Cutting Edge: In Vivo Trogocytosis as a Mechanism of Double Negative Regulatory T Cell-Mediated Antigen-Specific Suppression

Megan S. Ford McIntyre, Kevin J. Young, Julia Gao, Betty Joe and Li Zhang

J Immunol 2008; 181:2271-2275; doi: 10.4049/jimmunol.181.4.2271
http://www.jimmunol.org/content/181/4/2271

References
This article cites 27 articles, 17 of which you can access for free at:
http://www.jimmunol.org/content/181/4/2271.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Cutting Edge: In Vivo Trogocytosis as a Mechanism of Double Negative Regulatory T Cell-Mediated Antigen-Specific Suppression

Megan S. Ford McIntyre, Kevin J. Young, Julia Gao, Betty Joe, and Li Zhang

Recent data have demonstrated that treatment with αβ-TCR+CD3+CD4−CD8−NK1.1− double negative (DN) regulatory T cells (Tregs) inhibits autoimmune diabetes and enhances allotransplant and xenotransplant survival in an Ag-specific fashion. However, the mechanisms whereby DN Tregs suppress Ag-specific immune responses remain largely unknown. In this study, we demonstrate that murine DN Tregs acquire alloantigen in vivo via trogocytosis and express it on their cell surface. Trogocytosis requires specific interaction of MHC-peptide on APCs and Ag-specific TCR on DN Tregs, as blocking this interaction prevents DN Treg-mediated trogocytosis. Acquisition of alloantigen by DN Tregs was required for their ability to kill syngeneic CD8+ T cells. Importantly, DN Tregs that had acquired alloantigen were cytotoxic toward Ag-specific, but not Ag-nonspecific, syngeneic CD8+ T cells. These data provide new insight into how Tregs mediate Ag-specific T cell suppression and may enhance our ability to use DN Tregs as a therapy for transplant rejection and autoimmune diseases. The Journal of Immunology, 2008, 181: 2271–2275.

Regulatory T cells (Tregs) play an important role in controlling the development of various immune pathologies, including transplant rejection and autoimmune disease development (1, 2). Many subsets of Tregs have been identified, including CD4+CD25+ Treg1 cells, Th3 cells, CD8+ T cells, γδ-TCR+ cells, NK T cells, and NK-αβ-TCR+CD4−CD8− double negative (DN) Tregs (1–5). Both murine and human DN Tregs have been shown to suppress allogeneic immune responses in an Ag-specific fashion (6–8). DN Tregs have been demonstrated to enhance donor skin, islet, and heart graft survival (6, 8, 9) and play a role in preventing graft-vs-host disease (10, 11). DN Treg-mediated suppression requires cell-cell contact and occurs via direct cytotoxicity toward T cells (6–9). However, much remains unknown regarding the mechanisms whereby DN Tregs can interact with and kill Ag-specific syngeneic CD8+ T cells.

Studies originating in the early 1970s have shown that many lymphocytes, including T cells, are able to acquire foreign proteins, including membrane-bound proteins, from APCs (12, 13). Membrane acquisition, recently termed trogocytosis, can occur when T or B cells contact Ag-expressing cells in vitro (13–19). T cells have been shown to acquire MHC class I and II, CD80, CD86, and ICAM-1 via trogocytosis from APCs (14–17). Acquisition occurs in a rapid fashion and, depending on the cell type and the activation status, may require cell-cell contact (20, 21) or interaction with membrane exosomes that are shed from the APC (22). The mechanisms leading to trogocytosis are largely unknown, and the functional consequences of trogocytosis remain controversial. No previous experiments have investigated whether acquisition of alloantigens by a Treg population can augment its ability to suppress syngeneic immune responses in an Ag-specific fashion.

