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Lymph node stromal fiber ER-TR7 modulates CD4+ T cell lymph node trafficking and transplant tolerance¹

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Abstract

Background—Trafficking and differentiation of naïve CD4+ and regulatory T cells (Treg) within the lymph node (LN) are integral for tolerance induction. The LN is comprised of stromal fibers that dictate lymphocyte migration and LN structure, organization, and microanatomic domains. Distribution of the stromal fiber ER-TR7 changes within the LN following antigenic challenge, but the contributions of ER-TR7 to the resulting immune response remain undefined. We hypothesized that these stromal fiber structural changes affect T cell fate and subsequently allograft survival.

Methods—C57BL/6 mice were left naïve (untreated) or made immune or tolerant (donor-specific BALB/c splenocyte transfusion $-/+$ anti-CD40L mAb), or made tolerant and received anti-ER-TR7 mAb. Donor-specific T cell migration was visualized by adoptive transfer of CFSE-labeled TEa T cell receptor transgenic CD4+ cells. Immunohistochemistry was performed on lymph nodes to detect stromal fiber distribution, structure, CCL21 presence, and Treg and donor-specific cell location relative to high endothelial venules (HEV). Naïve, tolerant, and tolerant + anti-ER-TR7 mice received BALB/c heterotopic cardiac allografts and graft survival was monitored.

Results—ER-TR7 distribution changed following the induction of tolerance vs. immunity. Treating tolerant mice with anti-ER-TR7 altered HEV basement membrane structure and the distribution of CCL21 within the LN. These differences were mirrored by changes in the

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migration of naïve and Treg cells within and surrounding the HEV. Anti-ER-TR7 prevented tolerance induction and resulted in allograft inflammation and rejection.

Conclusions—These results identify ER-TR7 as an important component of LN structure in tolerance and a direct target for immune modulation.

Keywords

Tolerance; Lymph Node; Trafficking; T Cells

Introduction

Lymph nodes (LN) are highly complex and organized secondary lymph organs in which lymphocytes and antigen presenting cells (APC) interact along a stromal reticular network to control the generation of tolerance or immunity. The outermost cortex contains B cells, follicular dendritic cells, and macrophages. In the paracortex, fibroblastic reticular cells (FRC) form a field for T cell and dendritic cell (DC) interactions. Between the cortex and paracortex, a dense network of stromal fibers forms the cortical ridge (CR), a platform on which T cells, B cells, and DC interact to control the immune response (1). In the CR, high endothelial venules (HEV) allow for T cell entry into the LN from the blood (2). HEV are lined on the vascular lumen by blood endothelial cells (BEC) and on the abluminal side by basement membrane stromal fibers.

Leukocyte trafficking through the LN is integral for the induction of transplant tolerance (3–6). Tolerance-inducing plasmacytoid DC (pDC) acquire alloantigen in the periphery and migrate to the LN where they induce alloantigen specific Treg (3, 4, 7). Treg also migrate to the LN, where they suppress the priming of naïve antigen reactive T cells (3, 8, 9). Naïve CD4⁺ T cells, Treg and pDC all traffic through the HEV and enter into the CR where they co-localize to engage several mechanisms required for inducing tolerance (10, 11). The timing of these interactions is integral in determining CD4⁺ T cell fate, so that the generation of alloreactive CD4⁺ Treg or anergic and apoptotic alloreactive CD4⁺ T cells occur at different times during tolerance induction (4). Although the co-localization of naïve alloreactive CD4⁺ T cells, Treg, and pDC within the CR is integral for the generation of tolerance, precisely how these interactions are constrained to specific LN domains has not been determined.

