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Down regulation of RhoA and changes in T cell cytoskeleton correlate with the abrogation of allograft rejection

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Abstract

Proper actin cytoskeleton architecture and dynamics are indispensable for events in the immunological response such as T cell migration, redistribution of T cell receptors, and interaction with antigen presenting cells. Thus, T cell activation, downstream signaling events and effector functions are all actin-dependent. Actin cytoskeleton architecture and dynamics are regulated by proteins belonging to the superfamily of small GTP-binding proteins, such as RhoA GTPase. We previously showed that the administration of an MHC class I allochimeric molecule [α 1h1/u]-RT1.Aa, which contains donor-type (Wistar Furth, WF; RT1u) immunogenic epitopes displayed on recipient-type (ACI, RT1a) sequences, to the ACI recipient of heterotopic WF heart resulted in the restriction of the TCR repertoire, inhibition of T cell infiltration into the heterotopic cardiac allografts, abrogation of acute and chronic rejection, and induction of indefinite survival of the allograft. Here we show that the allochimeric molecule treatment caused down regulation of RhoA GTPase in T cells. This resulted in dramatic changes in the distribution of actin and the actin-binding protein, Hip55, in these cells, which in turn, inhibited T cell infiltration into the graft. This indicates that the immunosuppressive activity of the allochimeric molecule is achieved via downregulation of the RhoA pathway and disruption of the proper organization of T cell actin cytoskeleton to inhibit T cell functions such as motility and/or TCR signaling events.

Keywords

T cell; actin; RhoA; Hip55

1. Introduction

The immune response relies on the ability of T cells to move, scan and form the immunological synapse with antigen presenting cells (APCs). Interaction of a T cell with an APC involves several steps: active migration towards the APCs, adhesive contact, required to scan the surface of APC, and the polarization and redistribution of cytoskeleton, which allows the close apposition of cell membranes necessary for T cell receptor (TCR) interaction with the major histocompatibility complex (MHC). Thus, T cell activation,

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downstream signaling events and effector functions require a functional actin cytoskeleton, proper segregation of membrane, adhesion and intracellular signaling proteins [1–10].

The first studies to indicate that actin is required for T cell functions such as motility, adherence to target cells and cytotoxic activity were based on the use of actin-disrupting agents such as cytochalasin D. These, and more recent studies, indicate that actin filaments not only enhance T cell activation by promoting conjugate formation and the assembly of signaling complexes, but also facilitate the movement of molecules and internalization of the T cell receptor (TCR). Engagement of the T cell receptor triggers a series of signaling events that lead to the activation of T cells [1, 8, 9, 10]. One of the molecules that was recently discovered to be crucial for TCR signaling events is a novel actin-binding adaptor protein, HIP-55 (hematopoietic progenitor kinase 1 [HPK1]-interacting protein of 55 kDa, also called SH3P7 and mAbp1), [11]. Studies of HIP-55 knockout mice showed defective T cell proliferation, decreased cytokine production and decreased upregulation of activation markers induced by TCR stimulation [11, 12]. These results demonstrate the importance of HIP-55 as an actin adaptor protein in TCR signaling and the immune response.

The remodeling of the T cell actin cytoskeleton occurs in response to environmental stimuli [1, 4, 5, 7, 9, 10]. During maturation and activation, T cells migrate through vessel walls, interact with antigen presenting cells and adhere to target cells. All these steps depend upon a functional cytoskeleton network. Circulating T cells contain microvilli composed of parallel actin bundles. During migration through tissues, T cells polarize and form a posterior uropod as well as an anterior leading edge that is rich in a network of actin filaments that moves the cell forward. T cell activation requires contact between the T cell receptor and major histocompatibility complexes (MHC) expressed on APCs. Upon recognition of an APC bearing appropriate MHC-peptide complexes, the T cell rounds up, extends lamellipodia toward the APC and forms a tight contact junction that is rich in branched actin filaments. This process results in the formation of a flattened, F-actin-rich interface called the immunological synapse (IS) between the T cell and the APC. In addition, the actin cytoskeleton may act as a scaffold for the temporal and spatial distribution of T cell signaling components [2–6, 9].

Actin cytoskeleton architecture and dynamics, as well as other biological processes, such as cell cycle progression, gene transcription, and cell adhesion, are regulated by proteins belonging to the superfamily of small GTP-binding proteins such as Rho GTPases (RhoA, RhoB, and RhoC). Rho-GTPases have been implicated in the regulation of cell shape in a T cell line, in thymocyte homeostasis in transgenic mice and in the promotion of IL-2 production and calcium influx in Jurkat cells [13–20]. Thus, it is believed that Rho GTPases, acting through Rho kinase, have a role in T cell cellular immune responses by promoting structural rearrangements that are critical for T cell signaling. RhoA, the most extensively studied member of Rho family, is activated by cytokines, adhesion molecules, growth factors, hormones, integrins and G proteins. RhoA has a dual role in the formation of focal adhesions and the regulation of actin stress fibers [19, 20]. RhoA is also a positive regulator of T cell receptor responses in vivo [18]. It has been shown that T cells expressing active RhoA were hyperresponsive in the context of TCR-induced proliferation in vitro and in vivo. This indicates that RhoA function is not only important for pre-T cells but also plays a role in determining the fate of mature T cells. In addition, RhoA is involved in lymphocyte polarization, chemotaxis and leading edge retraction, all of which are absolutely required for T cell migration [19]. The most recent studies of the mechanism underlying the function of immunomodulatory drugs (IMiDs) such as lenalidomide and pomalidomide showed that pomalidomide reorganizes actin cytoskeleton and modifies interleukin-2 expression in human T cells through the modulation of activity of RhoA GTPase [13]. These authors propose that the reorganization of the cytoskeleton via modulation of GTPases activity represents a