In this study, we investigated the Ag specificity of trogocytosis mediated by DN Tregs and whether trogocytosis enables DN Tregs to recognize and suppress T cell responses in an Ag-specific fashion. We demonstrate that DN Tregs can acquire allo-MHC-peptides in vivo and maintain expression for a longer period of time than CD8+ T cells. DN Tregs acquire alloantigen in a TCR-specific fashion. Importantly, we establish for the first time that alloantigen acquisition by DN Tregs in vivo enables them to induce apoptosis of Ag-specific, but not Ag-nonspecific, syngeneic CD8+ T cells. These findings suggest that alloantigen acquisition allows DN Tregs to specifically recognize target CTLs to inhibit immune responses. Understanding the functions and mechanisms of DN Tregs may move us closer to realizing their potential as an Ag-specific immune therapy.

Materials and Methods

Mice

2C (H-2d, expressing the 1B2+ anti-L3 thymic TCR) breeders on a C57BL/6 background were kindly provided by Dr. D. H. Loh (Nippon Roche 2C TCR transgenic mice). Recipient mice were C57BL/6 (H-2b, expressing the 1B2 anti-L3 thymic TCR) breeders on a C57BL/6 background. All mice were maintained in accordance with the guidelines of the University of Toronto Animal Care Committee.

Received for publication May 16, 2008. Accepted for publication June 11, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the Canadian Institute of Health Research and the Canadian Cancer Society.

2 M.S.F. and K.J.Y. contributed equally to this study.

3 Address correspondence and reprint requests to Dr. Li Zhang, Toronto General Hospital Research Institute, Toronto Medical Discovery Tower 2-807, 101 College Street, Toronto, Ontario, Canada M5G 1L7. E-mail address: li.zhang@uhnres.utoronto.ca

4 Abbreviations used in this paper: Treg, regulatory T cell; 7-AAD, 7-aminoactinomycin D; DN, double negative; MCMV, mouse CMV.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00
Research Center), BALB/c-H-2b,K/Hk/Hkg mice, a BALB/c Ld-loss mutant, (H-2d, Ld0, The Jackson Laboratory) were bred with 2C mice to create 2C2, mice (B2``-TCR, H-2bd,Ld0,1B2) or with C57Bl/6 mice to create B6 × Dm2 (H-2bd, Ld-) mice. CB6F1/J mice (H-2bd), referred to as CBy and CBy-SCID mice, were created by crossing C57Bl/6 (H-2b) with BALB/c (H-2d) mice (Charles River) and B6.CB17-Prkdcscid/JesJ (H-2b) to CBBym. CB6.CB17-Prkdcscid/J (H-2b) mice (The Jackson Laboratory), respectively. All mice were housed in specific pathogen-free conditions at the University Health Network (Toronto, Canada). All experiments have been approved by the University Health Network animal care committee.

Results and Discussion

DN Tregs acquire alloantigen in vivo

Most reports that describe the acquisition of foreign proteins by T cells rely on data from in vitro studies. In this study we investigated the ability of DN Tregs to acquire MHC class I alloantigen in vivo. 2C2F1 (transgenic anti-Ld-TCR+, Dbd+/-, Kbd+/-, Ld-) mice were infused with splenocytes from MHC class I alloantigen-expressing CBy (Dbd+/-, Kbd+/-, Ld+) mice. Groups of mice were sacrificed at various time points and the spleen cells were stained to analyze their ability to acquire Ld alloantigen. As shown in Fig. 1a, 1 day following infusion a similar proportion of DN Tregs and CD8+ T cells acquired Ld. However, the expression of Ld on CD8+ T cells diminished after 2 days and was at a very low level after 4 days (Fig. 1a). In contrast, DN Tregs retained higher levels of Ld alloantigen throughout the course of the experiment (Fig. 1a). These data demonstrate for the first time that DN Tregs can acquire MHC class I-Ld alloantigen in vivo and retain expression of alloantigen for at least 7 days. The ability of DN Tregs to express acquired membrane fragments for an extended period of time may allow the small number of DN Tregs to interact with and kill many effector T cells during an immune response.