Precise LN architecture is essential for generating an appropriate immune response (12, 13). The LN (14–16) and HEV (17, 18) undergo structural changes or remodeling in response to antigenic challenge highlighting the importance of correct architectural configuration. The stromal network defines the LN physical shape, compartments, basement membranes, and lymphocyte migratory paths. This network is comprised of intertwined stromal fibers that include ER-TR7 (1, 19, 20), laminins, desmin, and collagens (21). This network may also dictate an “open” state that allows for naïve cells to travel quickly through the LN during homeostatic conditions, and a “closed” state that restricts cell travel following antigenic challenge (22). The stromal network also creates distinct domains for generating polarized immune responses. T follicular helper cells and memory CD8⁺ T cells develop and are activated in the interfollicular zone, Th1 priming occurs in the T cell area, Th2 priming

occurs near B cell areas, and Treg and Th17 cells are likely primed in the CR (21). The importance of the correct structure and organization of the LN is reinforced by findings that interruption of this organization results in impaired or inappropriate immune responses (12, 13). Although the physical structure of the LN regulates immune responses by dictating both the types and kinetics of cell subset contact, the individual contributions and regulation of specific stromal elements in immune responses remains undefined.

The ER-TR7 antigen is an integral building block of the LN stromal fiber network (20). ER-TR7 is produced by FRC and found on the FRC surface and intertwined with the reticular network, particularly in the CR (19, 20). FRC and T cell contact are required for the construction of this reticular mesh (19). Stromal fibers, including ER-TR7, directly affect T cell LN entry by forming “exit ramps” that allow for T cell egress from the HEV (13). Although the presence of ER-TR7 within the structural network of the LN is well defined, the antigen itself and how it functions to affect lymphocyte migration and resulting immune responses is unknown.

We investigated the function of ER-TR7 in the LN structural changes that accompanied allograft tolerance by administering an anti-ER-TR7 mAb at the time of tolerance induction. The tolerant LN structure was unique compared to naïve and immune LN. Anti-ER-TR7 altered LN and HEV structure, and the migration of antigen specific T cells and Treg through the HEV. Anti-ER-TR7 administered to tolerogen treated mice induced allograft inflammation and graft loss. Thus, manipulation of this stromal fiber altered T cell trafficking within the LN and alloantigenic responses.

Results

Anti-ER-TR7 alters LN remodeling and CCL21 chemokine gradients associated with the induction of tolerance

Previous studies revealed a significant increase in the area of the LN staining positive for the ER-TR7 epitope which accompanied the induction of tolerance, but not immunity (23). The change of ER-TR7 expression was one of several tightly regulated stromal morphological events that accompanied tolerization (23). These observations led us to hypothesize that anti-ER-TR7 antibody may bind to the stromal fibers and that such treatment would affect fiber morphology and/or fiber function and thus negatively affect the induction of alloantigenic tolerance. To investigate this hypothesis, C57BL/6 mice were made tolerant via i.v. administration of donor specific transfusion of BALB/c splenocytes (DST, 10^7 cells) plus anti-CD40L mAb (250 µg). To test the impact of anti-ER-TR7 mAb treatment on ER-TR7 remodeling associated with tolerance induction, tolerant mice were treated with 100 µg anti-ER-TR7 i.v. one day before the administration of tolerogen (tolerant + anti-ER-TR7). No adverse effects of anti-ER-TR7 treatment were observed; mice appeared healthy and active. Normal H&E histology was observed in the liver, kidney, hearts, lungs and spleen after transplantation. LN were of normal size and lymphocyte subset content after anti-ER-TR7 treatment (data not shown). For comparison, mice were made immune (+ DST, only) or left untreated (naïve). After 24h, LN were harvested and fluorescent immunohistochemistry used to determine the percent of the LN that was ER-TR7+ (Fig. 1A). Tolerance induction resulted in increased ER-TR7 in the basement membrane of HEV and in the CR region

compared to naïve and immune mice. Anti-ER-TR7 treatment of tolerant mice resulted in no change in the total percentage of the LN that was ER-TR7+, indicating increased LN ER-TR7 remodeling resulted from the combination of antigen stimulation in the presence of costimulatory blockade and anti-ER-TR7 did not change stromal fiber expression.