fundamental molecular mechanism by which immunomodulatory compounds influence the host immune response [13]. Another recent studies showed that transfection of the human Jurkat T cell line with a dominant negative, kinase-defective mutant of Rho kinase diminished Jurkat cell proliferation. Rho kinase inhibition attenuated the expression cytokines that characterize T cell activation, blocked actomyosin polymerization, and prevented aggregation of the TCR/CD3 complex with lipid rafts [17]. In addition, a Rho kinase inhibitor considerably prolonged the survival of fully allogeneic heart transplants in mice and diminished intragraft expression of cytokine mRNAs [17].

Transplantation of a genetically incongruous organ generates an immune response that leads to the destruction of the grafted tissue [21–24]. Because the immunosuppressants currently used to prevent rejection also impair the recipient's immune system, a major goal in transplantation is to prevent acute and chronic rejection by inducing tolerance while avoiding global immunosuppression [21–24]. We previously showed that the administration of an MHC class I allochimeric molecule, designated [α 1h1/u]-RT1.Aa, in rats that had undergone heterotopic cardiac allografts inhibited T cell infiltration into the allografts, abrogated acute and chronic rejection as well as induced indefinite survival of the allograft when administered in conjunction with a sub-therapeutic dose of cyclosporine A (CsA), [25–28]. In contrast, untreated rats, rats treated with a sub-therapeutic dose of CsA alone or allochimeric molecule alone rejected transplanted hearts in 7 days [25–28]. This allochimeric MHC molecule contained donor-type (Wistar Furth, WF; RT1u) immunogenic epitopes displayed on recipient-type (ACI, RT1a) sequences and was produced by altering the immunodominant determinant in the α 1-helical region of class I MHC RT1.Aa to that of RT1.A1 and RT1.Au sequences [25]. Recently, we also showed that the allochimeric molecule-treated rats had a restricted TCR repertoire, exhibiting altered dominant size peaks to represent preferential clonal expansion of V β 7, V β 11, V β 13, V β 14, and V β 15 genes. Moreover, we found a positive correlation between the alteration of the V β profile, restriction of the TCR repertoire, and the establishment of allograft tolerance. Our findings indicate that presentation of allochimeric MHC class I sequences that partially mimic donor and recipient epitopes may induce a unique tolerant state by selecting alloresponsive V β genes [29].

Although the cellular and molecular mechanism(s) underlying the immunosuppressive function of the allochimeric [α 1h1/u]-RT1.Aa molecule remain largely unknown, we recently performed a microarray analysis of splenic T cells and showed that the allochimeric molecule treatment caused downregulation of genes involved in actin filament polymerization (RhoA and Rac1), cell adhesion (Catn1, Vcam and CD9), vacuolar transport (RhoB, Cln8 and ATP6v1b2), and the MAPK pathway (Spred1 and Dusp6) involved in tubulin cytoskeleton reorganization as well as the interaction between actin and the microtubule cytoskeleton [30]. In the study presented here, we examined early (one and three days post-transplantation) molecular events and show that the downregulation of the RhoA pathway in T cells from allochimeric molecule-treated rats resulted in dramatic changes in actin and actin-binding protein Hip55 distribution in these cells. This indicates that the early molecular events involved in the immunosuppressive function of the allochimeric molecule include the inhibition of T cell functions, such as motility and/or TCR signaling processes, via downregulation of the RhoA pathway and the disruption of proper organization of the T cell actin cytoskeleton.

2. Materials and Methods

2.1. Animals

Adult male inbred Wistar Furth (WF; RT1.Au) and ACI (RT1.Aa) rats (180–250 g) were purchased from Harlan Sprague Dawely (Indianapolis, IN). Heterotopic cardiac transplants

were placed intra-abdominally as described previously (5). There were three experimental groups: 1) transplantation control group without any treatment, 2) transplantation in the presence of a sub-therapeutic dose of CsA (acute rejection), and 3) transplantation in the presence of sub-therapeutic dose of CsA supplemented with the allochimeric molecule (CsA + peptide). CsA treatment consisted of a three-day course of oral cyclosporine delivered by gavage feed (CsA, 10 mg/kg/day; day 0–2). The allochimeric peptide [α 1h1/u]-RT1.Aa (GenWay, San Diego CA; 1 mg/kg) was delivered through the portal vein into ACI recipients of WF hearts at the time of transplantation. Our previous studies [25–28] showed that in contrast to oral or subcutaneous delivery, only the portal vein delivery of allochimeric molecule was effective in inhibiting rejection. We believe that liver participation may play some unknown role in this effect. All experiments were performed according to The Methodist Hospital Research Institute's animal care and use NIH standards as set forth in the "Guide for the Care and Use of Laboratory Animals" (DHHS publication No. (NIH) 85–23 Revised 1985). The Institute also mandates concordance with the PHS "Policy on Humane Care and Use of Laboratory Animals" and the NIH "Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training."

