Real-time immune cell interactions in target tissue during autoimmune-induced damage and graft tolerance

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Real-time imaging studies are reshaping immunological paradigms, but a visual framework is lacking for self-antigen-specific T cells at the effector phase in target tissues. To address this issue, we conducted intravital, longitudinal imaging analyses of cellular behavior in nonlymphoid target tissues to illustrate some key aspects of T cell biology. We used mouse models of T cell–mediated damage and protection of pancreatic islet grafts. Both CD4⁺ and CD8⁺ effector T (Teff) lymphocytes directly engaged target cells. Strikingly, juxtaposed β cells lacking specific antigens were not subject to bystander destruction but grew substantially in days, likely by replication. In target tissue, Foxp3⁺ regulatory T (Treg) cells persistently contacted Teff cells with or without involvement of CD11c⁺ dendritic cells, an observation conciliating with the in vitro “trademark” of Treg function, contact-dependent suppression. This study illustrates tolerance induction by contact-based immune cell interaction in target tissues and highlights potentials of tissue regeneration under antigenic incognito in inflammatory settings.

Tissue damage by self-antigen–specific T lymphocytes causes autoimmune diseases such as type 1 diabetes. In these disorders, defective central tolerance (Mathis and Benoist, 2004) and peripheral regulation (Josefowicz et al., 2012) lead to initiation of autoantigen-specific responses in a cascade of molecular and cellular interactions between antigen-presenting cells and T lymphocytes. During the effector phase, activated CD4⁺ and CD8⁺ Teff cells migrate to target tissues to inflict damage. The immune destruction at this phase can be suppressed by CD4⁺Foxp3⁺ Treg cells (Josefowicz et al., 2012), as demonstrated in models of autoimmune diabetes (Chen et al., 2005; Feuerer et al., 2009). Extensive studies have contributed to the understanding of immune responses at the induction phase in lymphoid organs; however, the behavior of immune cells in nonlymphoid target tissues remains murky.

High-resolution imaging of live cells in lymphoid organs has elucidated key features of cellular dynamics during the initiation phase of immune responses (Germain et al., 2012). A major gap of knowledge remains, however, in understanding immune cell action and interaction in nonlymphoid target tissues, except in some infection models. In particular, noninvasive real-time evidence of how pathogenic immune cells at the effector phase engage target cells, how immune damage is controlled, and how target tissue cells respond remains scanty. This is largely a result of technical limitations that make most target tissues inaccessible to noninvasive visualization at cellular levels. Researchers often have to resort to surgical exposure of tissue or invasive insertion of a probe during imaging. Surgical wounds, however, create a two-pronged limitation on imaging analyses. First, they make...
RESULTS

Noninvasive imaging of T cells in ACE without hindrance by the putative immunoprivilege

To study CD4+ T cell responses in target tissue, we used CD4+ T_{eff} and T_{reg} cells from the NOD.BDC2.5 TCR transgenic mice (Katz et al., 1993), with a specificity against a natural antigen in the pancreatic islet β cells, chromogranin A (Stadinski et al., 2010). ACE offers the technical advantage of noninvasive access and high resolution in vivo imaging, but studies using ACE could be complicated by a status of immune privilege attributed to this compartment of the eye (Benhar et al., 2012). To test whether this impacts on the immune responses of antigen-specific T cells in the islet grafts in ACE, we compared the frequency of immune damage by BDC2.5 CD4+ T_{reg} cells against β cells in the ACE graft and that in the native pancreas. Donor pancreatic islets were injected into ACE through the cornea (Speier et al., 2008a,b; Abdulreda et al., 2011) at least 2 wk before T cell transfer, to ensure stable engraftment of the islets and complete healing of the minor injection wound.