The complexity of ER-TR7+ fiber branching around the HEV was also assessed 24h after administration of tolerogen (Fig. 1B). Increased complexity of ER-TR7+ fibers branching around the HEV was associated with tolerance but not immunity, suggestive of tolerance specific remodeling (Fig. 1B). The complexity of ER-TR7+ HEV branching observed in tolerant + anti-ER-TR7 LN was similar to that observed in naïve and immune LN, and significantly reduced in comparison to tolerant LN. Thus, while anti-ER-TR7 treatment did not alter the total amount of ER-TR7+ regions in the tolerant LN, it did alter the physical structure of the stromal fiber network encircling the HEV.

We hypothesized that anti-ER-TR7 antibody may further affect the microdomains established within the tolerant LN by altering the CCL21 chemokine gradient that is associated with the stromal network and that is necessary for T cell trafficking into and through the LN (24). The quantity and distribution of CCL21 within the LN was assessed following the various treatments (Fig. 1C). Anti-ER-TR7 increased the percentage of the LN staining positive for CCL21 (5.09% tolerant vs. 7.54% tolerant + anti-ER-TR7). However, there was a decrease in the percentage of the total CCL21 detected within the LN that was associated with the HEV (59.81% tolerant vs. 49.76% tolerant + anti-ER-TR7) and a significant increase in the percentage of total CCL21 detected within the LN that was associated with the CR (77.53% tolerant vs. 92.02% tolerant + anti-ER-TR7). Taken together, these data suggested that targeting ER-TR7 led to changes both in the LN physical structure and the chemokine gradient associated with the stromal networks.

Anti-ER-TR7 alters alloreactive and Treg cell migration to CR associated with tolerance induction

LN structure and chemokine gradients affect T cell migration into and through the LN (12, 13, 24), and CD4+ T cell and Treg location changes in the LN following tolerance induction (3). Since anti-ER-TR7 altered stromal fiber structure and the chemokine gradient, we hypothesized these changes would affect the migration of naïve alloreactive T cells and Treg through the HEV. To further investigate this we used local delivery of antibody to the LN by injecting anti-ER-TR7 into the footpad one day prior to the induction of tolerance (1 µg/footpad). Pilot experiments demonstrated that this small quantity of antibody was rapidly transported to and bound in the draining LN chain. Bound antibody was detected via immunohistochemistry in the draining popliteal, inguinal, and paraaortic LN; and local delivery of mAb resulted in the same changes in LN remodeling as observed above with systemic administration of anti-ER-TR7. These changes were not apparent systemically, and non-draining LN were similar to those of tolerant mice (data not shown). Previously, we (4, 23) and others (25–27) have used adoptive transfer of TEa T cell receptor transgenic CD4 T cells that recognize donor I-E^d presented by recipient I-A^b (28) to visualize antigen-reactive T cell responses. CFSE-labeled TEa T cell receptor transgenic CD4 T cells were administered i.v. to C57BL/6 mice at the time of tolerance or immunity induction. Four

hours after cell transfer, total numbers of TEa cells located within the HEV were studied by confocal microscopy. Microdomains within the HEV were defined as: a) intraendothelial; b) between endothelium and basement membrane; c) intrabasement membrane; d) touching the abluminal surface of the HEV; and e) in the CR (Fig. 2A, (23, 29)). TEa T cells were identified in the intraendothelial spaces of the HEV and in the CR in naïve and immune LN, while little association with other microdomains was observed (Fig. 2B). In the HEV of tolerant LN, TEa cells were located in the intraendothelial space, between the endothelium and basement membrane, within the basement membrane, and abluminally adjacent to the HEV. LN of tolerant + anti-ER-TR7 treated mice had fewer TEa cells entering the HEV compared to naïve, immune, and tolerant mice. A significant reduction in the number of cells located within or adjacent to the HEV of tolerant + anti-ER-TR7 treated mice was observed in every microdomain as compared to tolerant LN. Gross differences in the location of TEa cells in naïve, immune, and tolerant mice persisted for at least 3 days, suggesting these alterations were long-lasting (data not shown). Overall, anti-ER-TR7 significantly inhibited the entry of antigen specific CD4 T cells into the LN of tolerant treated mice.