2.2. T cell isolation and FACS analysis

Spleens from ACI host rats were harvested at one, three and seven days post-transplantation. Cell suspensions were made by passing spleen through a cell strainer using 3-cc syringe. Cells were treated with a lysing reagent (Becton Dickinson) to remove the red blood cells and then washed twice with complete media (10% FCS/1640 RPMI). The T cell population was purified via a positive T cell isolation kit using magnetic anti-T cell micro beads (Miltenyi Biotech) and purity of T cells was confirmed by FACS analysis. T cells were stained with antibodies from BD Pharmingen (Franklin Lakes, NJ), including FITC-conjugated mouse anti-rat CD3 and CD4, for 15 minutes at room temperature and then washed three times with PBS and analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

2.3. RhoA immunostaining

T cells purified from spleen (see above) were pelleted and fixed in 4% formalin in PBS-0.05% Tween for 30 min at room temperature. After washing three times (15 min each wash) in PBS-0.05% Tween, cells were blocked in Caseine blocking buffer (BioRad) with 0.05% Tween for 1 hr at room temperature. Subsequently, cells were incubated with a 1:200 dilution of anti-RhoA (67B9) rabbit monoclonal antibody (Cell Signaling, cat # 2117) in Caseine blocking buffer-0.05% Tween, overnight at 4°C. After extensive washing (3 × 1 hr each wash) in PBS-0.05% Tween, cells were incubated with a 1:200 dilution of FITC-conjugated anti-rabbit secondary antibody in Caseine blocking buffer-0.05% Tween overnight at 4°C. After extensive washing (3 × 1 hr each wash) in PBS-0.05% Tween, cells were mounted in Antifade with 10 µg/ml of Hoechst (both from Molecular Probes) and observed with a fluorescent microscope.

2.4. Actin and Hip55 staining

The actin staining method was adapted from the protocol of Kloc et al. (2004). Isolated T cells were pelleted and fixed in 4% formaldehyde (EM grade, Ted Pella) in PBS with 0.1% Triton X-100 for 30 min at room temperature. After two 15-min washes in PBS–Tween 20, they were blocked for 30 min in Caseine blocking buffer-0.05% Tween and then incubated overnight in the dark at 4°C in the Caseine blocking buffer-0.05% containing rhodamine-phalloidin (Molecular Probes, Eugene, OR; 5 µl of 200 U/ml methanolic stock solution per 200 µl of blocking buffer) alone or in the case of actin/Hip55 double staining, also containing a 1:200 dilution of anti-Hip55 goat polyclonal antibody (Abcam, cat# ab2836). After extensive washing (3 × 1 hr each wash) in PBS-0.05% Tween, cells were incubated

with a 1:200 dilution of FITC-conjugated anti-goat secondary antibody in Caseine blocking buffer-0.05% Tween overnight at 4°C. Subsequently, cells were washed twice for 15 min each in PBS-0.05% Tween in the dark, mounted in Antifade with 10 µg/ml of Hoechst (both from Molecular Probes) and observed with a fluorescent microscope.

2.5. Western blotting

Isolated splenic T cells were homogenized on ice in RIPA buffer (0.15 M NaCl, 1% deoxycholate Na salt, 1% Triton X-100, 0.1% SDS, 0.01 M Tris HCl, pH 7.2) in the presence of Complete proteinase inhibitor (Roche). After homogenization, cell debris was removed by centrifugation (1500 × g for 10 min at 4°C). The protein concentration was determined using Biorad protein assay reagents, and SDS-PAGE and western blotting with anti-RhoA or anti-Hip55 antibodies (see above) were performed as described in Kloc et al. [31]. For the measurement of protein band intensity Actin/ RhoA bands were visualized using Lumi-Light Western blotting substrate (Roche). Band intensity for each experimental group was determined using Quantity One 4.6.1 system (Biorad).

2.6. Histology

Allografts from control untreated animals and animals treated with a sub-therapeutic dose of CsA or with a sub-therapeutic dose of CsA supplemented with the allochimeric molecule (CsA + peptide, see above) were fixed in 10% formalin, embedded in paraplast, sectioned at 10 µm and stained with Hematoxylin-Eosin (Sigma) according to the manufacturer's protocol.

2.7. RNA isolation

For RNA isolation, isolated T cells were pelleted and immediately placed in RNA Later (Applied Biosystems/Ambion, Austin, TX). After overnight infiltration at 4°C, samples were kept in RNA Later at -70°C until the isolation of total RNA. T cell pellets were homogenized using a TissueLyser (Qiagen, Valencia, CA), and RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. The quantity, purity and integrity of RNA were evaluated using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA).

2.8. Quantitative RT PCR

Total RNA was isolated from purified splenic T cells using the RiboPure kit (Applied Biosciences, Foster City, CA). Complementary DNA (cDNA) was made using the High Capacity cDNA Reverse Transcription Kit (Applied Biosciences, Foster City, CA). The cDNA was used to determine the expression levels of the housekeeping gene, actin β (ACTβ). The RhoA primer (cat # PPR56555A) was purchased from SABiosciences (Frederick, MD, USA). Total RNA (1000 ng) was reverse transcribed (RT) and PCR amplified using the High Capacity cDNA Reverse Transcription Kits (Applied Biosystems) according to the manufacturer's protocol. The RT reaction consisted of a 10-min incubation at 25°C and a 120-min incubation at 37°C followed by a 5 min, 85°C termination step, and the resulting complementary DNA (cDNA) was stored at -20°C. For real-time PCR amplification, samples were run in duplicate, and only one gene was analyzed per reaction. The cDNA template reaction contained Assay On Demand Gene Expression primers (see below; Applied Biosystems, Foster City, CA) and TaqMan® Fast Universal PCR no AmpErase UNG master mix (Applied Biosystems, Foster City, CA). Reactions were heated to 95°C for 10 min, followed by 40 amplification cycles of 95°C for 15 s and 60°C for 1 min using an Applied Biosystems 7500 Standard System. The amount of target mRNA relative to housekeeping gene mRNA was expressed as fold increase or decrease. Relative changes

