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Research Letter In-Press Preview Immunology Transplantation

Conversion of CD73^{hi}FR4^{hi} anergic T cells to IFN-γ-producing effector cells disrupts established immune tolerance

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Anergic T cells (T_{AN}) marked by $CD73^{hi}FR4^{hi}$ have been shown to differentiate to immunosuppressive populations such as $FoxP3^+$ T_{REG} or IL-10-producing Tr1 cells (1, 2), and are therefore deemed harmless to stable immune tolerance. However, their potential to differentiate to pathological IFN-γ-producing effector cells has not been studied. We developed an allogeneic transplant tolerance model to investigate this possibility.

We used a BALB/c to C57BL/6(B6) islet transplant model. Donor-specific transplant tolerance was induced in recipients by infusing on days -7 and +1 donor splenocytes(SP) treated *ex vivo* with ethylenecarbodiimide(ECDI)(3), and is indefinitely maintained in unmanipulated recipients as described(4). We first examined the presence of $CD73^{hi}FR4^{hi}$ T_{AN} in tolerized recipients. As shown in Figure 1A, the majority of intragraft $CD44^+FoxP3^-$ CD4 T cells were $CD73^{hi}FR4^{hi}$ T_{AN}. These cells were also present in the spleen of tolerized recipients, albeit less prominently than in allografts. In contrast, there was a significantly smaller T_{AN} population in the spleen of non-tolerized mice.

We next perturbed this stable tolerance on day 95 post-transplantation by acute murine cytomegalovirus (MCMV) infection previously shown to precipitate allograft rejection in ~60-70% recipients over the ensuing 5-6 weeks(5). Interestingly, at the time of rejection, we observed a significant reduction of the number of intragraft $CD73^{hi}FR4^{hi}$ T_{AN} cells, along with a significantly reduced level of CD73 and FR4 expression on remaining T_{AN} cells (Figure 1B). We confirmed that intragraft $FoxP3^+$ T_{REG} cells, known to similarly express CD73 and FR4, continued to exhibit the same level of CD73 and FR4 pre- and post-MCMV infection (Supplemental Figure 1).

Next, we determined the fate of $CD73^{hi}FR4^{hi}$ T_{AN} cells in response to MCMV infection. *In vitro*, we FACS-sorted T_{AN} cells ($CD3^+CD4^+CD44^+CD25^-CD73^{hi}FR4^{hi}$) from

the spleen of tolerized B6 mice (verified to be indeed anergic (Supplemental Figure 2)). We cultured them for 5 days with B6 bone marrow-derived dendritic cells (BMDCs) with/without MCMV pre-treatment and with/without pulse with BALB/c cell lysate. As shown in Figure 1C, T_{AN} cells co-cultured with MCMV-infected DCs with/without pulse with BALB/c lysates showed a significant down-regulation of CD73 and FR4, acquired cell surface CD25 expression (data not shown) and began to prominently express the Th1 cytokine IFN-γ among the proliferating (Ki-67$^+$) subset. Interestingly, LPS-treated DCs, in contrast to MCMV-treated DCs, did not lead to any down-regulation of CD73 or FR4 on T_{AN} cells (Supplemental Figure 3). *In vivo*, we similarly FACS-sorted T_{AN} cells for CD45.2 mice and transferred them to CD45.1 mice, followed by MCMV infection a day later. As shown in Figure 1D, 1 week after MCMV infection, a substantial portion of the CD45.2$^+$ T_{AN} cells became CD73$^-$FR4$^-$ and began to produce IFN-γ; in contrast, without MCMV infection, the CD45.2$^+$ T_{AN} cells remained CD73^{hi}FR4^{hi}. Collectively, these data support that MCMV infection reverts the anergic phenotype of T_{AN} cells, likely via DCs, and promote their differentiation to IFN-γ-producing cells.

Lastly, we determined the functional significance of T_{AN} cells in MCMV-mediated disruption of stable tolerance. First, we depleted T_{AN} cells in stably-tolerized recipients with a course of anti-FR4 (clone TH6) from day 95 to 120 (100 μg i.v. every 5 days x 6 doses), followed by MCMV infection on day 125. Using a different clone of anti-FR4 (12A5), we confirmed that this course of anti-FR4 indeed effectively depleted CD4$^+$FR4$^+$ but not other cells (Supplemental Figure 4). As shown in Figure 1E left panel, none of the recipients treated with anti-FR4 experienced allograft rejection (followed up to ~day 150). Furthermore, this treatment with anti-FR4 prior to MCMV infection completely

prevented MCMV-precipitated rejection in previously tolerized recipients (Figure 1E right panel).

To further corroborate above findings from the anti-FR4 experiment, we adoptively transferred sorted T_{AN} cells to B6.RAG$^{-/-}$ mice bearing BALB/c islets, followed by MCMV infection and infusion of naïve CD8 T cells (experimental scheme is shown in Figure 1F). We first observed that T_{AN} cells converted to CD73^{-}FR4^{-} T cells following MCMV infection (Figure 1G). In addition, mice receiving T_{AN} cells rejected the BALB/c islet allograft following MCMV infection and naïve CD8 T cell infusion; whereas mice not receiving T_{AN} cells did not, despite identical MCMV infection and naïve CD8 T cell infusion subsequently (Figure 1H). These data substantiate that the T_{AN} cells are crucial in driving the rejection in MCMV-infected mice.