Although DN Tregs were shown to acquire alloantigen in vivo, the percentage of cells that acquired Ld was very low. This may be due to either a limited overall alloantigen exposure in this model or because only a small subset of DN Tregs is able to acquire alloantigen as a result of allogeneic lymphocyte infusion (Fig. 1a). These data demonstrate that a high percentage of the overall DN Treg population is able to acquire alloantigens in vivo when unlimited Ag is present. Furthermore, alloantigen expression was higher on the surface of Ld-specific TCR-expressing (B2+T) DN Tregs when compared with DN T cells that do not express the Ld-specific TCR.
were repeated three times. MFI, Mean fluorescence intensity.

membrane fragments (PKH26) was assessed over time by using flow cytometry analysis. b, 2CF1 DN Treg clones were pretreated with either TCR-specific 1B2 (Fab’2) mAbs (●) or L4d+ tetramers in the presence of either the 2C31-TCR-specific peptide QL9 (●) or the non-specific peptide, MCMV (●). The ability of DN Tregs to acquire membrane fragments (PKH26) was assessed over time using flow cytometry. The results of a and b represent five replicate cultures and were repeated three times. MFI, Mean fluorescence intensity. c, 2CF1, DN Tregs were cocultured with CBy splenocytes in the presence of 20 μM QL9, p2Ca, or P1A peptide or no peptide as a control. Two hours later, the expression of L4d on the surface of 1B2− DN Tregs was assessed. This experiment was repeated three times and representative results are shown. p = 0.005 for the percentage of L4d+ DN plus QL9 vs P1A. d, i-iii, Drosophila Schneider cells that were transfected with GFP-L4d (diffuse green fluorescence) were preincubated for 30 min with 10 μM MCMV peptide (i) or QL9 peptide (ii-iii) and used as APCs. 2CF1 DN Treg clones were stained with 1B2-TCR-FITC mAbs (focal red) following coinubcation with peptide-treated APC at 37°C for 30 min. Cells were then examined by fluorescence confocal microscopy. The images from (i) L4d-GFP and (c) 1B2-PE were overlaid to show the colocalization (ii-iii) yellow) of GFP-L4d fluorescence with the 1B2 TCR on the surface of DN Tregs.

(1B2−) at all time points assessed (Fig. 1, b and c). These data suggest that alloantigen acquisition mediated by DN Tregs may occur in a TCR-alloantigen-specific fashion in vivo.

Trogocytosis of alloantigens requires specific TCR-MHC interaction
To confirm that DN Tregs mediate trogocytosis in an Ag-specific manner, APCs from either H-2d+ or H-2s− mice were labeled with the membrane-specific dye PKH26, coincubated with 1B2+ DN Tregs, and alloantigen acquisition was measured. As shown in Fig. 2a, 1B2+ DN Tregs that were incubated together with H-2d+ but not H-2s+ APCs acquired membrane fragments, suggesting that the acquisition of membrane fragments by DN Tregs requires TCR-specific alloantigen expression by APCs.

To further determine whether TCR-specific interaction with MHC expressed on an APC is required for DN Treg acquisition we used MHC tetramers and mAbs to block the L4d-specific TCR on the DN Tregs. L4d tetramers loaded with QL9, but not those loaded with MCMV peptides, can interact specifically with the 2C31 1B2+ TCR. 1B2+ DN Tregs were preincubated with either 1B2 Fab’ or L4d tetramers that were loaded with QL9 or MCMV peptides. As shown in Fig. 2b, blocking either the TCR on DN Tregs with 1B2 Fab’ or L4d-QL9 tetramers abrogated the acquisition of membrane fragments from H-2d+ APCs, whereas preincubation with L4d MCMV tetratramers could not block DN Tregs from acquiring L4d peptides from APC. These data further demonstrate the importance of the TCR on DN Tregs for the acquisition of MHC-peptide complexes from APCs.