were measured using real-time PCR in the 7500 Fast or Standard Real-Time PCR System (version 1.3.1). To calculate the relative quantity (RQ) of particular gene, we used the 2-delta delta ct method in the software. The data are presented as the fold change (RQ values) in gene expression level normalized to an endogenous reference gene.

3. Results

3.1. Down regulation of RhoA by allochimeric molecule treatment

Recently, using microarray analysis of gene expression in splenic T cells, we showed that genes belonging to the RhoA pathway and involved in actin filament polymerization were among the genes in which transcription was downregulated at one day and three days post-transplantation in the CsA + peptide treatment group [30]. Here we were interested to see if the transcriptional downregulation of the RhoA gene results in the downregulation of its protein product. Immunostaining (Fig. 1) and western blotting (Fig. 2) with an anti-RhoA antibody in T cells isolated from the spleens of rats in the three treatment groups (untreated control, sub-therapeutic dose of CsA, and sub-therapeutic dose of CsA in conjunction with the allochimeric molecule treatment) showed that the level of RhoA protein was dramatically reduced in the allochimeric molecule treatment at day 1 and 3 post-transplantation (Fig. 1 and 2). Immunostaining showed that in T cells isolated from control, untreated rats at day 1 and 3 post-transplantation, the RhoA protein was uniformly distributed within the T cell cytoplasm, with a slightly higher concentration of RhoA protein at the T cell periphery and underneath the cellular membrane (Fig. 1A1, A3 and E1). In T cells isolated from rats treated with a sub-therapeutic dose of CsA, RhoA protein was still uniformly distributed in the cytoplasm, but it had formed distinct patches at the cell membrane and cell periphery (Fig. 1B1, B3, and F1). In contrast, in the T cells isolated from allochimeric molecule-treated rats, RhoA protein was not detectable in the T cell cytoplasm and only occasionally was visible in the form of one or two patches at the cell periphery (Fig. 1C1, C3, D1, D3 and G1). This indicates that allochimeric treatment results in drastic reduction of RhoA protein level and dramatic changes in its cellular distribution in splenic T cells. This conclusion was also supported by qRT-PCR results (30, and Fig. 2) and western blotting analysis (Fig 2).

3.2. Allochimeric molecule treatment changes cortical actin distribution in T cells

Knowing that RhoA is a regulator of the actin cytoskeleton, we were interested in exploring whether the observed downregulation of RhoA at the RNA and protein levels in T cells from allochimeric molecule-treated rats resulted in any morphologically detectable changes in the distribution of actin in these cells. Rhodamine-phalloidin staining of actin in T cells at one and three days post-transplantation is shown in Fig. 3. We found that in T cells from control, untreated rats, actin formed a uniform cortical layer under the cellular membrane (Fig. 3A and I). In T cells from rats treated with a sub-therapeutic dose of CsA, the cortical layer of actin had a patchy appearance (Fig. 3B and J). In T cells from allochimeric molecule-treated rats, the distribution of actin changed dramatically; it formed large aggregates that were unevenly distributed below the cellular membrane (Fig. 3C, D1–H, and K). These observations indicate that the decrease in RhoA expression results in dramatic changes in the distribution of T cell cortical actin.

3.3. Allochimeric molecule treatment causes dissociation of the actin-binding adaptor protein Hip55 from actin

Recent studies showed that a novel actin-binding adaptor protein, Hip55, colocalizes with actin, and via regulation of actin dynamics, has a crucial role in TCR signaling and the immune response [11, 12]. These data prompted us to investigate the distribution of Hip55 protein in splenic T cells from untreated, CsA-treated, and CsA plus allochimeric molecule-

treated rats. To visualize co-distribution of actin and its binding protein, Hip55, we performed rhodamine-phalloidin staining for actin in conjunction with immunostaining using anti-Hip55. We found that in T cells from untreated rats and rats treated with a sub-therapeutic dose of CsA, Hip55 showed distinct colocalization with actin. Both were localized at the T cell periphery (Fig. 4A1–B3). In addition, we found that in T cells from control, untreated rats, actin and Hip55 colocalized as a uniform cortical layer under the cellular membrane (Fig. 4A1–A3). In T cells from rats treated with a sub-therapeutic dose of CsA, in which the cortical layer of actin had a patchy appearance (Fig. 3B and I and Fig. 4B2), the Hip55 protein colocalized with actin patches (Fig. 3B1–B3). In stark contrast, in the T cells from allochimeric-treated rats, both actin and Hip55 formed distinct patches and aggregates. There was an obvious, partial dissociation of actin from its binding partner, Hip55, and Hip55 formed distinct actin-free patches and aggregates (Fig. 4C1–D3). We calculated the number of aggregates in 100 cells, which were co stained for actin and Hip55 and calculated the number of aggregates stained exclusively with Hip55. In both control and cyclosporine alone all actin positive aggregates (on average 21 aggregates per cell) were also Hip55 positive, which indicates perfect colocalization of these two binding partners. However, in allochimeric molecule treatment there were on average 2.3 aggregates per cell, which were exclusively Hip55 positive (i.e. actin negative). Western blot analysis using the anti Hip55 antibody indicated that the level of Hip55 protein in T cells from allochimeric molecule-treated rats remained unchanged when compared to CsA alone or untreated control animals (not shown). These observations indicate that in the allochimeric treatment, although the level of Hip55 protein remains unchanged, the destabilization of the normal cytoarchitecture of actin correlates with its dissociation from its binding partner, Hip55.