Findings in this study refute the notion that CD73^{+}FR4^{+} T_{AN} cells are simply passive cells innocently present during immune tolerance, instead support that when appropriately stimulated, these cells can differentiate to IFN-γ-producing Th1 cells to promote immunity. More importantly, we developed a therapeutic strategy for preserving the stability of tolerance by pre-emptively depleting T_{AN} cells prior to immune perturbation. In our model, the CD73^{+}FR4^{+} cells expressed lower levels of the inhibitory molecules CTLA4 and TIGIT in comparison to FoxP3^{+} T_{REG} cells (data not shown), suggesting cell-intrinsic factors that determine their fate in response to external stimuli. Our data also point to cell-extrinsic factors originating from their interacting partners, specifically DCs, that play a critical role in their differentiation to effector cells. Collectively, our studies underscore the potential detriment of what may seem benign anergic T cells in tolerant

recipients, and support future studies in identifying crucial elements in their instability and therapeutic targets in preventing their differentiation to pro-inflammatory cells.

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Figure

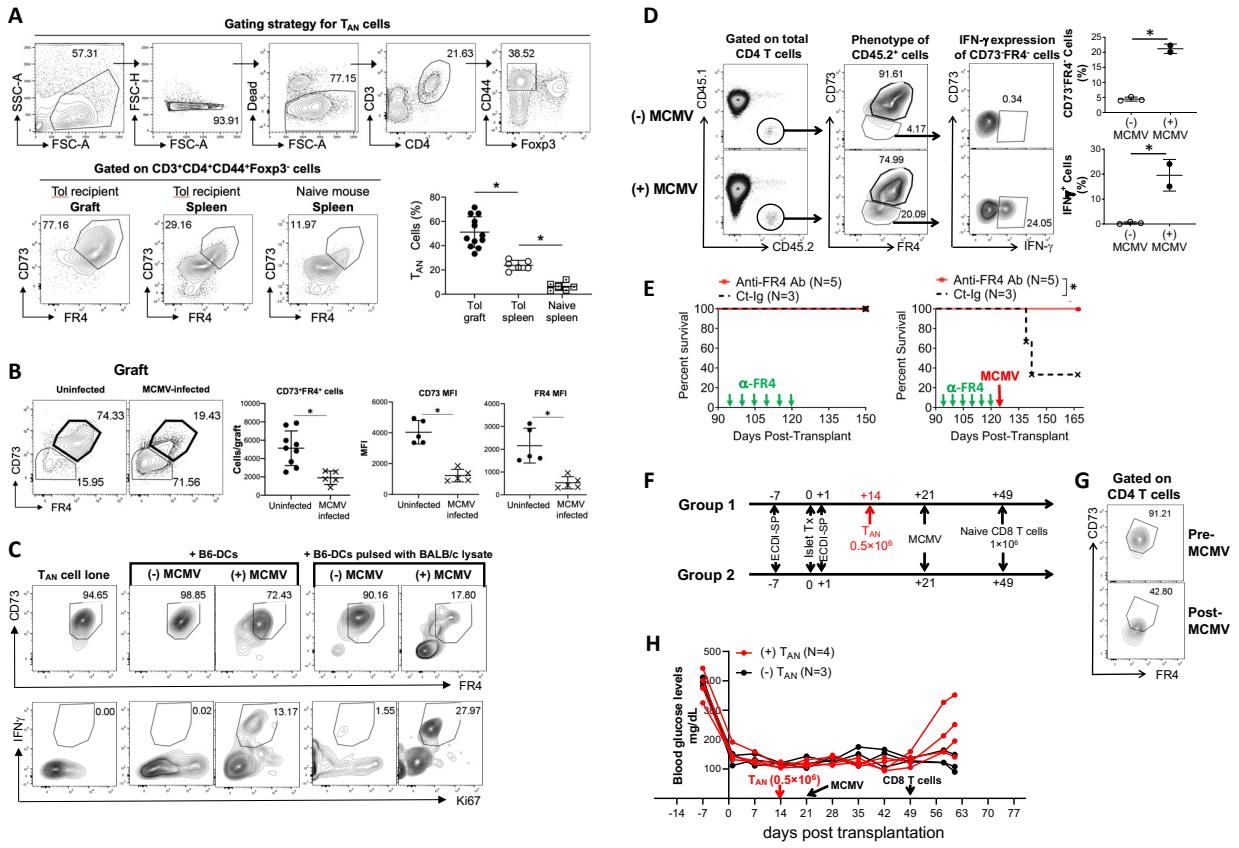

Figure 1. Conversion of CD73^{hi}FR4^{hi} anergic T cells to IFN-γ-producing cells disrupts established transplantation tolerance. **A.** T_{AN} cells are enriched in islet allografts and spleens of tolerized recipients. N=6-12 per group. **P*<0.05. **B.** Loss of T_{AN} cells in islet allografts following MCMV infection of stably tolerized recipients. N=3-9 per group. **P*<0.05. **C.** T_{AN} cells from tolerized recipients are induced to produce IFN-γ by MCMV-infected DCs (representative of 2 independent experiments). **D.** *In vivo* conversion of adoptively transferred CD45.2 T_{AN} cells following MCMV infection of CD45.1 hosts. N=2-3 per group. **P*<0.05. **E.** Depletion of T_{AN} cells prevents MCMV-mediated transplant tolerance disruption. N=3-5 per group. **P*<0.05. **F.** Experimental scheme in RAG$^{-/-}$ mice. **G.** *In vivo* conversion of adoptively transferred T_{AN} cells following

MCMV infection of $RAG^{-/-}$ (representative of N=4). **H.** Rejection of islet allografts in $RAG^{-/-}$ following T_{AN} adoptive transfer. N=3-4 per group.