells exist to recognize I-A^d and I-E^d on dAPCs to develop into IFN- γ driven cytotoxic responses. These reactions are initiated and sustained by dAPCs (Fig. 1). When placed *in vivo*, dAPCs migrate from skin explants to stimulate recipient T cells in regional lymph nodes and ultimately populate the rest of the host. The extent of anti-graft T cell activation correlates with MHC-II+ dAPCs migrated to graft-draining lymph nodes [8]. Donor MHC antigens (released by dead graft cells) are also presented by graft-infiltrated host APCs. While this form of indirect recognition contributes to rejection [2], the initial rejection intensity largely depends on dAPCs.

Viability of skin allografts are difficult to sustain in allogeneic hosts owing to their stronger antigenicity. Attenuating acute rejection of skin-derived allogeneic antigens would improve the viability of such transplants functioning as wound dressings. Cytotoxic T cell responses constitute the major mechanisms of acute rejection. High doses of calcineurin inhibitors (cyclosporine A and tacrolimus) and glucocorticoids suppress the activation of all lymphocytes, not just allospecific T cells.[3, 4] An immune-compromised state is not conducive to healing in patients with burn wounds [32]; the incidence of opportunistic infections in burn victims receiving skin allografts and standard immunosuppressants is predictably high.[32]

Local-regional delivery of immunosuppressants was first reported by Billingham *et al.* in 1951 [33]. Using cortisone acetate in a topical formulation the investigators were able to prolong skin allograft survival in a rabbit model. Importantly, the same dose, which was relatively low, was ineffective when administered systemically. The next documented attempt came 35 years later when another group applied topical cyclosporine A formulated in DMSO to rats received skin allografts [34]. While long-term graft survival was attained, interpreting results of the topical formulations was complicated by the large dose used (5 mg per kg daily); indeed, the authors noted significant systemic drug absorption. In general, the rationale for topical delivery of calcineurin inhibitors and steroidal anti-inflammatory drugs are weakened by their lipophilicity and penetration into the vasculature of deeper tissues. Furthermore, Zhao and colleagues have reported bacterial infiltrates in skin allografts of rats received topical cyclosporine A and fluocinolone acetonide [35]. Thus topical or local drug delivery *per se* does not necessarily circumvent the toxicities of systemic immunosuppressants.

Rather than suppressing all host leukocytes, depleting dAPCs may impute a targeted mechanism to attain specific immunosuppression. The concept has been explored as potential anti-rejection therapies. Odling and coworkers treated donor skins *ex vivo* with low-dose ultraviolet light radiation or chemical carcinogens prior to grafting in mice to reduce dAPC activity [36, 37]. Depletion has also been done using anti-cell antibodies and

genetically engineered knock-out mice defective in select populations of APCs [16–18]. Moreover, skin grafts live longer when lymphatic drainage is temporarily impeded [2, 38, 39].

In this paper we present evidence supporting a local strategy that builds upon SAPs to facilitate stringent allogeneic skin transplantation. The goal is to target unique cell-surface molecules on migratory donor immune cells. The design of the membrane is such that IgG would be bound via the Fc domain thereby precluding from activating FcγRs on leukocytes. But we recognize that dissociated IgG may elicit such cytotoxic mechanisms and eliminate dAPCs either in graft bed or in lymphatics. On the other hand, a fraction of the dAPCs would have their MHC-II blocked by the antibodies, thus inhibiting signal 1 in recognition by allospecific T cells. The data presented herein demonstrate the utility of an anti-MHC-II material system in mitigating skin allograft rejection. The anti-dAPCs membrane was shown to form *in situ*, in both normal skins and on transplant graft bed. The membranes remained in place for 6 days, exceeding the peak (5 days) egression of dAPCs from ear skins *in vitro* [12]. Taken together, the data show that anti-MHC-II membrane is stable at host-graft interface and can attenuate allogeneic T cell responses by neutralizing dAPCs *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A generalized depiction of cellular mechanism of acute rejection of allografts. Shortly after the transplantation, dAPCs in the allograft are activated (1) and migrate towards draining lymph nodes (2). Host T cells in the draining lymph nodes are activated via direct allorecognition by dAPCs (3). Activated T cells will migrate to the allograft and mediate graft rejection (4).

Figure 2.

Stability of the antibody display system in BALB/c mouse footpads. (a) Representative *ex vivo* images of footpads injected with solution containing EAK16-II, EAKIIIH6, α H6-IgG, pAG, and IgG⁸⁰⁰ (“System + IgG⁸⁰⁰”) or solution of EAK16-II, EAKIIIH6, α H6-IgG, and IgG⁸⁰⁰ (“System w/o pAG + IgG⁸⁰⁰”) or IgG⁸⁰⁰ alone in PBS (“Free IgG⁸⁰⁰”). (b) Fluorescent intensity in footpads quantified using Odyssey. Statistical significance (compared to “System + IgG⁸⁰⁰”) was analyzed by one way ANOVA with Tukey’s multiple

comparison (0.5 h, 6 h, and 24 h) or student's t test (48 h and 72 h) (n=3–6, ***: $p < 0.001$, **: $p < 0.01$).