3.4. Allochimeric molecule treatment inhibits T cells infiltration into the allograft

Although we previously had shown that the allochimeric molecule treatment inhibits T cell infiltration into the allograft, abrogates acute and chronic rejection, and induces an indefinite survival of heart allografts in rats, these studies were focused on long-term survival events at >100 days post-transplantation [25–28]. Here we were interested in early post-transplantation events and in examining whether the allochimeric molecule treatment induces changes in the ability of lymphocytes to infiltrate the graft at day 3 and day 7 post-transplantation. Histological analysis of transplanted hearts showed that allochimeric molecule treatment dramatically reduced lymphocyte infiltration into the graft (Fig. 5), and this was especially prominent at day 7 post-transplantation, when untreated rats and rats treated with a sub-therapeutic dose of CsA reject the allograft (Fig. 5 D–F). FACS analysis of T cells isolated from heart allografts confirmed this finding and showed that CD3⁺, CD4⁺ and CD8⁺ T cell populations are reduced in response to CsA treatment (7.4%, 4.7% and 12.9%, respectively) and drastically reduced with the allochimeric molecule treatment (4.1%, 2.4% and 9.6%, respectively) in comparison to untreated control (12.2%, 5.6% and 21.9%, respectively). All these data indicate that allochimeric molecule treatment drastically inhibits ability of T cells to infiltrate allograft tissue.

4. Discussion

We showed previously that the administration of an MHC class I allochimeric molecule, [α1h1/u]-RT1.Aa, inhibited T cell infiltration into heterotopic cardiac allografts, abrogated acute and chronic rejection, and induced indefinite survival in rats when administered in conjunction with a sub-therapeutic dose of cyclosporine A (CsA) [25–28]. In addition, we recently showed that the allochimeric molecule-treated rats displayed a restricted TCR repertoire that correlated with the establishment of allograft tolerance [29]. These studies focused on long-term survival and end-point events occurring at >100 days post-transplantation, and the molecular mechanisms and early post-transplantation events

responsible for the immunosuppressive function of the allochimeric molecule remained a mystery. To define the mechanisms and molecular pathways affected in early post-transplantation events in the same experimental model system, we recently performed T cell gene expression microarray analysis at days 1–7 post-transplantation and showed that there is downregulation of Rho GTPase pathway genes in splenic T cells from allochimeric molecule-treated rats [30].

Higher vertebrates have three Rho GTPases, RhoA, RhoB, and RhoC, that, although highly homologous in structure, have different cellular functions [32]. These differences are reflected in their localization: RhoB localizes mainly on late endosomes and lysosomes, and RhoA and RhoC are found in the cytoplasm or at the plasma membrane [32]. RhoA directly stimulates actin polymerization and addition of actin monomers to the barbed, or fast-growing, end of actin filaments [1, 10, 14, 16, 32]. In migrating cells, RhoA has been shown to have a key role in the turnover of cell-extracellular matrix adhesions at the cell rear [1, 10].

Regulation of actin polymerization is critical for many different functions of T lymphocytes, including cell polarization, migration and chemotaxis [1, 10, 19, 20]. Recent studies on Jurkat T cells showed that RhoA (acting through its effector mDia) is involved in T lymphocyte migration and activation via regulation of actin polymerization [17, 19]. Studies of T cells from transgenic mice expressing an active mutant of RhoA showed that RhoA activation is sufficient to stimulate integrin-mediated cell adhesion in thymocytes and that RhoA can strikingly potentiate TCR-mediated responses in primary lymphocytes. Loss of function of RhoA blocks pre-T cell differentiation and survival, indicating that RhoA is a critical signaling molecule during early thymocyte development [18]. This study showed that cells expressing active RhoA were hyperresponsive to TCR-induced proliferation in vitro, and in vivo, active RhoA augmented positive selection of thymocytes expressing defined TCR complexes [18]. All of these studies indicate that RhoA has a role in determining the fate and functions of T cells. In addition, RhoA, via cytoskeletal rearrangements and tetraspanin CD82, participates in early TCR signaling events and selective inhibition of RhoA using toxins and dominant-negative mutants diminished the CD82-induced T cell activation [20]. Another study showed that transfection of the human Jurkat T cell line with a dominant negative, kinase-defective mutant of Rho kinase diminished Jurkat cell proliferation. In addition, inhibition of Rho kinase substantially attenuated the cytokine gene expression characteristic of T cell activation, blocked actomyosin polymerization, and prevented aggregation of the TCR/CD3 complex [17]. All of these changes resulted in the drastic diminution of immune responses in vivo, as treatment with a Rho kinase inhibitor considerably prolonged the survival of fully allogeneic heart transplants in mice [17].