We next assessed the impact of anti-ER-TR7 on the location of Treg within and surrounding the HEV (Fig. 2C and D). In naïve mice, and 24h following the induction of immunity, few Treg were located between the endothelium and basement membrane or adjacent to the abluminal surface of the HEV. In contrast, tolerance induction resulted in an increase of Treg in both locations. Conversely, tolerant + anti-ER-TR7 LN had fewer Treg associated with the HEV, both in total number and in each microdomain. Taken together, these findings showed that anti-ER-TR7 inhibited the association of both naïve alloreactive CD4+ T cells and Treg with HEV.

Anti-ER-TR7 inhibits induction of tolerance

Treatment of tolerant mice with anti-ER-TR7 was sufficient to alter LN and HEV remodeling, and the location of naïve and Treg cells within and surrounding the HEV. We next assessed the immunological impact of these modifications on allograft survival. Mice were tolerized and treated with anti-ER-TR7 at various times relative to transplantation. Graft function was monitored, and mice were euthanized on the day of transplant rejection or at d25 (Fig. 3A). Independent of the timing or dosage of antibody, similar impacts on graft survival were observed. Forty percent of the tolerant + anti-ER-TR7 recipients rejected their grafts by d25, and grafts from anti-ER-TR7 treated recipients had abnormal histology with increased inflammation evidenced by vascular occlusion, myocyte death, and mononuclear cell infiltration (Fig. 3B and C). For comparison, allograft survival and histology were assessed in unmodified and tolerant recipients utilized in a concurrent study (23), and tolerant recipients were treated with a control isotype rat IgG2a (rIgG2a) antibody (Fig. 3). These data demonstrated that anti-ER-TR7 prevented the induction of tolerance.

Discussion

Although it was first described almost 30 years ago (20), and it is widely used as marker of LN structure and morphogenesis, the binding target and function of the epitope recognized

by anti-ER-TR7 remains unknown. These experiments sought to address the hypothesis that the proper structure and function of ER-TR7 is necessary to induce allograft tolerance by treating tolerant mice with anti-ER-TR7 mAb.

Appropriate LN structure, and T cell migration within this structure, are integral for the induction of allograft tolerance. This network is, in part, comprised of the stromal fiber ER-TR7 secreted by FRC (19) and the disruption of this network results in insufficient or improper immune responses to immune challenge (12, 30–32). FRC build, remodel, and maintain the fiber network that gives the LN shape, defines microdomains within the LN, and provides a platform and directional cues via cytokines and chemokines for lymphocyte migration (19, 33, 34). FRC and ER-TR7 also surround the HEV and serve as an early site of contact for T cells entering the LN (13).

Both LN and HEV undergo structural changes following immune challenge, and these changes are integral to pathogen clearance and proper immune function (12, 30). The finding that T cells isolated from the LN of naïve, immune, tolerant, and tolerant + anti-ER-TR7 treated mice had equal responses to *ex vivo* alloantigen re-stimulation (data not shown) reflects the importance of the physical environment in which these cells were primed and resided as determinants of immune competence. Remodeling of the stromal fiber network and the chemokine gradient during tolerization allowed for the precise interactions of alloantigen specific cells, pDC and Treg to mediate tolerance. Treatment of tolerant mice with anti-ER-TR7 led to alterations in ER-TR7+ fiber complexity around the HEV and a re-distribution of CCL21 away from the HEV and accumulation within the CR. These structural and gradient changes were associated with re-distribution of naïve alloantigen specific cells and Treg away from the HEV. These changes in T cell trafficking through the HEV may have been due to the decreased complexity of branching, which may have indicated a disrupted stromal network or fewer “exit ramps” through which cells could exit the HEV and enter the LN (13). The disrupted CCL21 chemokine gradient may also have altered the microdomains to which newly emigrated cells homed (24). The interruption of the stromal network, chemokine gradient, and naïve and Treg cell trafficking resulted in altered immune responses and induced graft inflammation and infiltration instead of tolerance.