We next determined whether the affinity of the peptide-MHC complex for the TCR on DN Tregs would affect the acquisition of alloantigen. Primary 1B2− DN Tregs were cocultured with CBy L4d+ APC in the presence of peptides that bind to L4d molecules but have differing affinities for the anti-L4d TCR. Two hours later, the percentage of DN Tregs that had acquired L4d molecules was assessed by flow cytometry. As some endogenous peptide-MHC complexes can bind to the anti-L4d TCR (23), DN Tregs acquire L4d when cocultured with L4d+ APCs in the absence of additional peptides. Addition of P1A, which has no detectable affinity to the anti-L4d TCR (23), reduced the acquisition of L4d (Fig. 2c), suggesting that this peptide prevents the acquisition of endogenous peptide-MHC by DN Tregs. Importantly, addition of either the high affinity peptide QL9 (binding affinity: $K_D = 2 \times 10^{-7} \text{M}^{-1}$) or the medium affinity peptide p2Ca ($K_D = 2 \times 10^{-6} \text{M}^{-1}$) (23), resulted in significantly higher levels of L4d acquisition when compared with those cocultured in the presence of P1A or without additional peptides (Fig. 2c). These data suggest that DN Tregs have an increased ability to acquire L4d peptide complexes that bind with high affinity to their TCR.

One limitation of using flow cytometry to assess alloantigen acquisition is the inability to differentiate whether it is the T cell that is expressing the APC-associated molecules or the alloergic APC that is expressing the T cell-associated markers. Moreover, whether DN Tregs directly interact with APCs to acquire alloantigens is not known. To overcome this limitation, we used confocal microscopy to directly visualize the acquisition of alloantigens mediated by 1B2+ DN Tregs. 1B2+ DN Treg clones were cocultured with APCs that had been transfected with the fluorescent marker GFP linked to MHC class I-L4d (15) in the presence of MCMV (Fig. 2dii) or QL9 (Fig. 2di). Cells were then stained with the anti-L4d TCR-specific mAb 1B2-PE and visualized by fluorescence confocal microscopy. After 30 min, APCs could be seen interacting directly with DN Tregs (Fig. 2di and dii). However, only DN Tregs that had been cocultured with QL9 (Fig. 2diii), but not with MCMV (Fig. 2di), expressed GFP on their cell surfaces. Furthermore, when the two fluorescent channels for green (Fig. 2div) and red (Fig. 2dv) are overlaid, the 1B2+ TCR on DN Tregs is seen to colocalize with GFP-L4d molecules (Fig. 2dvi). These results further confirmed that it is the DN Treg that acquires alloantigens as opposed to the APCs acquiring TCR molecules. Collectively, our data demonstrate that acquisition of MHC-peptide complexes is mediated in an Ag-specific fashion through the interactions between the TCR on DN Tregs and specific peptide-MHC on APCs.

DN Treg trogocytosis of alloantigen is necessary for their ability to induce apoptosis in an Ag-specific fashion
Previous work has shown that human DN Tregs that had acquired alloantigen-peptide complexes in vitro are able to induce
apoptosis of alloimmune target cells in an Ag-specific fashion (7). In this study we determined whether in vivo acquisition and expression of alloantigen is critical for DN Tregs to kill activated syngeneic CD8\(^+\) T cells. 2C\(_{E3}\) DN Tregs that had been i.v. injected into allogeneic L\(^d\)-high Cby-SCID mice and had acquired and expressed L\(^d\) on their surfaces were sorted purified into either L\(^d\)-high- or L\(^d\)-low-expressing populations and used as putative effector cells to kill syngeneic CD8\(^+\) T cells. As shown in Fig. 3a, the L\(^d\)-high DN Tregs, but not the L\(^d\)-low DN T cells, were able to induce CD8\(^+\) T cell apoptosis in a dose-dependent manner. These data demonstrate that only those DN Tregs that are able to acquire L\(^d\) in vivo and express the acquired alloantigens on their surfaces at a high level can kill syngeneic CD8\(^+\) T cells. Furthermore, it suggests that acquisition and expression of alloantigen by DN Tregs is required for their suppressive function, perhaps by facilitating Ag-specific target cell recognition.