Another molecule that has recently been discovered to be crucial for TCR signaling events and T cell functions is a novel, actin-binding adaptor protein, HIP-55 (hematopoietic progenitor kinase 1 [HPK1]-interacting protein of 55 kDa; also called SH3P7 and mAbp1). Studies of HIP-55 knockout mice showed defective T cell proliferation, decreased cytokine production and decreased upregulation of activation markers induced by TCR stimulation [11, 12]. Using RNA interference and overexpression experiments, the HIP-55-HPK1 complex was found to negatively regulate nuclear factor of activated T cells (NFAT) activation by the T cell antigen receptor [11]. Moreover, HIP-55 promoted down-modulation of the TCR, resulting in decreased TCR expression, and actin-depolymerizing factor homology domains were required for this function [11]. These results suggest that HIP-55 negatively regulates TCR signaling through downregulation of TCR expression and modulates T cell activation by coupling the actin cytoskeleton and the TCR to gene activation [11]. Another study showed that HIP-55 knockout T cells are defective in

proliferation, have decreased cytokine production, and decreased upregulation of activation markers induced by TCR stimulation. These phenotypes of HIP-55 knockout mice were accompanied by reduced immune responses, including antigen-specific antibody production and T cell proliferation [12]. These results demonstrate the importance of HIP-55 as an actin adaptor protein in the creation of adequate immune responses.

5. Conclusions

Here we showed that allochimeric treatment caused downregulation of RhoA GTPase at the RNA and protein levels in splenic T cells. This, in turn, resulted in profound changes in the distribution of actin and the actin-binding protein Hip55 in these cells. Actin, which normally forms a uniform cortical layer underlying the cellular membrane and colocalizes with its binding partner, Hip55, formed irregularly spaced patches and aggregates that were dissociated from Hip55. Moreover, we found that all of these changes correlated with highly diminished infiltration of T cells into the allografts. This indicates that the presentation of allochimeric MHC class I sequences that partially mimic donor and recipient epitopes may induce a unique tolerant state leading to the abrogation of acute and chronic rejection. We suggest that the mechanism underlying this phenomenon involves the inhibition of the RhoA/actin/Hip55 pathway, which disrupts cytoarchitecture, restricts TCR repertoire, and inhibits the motility of T cells, preventing them from exerting a normal immunological response.

References

1. Burkhardt JK, Carrizosa E, Shaffer MH. The actin cytoskeleton in T cell activation. *Annu. Rev. Immunol.* 2008; 26:233–259. [PubMed: 18304005]
2. Al-Alwan MM, Liwski RS, Haeryfar SMM, Baldridge WH, Hoskin DW, Rowden G, West KA. Cutting edge: Dendritic cell actin cytoskeletal polarization during immunological synapse formation is highly antigen-dependent. *J. Immunol.* 2003; 171:4479–4483. [PubMed: 14568920]
3. Brossard C, Feuillet V, Schmitt A, Randriamampita C, Romao M, Raposo G, Trautmann A. Multifocal structure of the T cell-dendritic cell synapse. *Eur. J. Immunol.* 2005; 35:1741–1753. [PubMed: 15909310]
4. Davis DM, Dustin ML. What is the importance of the immunological synapse? *Trends Immunol.* 2004; 25:323–327. [PubMed: 15145322]
5. Dustin ML. Cell adhesion molecules and actin cytoskeleton at immune synapses and kinapses. *Curr. Opin. Cell Biol.* 2007; 19:529–533. [PubMed: 17923403]
6. Dustin ML. Synaptic asymmetry to go. *Cell.* 2008; 132:733–734. [PubMed: 18329360]
7. Dustin ML, Cooper JA. The immunological synapse and the actin cytoskeleton: molecular hardware for T cell signaling. *Nature Immunol.* 2000; 1:23–29. [PubMed: 10881170]
8. Huang Y, Burkhardt JK. T-cell-receptor-dependent actin regulatory mechanisms. *J. Cell Sci.* 2007; 120:723–730. [PubMed: 17314246]
9. Sancho D, Vicente-Manzanares M, Mittelbrunn M, Montoya MC, Gordon-Alonso M, Sarrador JM, Sanchez-Madrid F. Regulation of microtubule-organizing center orientation and actomyosin cytoskeleton rearrangement during immune interactions. *Immunol. Rev.* 2002; 189:84–97. [PubMed: 12445267]
10. Vicente-Manzanares M, Sánchez-Madrid F. Role of the cytoskeleton during leukocyte responses. *Nat. Rev. Immunol.* 2004; 4:110–122. [PubMed: 15040584]
11. Le Bras S, Foucault I, Foussat A, Brignone C, Acuto O, Deckert M. Recruitment of the actin-binding protein HIP-55 to the immunological synapse regulates T cell receptor signaling and endocytosis. *J. Biol. Chem.* 2004; 279:15550–15560. [PubMed: 14729663]
12. Han J, Shui W Jr, Zhang X, Zheng B, Han S, Tan T-H. HIP-55 is important for T-cell proliferation, cytokine production, and immune responses. *Mol. Cell. Biol.* 2005; 25:6869–6878. [PubMed: 16055701]