These findings demonstrate a previously unappreciated, active functional role for ER-TR7 in the shaping of immune responses. Although the precise target and function of the ER-TR7 epitope remains unknown, the present findings suggest that ER-TR7 itself has an important functional role in defining lymphocyte movements required for tolerance.

Materials and Methods

Mice

C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were purchased from Jackson Laboratory (Bar Harbor, ME) at 8–12 weeks of age. T cell transgenic mice expressing the TEa TCR (recognizes I-A^d peptide in the context of I-A^b) were from A.Y. Rudensky (Memorial Sloan Kettering Cancer Center, New York, NY (28)). Animals were housed under specific

pathogen-free conditions. All procedures were performed in accordance with protocols approved by the Institutional Animal Care and Utilization Committee.

Tolerance induction and anti-ER-TR7 treatment

Lymphocytes were isolated from BALB/c spleens for DST. Mice were tolerized i.v. at d-7 relative to transplant with 10^7 DST and anti-CD40L mAb (MR1; 0.25 mg; BioXCell, West Lebanon, NH). Anti-CD40L was also administered d-4, 0 and +4 (4, 35–40). Immune mice received 10^7 DST only. Tolerized mice were treated with anti-ER-TR7 mAb (ER-TR7; AbD Sertotec, Raleigh, NC), 50 – 200 μ g i.v. d-6 or +1 relative to transplant, or 1 μ g via footpad injection at d-8. Control mice were tolerized and treated with rat IgG2a (clone A23, BioXCell, Lebanon, NH), 200 μ g i.v. d-6 or +1 relative to transplant.

Cell preparations and T cell adoptive transfer

Lymphocytes were isolated from TEa Tg mice LN and spleen. CD4⁺ T cells were isolated and labeled with CFSE per manufacturer's recommendation (EasySepTM Mouse CD4⁺ T cell Enrichment kit, StemCell Technologies, Vancouver, BC, Canada, and Life Technologies, Carlsbad, CA). Typically, purity of ~85–99% was confirmed by flow cytometry. Mice were given 2×10^7 cells i.v. concurrent with DST. Naïve mice received CFSE labeled TEa Tg cells only without further treatment.

Cardiac allograft procedure

C57BL/6 mice received heterotopic cardiac allografts from sex-matched, donor BALB/c mice as described (41). Graft function was monitored daily by abdominal palpitation. At either the time of rejection or d25 post-transplant, mice were euthanized and the donor heart excised and prepared for H&E staining. Parenchymal rejection (PR) scoring was based on a modified protocol (42).

Immunohistochemistry

LN were excised and frozen in OCT (Sacura Finetek, CA, USA). LN cryosections were cut in triplicate at 5 μ m using a Microm HM 550 cryostat (ThermoFisher Scientific, Waltham, MA). Sections were fixed with cold acetone and washed in PBS (Lonza Walkersville, MD, USA). Primary rat anti-mouse ER-TR7 (ER-TR7, isotype IgG2a; AbD Serotec), rat anti-mouse PNA^d (MECA-79, isotype IgM; BD Pharmingen, San Diego, CA), goat anti-mouse CCL21 (polyclonal, R&D Systems, Minneapolis, MN), and rabbit anti-mouse FoxP3 (polyclonal, rabbit IgG; abcam® Cambridge, MA) antibodies were diluted 1:50 - 1:400 in PBS, added to slides and incubated 1 hour. Sections were washed with PBS and secondary antibodies diluted 1:50 – 1:400 in PBS added for 30 minutes. Slides were washed in PBS and imaged with a fluorescent microscope. Slides for confocal microscopy were fixed with 4% paraformaldehyde and 1% glycerol in PBS. Images were analyzed visually and with Volocity image analysis software (PerkinElmer, Waltham, MA). Percent of LN staining positive for ER-TR7 and CCL21 was calculated by dividing the total area staining positive for this protein by the total LN area, quantified by Volocity software measuring both positive staining within the tissue and tissue autofluorescence. 1000x and 630x images were used for TEa enumeration; image fields of equal size were used for enumeration (Fig. 2).