Figure 3.

Real-time monitoring of model antibody localization *in vivo*. (a) Representative real time images of BALB/c mice injected with solution containing EAK16-II, EAKIIIH6, αH6-IgG, pAG, and IgG⁸⁰⁰ ("System + IgG⁸⁰⁰") or IgG⁸⁰⁰ alone in PBS ("Free IgG⁸⁰⁰") at the lower flank. (b) Fluorescence at the site of injection (n=2). BALB/c mice were used to track stability due to lack of pigmentation (as in C57BL/6 mice). Once the kinetics in subcutaneous space was established, the study moved to C57BL/6 as hosts for allografts.

Figure 4.

Real-time monitoring of model antibody in graft bed *in vivo*. (a) Representative real time images of recipient C57BL/6 administrated with solution containing EAK16-II, EAKIIIH6, α H6-IgG, pAG, and IgG⁸⁰⁰ ("System + IgG⁸⁰⁰") or IgG⁸⁰⁰ alone in PBS ("Free IgG⁸⁰⁰") at the graft bed prior to the skin graft. (b) Fluorescence at the graft site. Student's t test was used for statistical analysis at each time point (n=3–6, ***: $p < 0.001$, **: $p < 0.01$).

Figure 5.

Detection of I-A^d dAPCs in draining lymph nodes. α I-A^d was administrated at the graft bed with the self-assembling components (" α I-A^d membrane") or PBS (" α I-A^d IgG, local") prior to the skin graft. Single cell suspensions enriched for APCs were prepared from draining lymph nodes of recipient C57BL/6 mice. Cells were stained with Allophycocyanin conjugated anti-mouse MHC-II I-A^d antibody, and flow cytometry was used to probe the frequency of I-A^d dAPCs. (a) Representative dAPCs frequency on day 2. Student's t test was used to confirm the statistical difference on day 1 (b, n=4, ns: not significant), day 2 (c, n=3, *: $p < 0.05$), and day 3 (d, n=3, ns: not significant).

Figure 6. Immobilization of dAPCs on $\alpha I-A^d$ membrane *in vivo*. Dorsal ear skin from BALB/c mouse was incubated with 2 mg/mL PFC nanoemulsion to label dAPCs *ex vivo*. Membranes with $\alpha I-A^d$ IgG or without (control) were administrated on graft beds before placement of ear skins. Signal was monitored using a Li-Cor Pearl imager 2 days after transplantation. (a) Representative NIR images at day 2. (b) Student's t test was used to confirm the statistical difference. $n = 3$, **** $p < 0.001$.

Figure 7.

Immobilization of dAPCs on membrane imaged using confocal microscopy (40×). dAPCs were induced to egress using CCL21. Cells adsorbed on membranes with αI-A^d (“αI-A^d membrane”) or without (“Control membrane”) were stained for DNA (visualized using a DAPI filter) and fixed. Cells ingested with nanoemulsions were illuminated with DiR (red). Membranes were imaged with a Cy3 filter set (orange).

Figure 8.

Concentration of IFN- γ produced by recipient C57BL/6 mouse splenic T cells cultured with naïve BALB/c APCs. Membranes or other treatments were established in graft beds prior to the skin graft: “ α I-A^d membrane” (EAK16-II, EAKIIH6, α H6-IgG, pAG, and α I-A^d IgG), “ α I-A^d IgG, local” (α I-A^d IgG in PBS), “EAK16-II/EAKIIH6” (SAPs without other components), “Irrelevant IgG” (EAK16-II, EAKIIH6, α H6-IgG, pAG, and anti-I-A^b IgG), and “Saline” (isotonic saline solution). “ α I-A^d IgG, I.V.” represents α I-A^d IgG administrated through tail vein. “Isograft” refers to C57BL/6 to C57BL/6 transplantation. (a) Schematic of the experimental procedures. 7 days after the transplantation, T cells were prepared from spleen of recipient C57BL/6 mice and cultured with naïve BALB/c mouse APCs. The cell culture medium was collected by centrifugation and concentrations of IFN- γ were quantified by ELISA. (b) Concentration of IFN- γ produced by recipient C57BL/6 mouse splenic T cells. One way ANOVA with Tukey’s multiple comparison was used for

statistical analysis (n = 3, compared to α I-A^d membrane group, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, ns: not significant).