13. Xu Y, Li J, Ferguson GD, Mercurio F, Khambatta G, Morrison L, Lopez-Girona A, Corral LG, Webb DR, Bennett BL, Xie W. Immunomodulatory drugs reorganize cytoskeleton by modulating Rho GTPases. *Blood*. 2009; 114:338–345. [PubMed: 19417207]
14. Pernis AB. Rho GTPase-mediated pathways in mature CD4+ T cells. *Autoimmun. Rev.* 2009; 8:199–203. [PubMed: 18723130]
15. Lee JH, Katakai T, Hara T, Gonda H, Sugai M, Shimizu A. Roles of p-ERM and Rho-ROCK signaling in lymphocyte polarity and uropod formation. *J. Cell. Biol.* 2004; 167:327–337. [PubMed: 15504914]
16. Nijhara R, van Hennik PB, Gignac ML, Kruhlak MJ, Hordijk PL, Delon J, Shaw S. Rac1 mediates collapse of microvilli on chemokine-activated T lymphocytes. *J. Immunol.* 2004; 173:4985–4993. [PubMed: 15470041]
17. Tharaux P-L, Bukoski RC, Rocha PN, Crowley SD, Ruiz P, Nataraj C, Howell DN, Kaibuchi K, Spurney RF, Coffman TM. Rho kinase promotes alloimmune responses by regulating the proliferation and structure of T cells. *J. Immunol.* 2003; 171:96–105. [PubMed: 12816987]
18. Corre I, Gomez M, Vielkind S, Cantrell DA. Analysis of thymocyte development reveals that the GTPase RhoA is a positive regulator of T cell receptor responses in vivo. *J. Exp. Med.* 2001; 194:903–913. [PubMed: 11581313]
19. Vicente-Manzanares M, Rey M, Pérez-Martínez M, Yáñez-Mó M, Sancho D, Cabrero JR, Barreiro O, de la Fuente H, Itoh K, Sánchez-Madrid F. The RhoA effector mDia is induced during T cell activation and regulates actin polymerization and cell migration in T lymphocytes. *J. Immunol.* 2003; 171:1023–1034. [PubMed: 12847276]
20. Delaguillaumie A, Lagaudrière-Gesbert C, Popoff MR, Conjeaud H. Rho GTPases link cytoskeletal rearrangements and activation processes induced via the tetraspanin CD82 in T lymphocytes. *J. Cell Sci.* 2002; 115:433–443. [PubMed: 11839793]
21. Dunn DL. Problems related to immunosuppression. Infection and malignancy occurring after solid organ transplantation. *Critical Care Clinics*. 1990; 6:955–977. [PubMed: 2265389]
22. Trivedi HL. Immunobiology of rejection and adaptation. *Trans. Proc.* 2007; 39:647–652.
23. Butcher GW, Howard JC. Genetic control of transplant rejection. *Transplantation*. 1982; 34:161–166. [PubMed: 6815843]
24. Colovai AI, Renna-Molajoni E, Cortesini R, Suci-Foca N. New approaches to specific immunomodulation in transplantation. *Int. Rev. Immunol.* 1996; 13:161–172. [PubMed: 8782739]
25. Ghobrial RM, Hamashima T, Wang M, Stepkowski SM, Kahan BD. Induction of transplantation tolerance by chimeric donor/recipient class I RT1.Aa molecules. *Transplantation*. 1996; 62:1002–1010. [PubMed: 8878396]
26. Semiletova N, Shen X-D, Fishbein MC, Gao F, Slomowitz SJ, Jiao Q, et al. Posttransplant administration of allochimeric major histocompatibility complex class-I-molecules induces true transplantation tolerance. *Transplantation*. 2003; 75:550–553. [PubMed: 12605125]
27. Semiletova NV, Shen X-D, Baibakov B, Feldman DM, Mukherjee K, Frank JM, et al. Inhibition of Chronic Rejection by Antibody Induced Vascular Accommodation in Fully Allogeneic Heart Allografts. *Transplantation*. 2005; 80:1535–1540. [PubMed: 16371921]
28. Singer JS, Mhoyan A, Fishbein MC, Shen X-D, Gao F, Zhao D, et al. Allochimeric class I MHC molecules prevent chronic rejection and attenuate alloantibody responses. *Transplantation*. 2001; 72:1408–1416. [PubMed: 11685113]
29. Liu D, Shen XD, Zhai Y, Lam W, Liao J, Busuttill RW, Ghobrial RM. Intragraft selection of the T cell receptor repertoire by class I MHC sequences in tolerant recipients. *PLoS One*. 2009; 4:e6076. [PubMed: 19562081]
30. Lisik W, Tejpal N, Gong Y, Skelton TS, Ganachari M, Bremer EG, Kloc M, Ghobrial RM. Down regulation of genes involved in T cell polarity and motility during the induction of heart allograft tolerance by allochimeric MHC I. *PLoS One*. 2009; 4:e8020. [PubMed: 19956540]
31. Kloc M, Reddy B, Crawford S, Etkin LD. A Novel 110-kDa maternal CAAX box-containing protein from *Xenopus* is palmitoylated and isoprenylated when expressed in baculovirus. *J. Biol. Chem.* 1991; 266:8206–8212. [PubMed: 2022638]
32. Wheelera AP, Ridley AJ. Why three Rho proteins? RhoA, RhoB, RhoC, and cell motility. *Exp. Cell Res.* 2004; 301:43–49. [PubMed: 15501444]