Immunohistochemistry stains have been controlled with secondary antibodies with and without isotype control primary antibodies.

Statistical analysis

Data were graphed and analyzed with GraphPad Prism version 4.0c or 6 software. Student's unpaired 2 tailed *t* tests or Log-rank (Mantel-Cox) test were used, as appropriate.

Abbreviations

APC	antigen presenting cells
BEC	blood endothelial cells
CR	cortical ridge
DC	dendritic cell
DST	donor specific transfusion
ER-TR7	Erasmus University Rotterdam - thymic reticulum antibody 7
FRC	fibroblastic reticular cells
HEV	high endothelial venules
iTreg	induced regulatory T cells
LN	lymph node
pDC	plasmacytoid DC
Treg	regulatory T cells

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Figure 1.

Anti-ER-TR7 affects LN remodeling associated with the induction of tolerance. LN from naïve, immune (+ DST), tolerant (+ DST + anti-CD40L), and tolerant + anti-ER-TR7 treated C57BL/6 mice 24h after DST +/- anti-CD40L treatment. (A) Representative images (left panels, 100x), and the percent of LN staining ER-TR7+ (right panel). (B) LN analyzed for ER-TR7+ branching from the abluminal surface of the HEV basement membrane. Representative images (left panels, 630x) and enumeration per HEV (right panel). (C) LN analyzed for presence and distribution of CCL21 within the total LN (%LN CCL21+), and the percentage of total CCL21 detected within the LN associated with HEV (%CCL21 with HEV) and CR (%CCL21 with CR). Representative images (left panels, 100x) and quantification (right panel). Data represented as the mean $\pm$ SEM. $n = 3-5$ mice/treatment; 1-4 LN/mouse; 1-4 sections/LN; * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

Figure 2.

Anti-ER-TR7 inhibits alloreactive cell location within HEV. CFSE labeled CD4⁺ TEa cells transferred to naïve, immune, tolerant, and tolerant + anti-ER-TR7 mice. Recipients euthanized 4h after cell transfer, and LN analyzed for ER-TR7⁺ fibers and PNAd⁺ HEV. Cell location defined as intraendothelial (surrounded by PNAd), within endothelium/basement membrane (surrounded by PNAd+ER-TR7), within basement membrane (surrounded by ER-TR7), touching the abluminal HEV surface, and within cortical ridge (not touching HEV). Representative images, 630x (A) and quantification (B). (C-D) Foxp3⁺ Treg cells in naïve, immune, tolerant, and tolerant + anti-ER-TR7 LN. Representative images, 630x (C) and quantification (D). Arrows denote cells in specific locations. Data are represented as the mean $\pm$ SEM. n = 3–5 mice/treatment; 1–4 LN/mouse; 2–36 HEV/LN; * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

Figure 3.

Anti-ER-TR7 inhibits tolerance induction. Tolerant mice received one dose of anti-ER-TR7 as follows: 100 μ g d-6 (n=1), 200 μ g d-6 (n=2), 50 μ g d+1 (n=2), 100 μ g d+1 (n=3), or 200 μ g d+1 (n=2), relative to transplantation. An additional cohort of tolerant mice received one dose of rat IgG2a (rIgG2a) as follows: 200 μ g d-6 (n=2), 200 μ g d+1 (n=2), relative to transplant. Recipients received BALB/c heterotopic cardiac allografts on d0. Graft function monitored and recipients euthanized at time of transplant rejection or d25, and graft survival recorded (A). Grafts analyzed by H&E staining and PR score determined (B). (C) Black arrow indicates area of monocytic infiltrate, blue arrow indicates graft vascular occlusion in representative H&E staining images (600x, C). Data represented as percent survival (A) or mean $\pm$ SEM (B). n = 4–10 mice/group. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.