We next wanted to investigate whether DN Tregs that have acquired alloantigen can induce apoptosis of target CD8\(^+\) T cells in an Ag-specific fashion. To do this, 2C\(_{E3}\) DN Tregs that had acquired L\(^d\) alloantigen in vivo were cocultured with syngeneic CD8\(^+\) T cell targets that had been activated by either the same alloantigen that DN Tregs had acquired (L\(^d\)) or a third party alloantigen (H-2\(^k\)) and the expression of annexin V (apoptosis marker) and 7-AAD (cell viability marker) were assessed. As shown in Fig. 3b and c, annexin V and 7-AAD increased in a dose-dependent fashion on CD8\(^+\) T cells that had been activated with L\(^d\) alloantigen before coculture with DN Tregs that had acquired L\(^d\) in vivo. However, CD8\(^+\) T cells that had been activated with H-2\(^k\) alloantigen did not increase the expression of annexin V or 7-AAD when cocultured with DN Tregs that had acquired L\(^d\) alloantigen. This suggests that DN Tregs that have trogocytosed alloantigen are only able to induce apoptosis of syngeneic CD8\(^+\) T cells that had been activated by the same alloantigen that has been acquired by the DN Treg and not those activated by a third party alloantigen. These data demonstrate for the first time that trogocytosis of alloantigen by peripheral DN Tregs is required for their ability to kill syngeneic CD8\(^+\) T cells and that DN Tregs that have acquired alloantigen induce cytotoxicity in an Ag-specific fashion. DN Tregs have been shown to increase in tolerant allografts and xenografts and to suppress allograft and xenograft rejection by suppressing CD4\(^+\) T cells (9, 24, 25). Further studies are required to determine whether trogocytosis occurs in transplanted organs and tissues and whether it plays a role in the ability of DN Tregs to suppress CD4\(^+\) T cell proliferation and survival.

Previous studies have shown that acquisition of Ag by CD8\(^+\) T cells sensitizes them to apoptosis by neighboring activated CD8\(^+\) T cells (6, 26). Tsang et al. show that CD4\(^+\) T cells that had acquired alloantigen were able to induce either the proliferation of naive T cells or the apoptosis of activated T cells (26). Interestingly, a recent study suggests that the acquisition of HLA-G by human CD4\(^+\) and CD8\(^+\) T cells confers suppressive function (21). However, it is not yet known whether FoxP3\(^+\) CD4\(^+\) Tregs acquire alloantigens as a mechanism of suppression of CD4\(^+\) T cell proliferation. In our study we demonstrated that Ag-specific acquisition in vivo and the expression of alloantigen by DN Tregs allows them to specifically trap and kill syngeneic CD8\(^+\) T cells that can interact with the acquired alloantigen on the DN Tregs. These studies suggest that trogocytosis of Ag allows cells to either prime or inhibit immune responses, perhaps depending on the type of cell that has acquired the alloantigen as well as the types of Ag acquired and/or the type of target cell.

Taken together, our data presented here suggest the following model to explain how DN Tregs are able to specifically suppress syngeneic CD8\(^+\) T cells that carry the same TCR specificity. DN Tregs acquire foreign MHC-peptides from APC in a TCR-specific fashion and express them on their cell surfaces. CD8\(^+\) T cells that have the same TCR specificity as DN Tregs will recognize the molecules that have been acquired and expressed on the DN Treg surface. This Ag-specific recognition will bring DN Tregs into close contact with the CD8\(^+\) T cells and lead to the death of Ag-specific CD8\(^+\) T cells. The prolonged expression of acquired MHC molecules by DN Tregs could provide a window of opportunity for the low numbers of DN Tregs to kill many Ag-specific CD8\(^+\) T cells, making them highly potent Ag-specific Tregs. These findings unravel some of the critical mechanism whereby DN Tregs can suppress graft rejection in an Ag-specific fashion (6, 8, 9) and may facilitate
the use of DN Tregs as an Ag-specific immune therapy for graft rejection, autoimmune type 1 diabetes, and graft-vs-host disease (10, 11, 27).

Acknowledgments

We thank Dr. Zeling Cai for generously providing the Drosophila cell line, Dr. Mark Peterson for assistance with confocal microscopy, and Joyce Pun and Michelle Tsang for cell sorting.

Disclosures

The authors have no financial conflict of interest.

References