Fig. 1. Allochimeric molecule treatment downregulates RhoA protein in T cells

Immunostaining of T cells isolated from spleen with anti-RhoA primary antibody and FITC-conjugated secondary antibody (green). T cell nuclei are counterstained with Hoechst (blue). (A1–A3) T cells from control, untreated animals at day 1 and (E1–E2) at day 3 post-transplantation. (A1, E1) Strong signal of RhoA localization is visible in the cytoplasm and at the cellular membrane. (A2, E2) T cell nuclei stained with Hoechst. (A3) Merged image of T cells with RhoA (green) and Hoechst (blue) staining. (B1–B3) T cells from animals treated with a sub-therapeutic dose of CsA at day 1 and (F1–F2) at day 3 post-transplantation. (B1, F1) Patches of RhoA protein (arrows) are visible at the cellular membrane. (B2, F2) T cell nuclei stained with Hoechst. (B3) Merged image of T cells with RhoA (green) and Hoechst (blue) staining. (C1–D1) T cells from animals treated with a sub-therapeutic dose of CsA in conjunction with the allochimeric molecule at day 1 and (G1–G2) at day 3 post-transplantation. (C1, D1, G1) T cells are either negative for RhoA staining or very weakly positive with occasional patches of RhoA protein visible (G1, arrow) at the cellular membrane. (C2, D2, G2) T cell nuclei stained with Hoechst. (C3, D3) Merged image of T cells with RhoA (green) and Hoechst (blue) staining. Bar is equal to 10 μ m.

Fig. 2. Down regulation of RhoA RNA and protein in T cells from allochimeric molecule-treated rats

(A). Graph of expression of RhoA RNA measured by qRT-PCR and (B). Western blot of RhoA (24 kDa) and actin (42 kDa, loading control) in T cells from untreated, CsA, and CsA in conjunction with allochimeric molecule (CsA+P) treated rats showing drastic downregulation of RhoA expression at the RNA and protein levels in the allochimeric treatment. (C) The expression level of RhoA determined on Lumi-Light Western blots using Quantity One 4.6.1 system and normalized to that of β actin loading marker to produce ratio of RhoA/ β actin, shows a drastic down regulation of RhoA protein level in CsA + allochimeric molecule treatment.

Fig. 3. Allochimeric molecule treatment induces changes in cortical actin distribution in T cells (A–C, D1, D3 E–K) Rhodamine-phalloidin staining (red) of actin in T cells isolated from spleen. (D2, D3, I–K) T cell nuclei are counterstained with Hoechst (blue). (D3, I–K) Merged images of actin (red) and nuclear (blue) Hoechst staining. (A, I) T cells from control, untreated animals at day 1 and (I) at day 3 post-transplantation. (A, I) Uniform layer of cortical actin is visible underneath the cellular membrane. (B, J) T cells from animals treated with a sub-therapeutic dose of CsA at day 1 and (J) at day 3 post-transplantation. (B, J) Actin distribution becomes patchy; small aggregates of actin underlie the cellular membrane. (D1–H) T cells from animals treated with a sub-therapeutic dose of CsA in conjunction with allochimeric molecule at day 1 and (J) at day 3 post-transplantation. Actin

forms large aggregates unevenly distributed below the cellular membrane. Bar is equal to 10 μm .

Fig. 4. Dissociation of actin-binding adaptor protein Hip55 from actin in T cells from allochimeric molecule treated rats

(A1, B1, C1 and D1) Immunostaining of T cells with anti-Hip55 antibody and FITC-conjugated secondary antibody (green) at day 1 post-transplantation. (A2, B2, C2, D2) Rhodamine-phalloidin staining (red) of actin in T cells. (A3, B3, C3, D3) Merged images of actin (red) and Hip55 (green) staining. (A1–A3) T cells from control, untreated animals. A uniform layer of cortical actin is visible underneath the cellular membrane and colocalizes with the actin-binding protein, Hip55. (B1–B3) T cells from animals treated with a sub-therapeutic dose of CsA. Actin and Hip55 distribution becomes patchy; small aggregates (on average 21 aggregates per cell), containing colocalized actin and Hip55, underlie the cellular

membrane. (C1–D3) T cells from animals treated with a sub-therapeutic dose of CsA in conjunction with allochimeric molecule. Actin and Hip55 form large aggregates unevenly distributed below the cellular membrane. Some of these aggregates show colocalization of actin and Hip55 (thick arrows), and some Hip55-positive aggregates (on average 2.3 aggregates per cell) do not contain actin (thin arrows), which indicates that actin partially dissociates from its binding partner, Hip55. Bar is equal to 10 μm .

Fig. 5. Allochimeric molecule treatment inhibits lymphocyte infiltration into the allograft
 Light microscopy images of heart allograft sections stained with Hematoxylin-Eosin showing blue nuclei and red cytoplasm at 3 days (A–C) and 7 days (D–F) post-transplantation. At day 3 post-transplantation, the infiltration of lymphocytes into the graft tissue is very low in CsA alone (A) and CsA+allochimeric molecule (B) treatment, and prominent in untreated, rejecting animals (C). At day 7 post-transplantation, when both untreated and sub-therapeutic dose of CsA treated rats reject the allograft, dramatic infiltration of lymphocytes is visible in CsA alone (D) and untreated (F) animals. In contrast, very low lymphocyte infiltration is present in the allograft from CsA+ allochimeric molecule (E) treated rats. Bar is equal to 20 μ m.