Although previous studies in other settings showed that immune cells in ACE could be impacted by the eye-associated immunoprivilege (Benhar et al., 2012), in our model we found that the β cell–specific CD4+ BDC2.5 T_{reg} cells destroyed the islets in the pancreas and the islet grafts in ACE at a similar tempo. Importantly, the protective T_{reg} cells acted with a similar efficacy (~50%) in controlling T_{eff} cell damage in ACE and in the endogenous pancreas (Fig. 1, A–D). Antigen-specific T_{reg} cells have been shown to be potent suppressors in several autoimmune settings, including models of type 1 diabetes (Tisch and Wang, 2008; Shevach, 2011). The main reason that only 50% of mice were protected in our experiments was likely because of the potency of the antigen-specific T_{eff} cells.
which were purified BDC2.5 CD4+CD25−Foxp3− cells. Nonetheless, these results are consistent with a previous study that found rejection of fully MHC-mismatched islets in ACE occurred similarly to that in a conventional extra-pancreatic implant site, the kidney sub-capsular space (Abdulreda et al., 2011). Therefore, the overall kinetics of immune destruction and protection of engrafted islet tissue in ACE was comparable to that in the native pancreas, and thus the islet grafts in ACE could serve as a surrogate in noninvasive and longitudinal imaging studies of basic T cell biology at the effector phase in the nonlymphoid target tissue.

**Direct contact between antigen-specific CD4+ T eff cells and their target cells**

CD4+ T cells are categorized into several helper and regulatory subsets. Their function as killers has also been shown (Hahn et al., 1995). The in vivo capacity of CD4+ T eff cells killing target β cells was shown in Fig. 1 (A–D). How CD4+ T cells kill remains to be fully examined. Most target tissues do not express MHC class II molecules, which are necessary for antigen-specific, cognate interaction with CD4+ T cells. To study tissue destruction by antigen-specific CD4+ T eff cells, pancreatic islets tagged with cyan fluorescence proteins (CFP; Hara et al., 2006) were grafted in ACE. CD4+Foxp3− BDC2.5 T eff cells marked with green fluorescence proteins (GFP) were injected into the intra-oculargraft-bearing animals. The GFP+CD4+ T eff cells appeared in the islet grafts and engaged in direct contact with their target β cells. We then used Annexin V in situ cyto-labeling to visualize apoptosis of β cells, by injecting allo-phycocyanin (APC)-conjugated Annexin V into ACE. The use of this in vivo assay for β cell apoptosis was described in detail in previous works (Speier et al., 2008a,b). Apoptosis signals were present in either the contact zone between T eff cells and target islet cells, or on the target islet cells with T eff cells in the vicinity but not in direct contact (Fig. 1 E and Video 1). We also examined the involvement of myeloid cells at the inflammatory site, by in situ immunoctolabeling (Abdulreda et al., 2011) with anti-CD11b antibodies. We could not detect CD11b+ cells in most of the areas wherein T eff cells interacted with target β cells. A low frequency of CD11b+ cells were found but usually in the periphery of damaged grafts. Importantly, T reg cells colocalized in the protected clusters of β cells that persisted amid areas of immune damage (Fig. 2, A–C). Overall, the majority of Annexin V signals associated with β cells rather than T eff cells and the amount of the apoptotic signals on β cells positively correlated with the number of T eff cells at the inflammatory site (Fig. 2, D and E). The exact molecular cause of the immunopathology by the CD4+ T eff cells remains unclear. IFN-γ and IL-17A could be detected by flow cytometry in substantial proportions of the CD4+ T eff cells in the draining cervical lymph nodes of the eyes (14 ± 2 and 6 ± 1%, respectively; mean ± SEM; n = 8 mice). However, further studies are needed to determine whether these or other cytokines have a pathogenic role. The imaging data suggest that, although not exclusive, direct contact may be involved in CD4+ T eff cell killing of target cells, even in the absence of CD8+ T eff cells. The contact-dependent mode is a hallmark of cytotoxicity by CD8+ T eff cells.
Direct engagement between CD8⁺ T-effector cells and target cells: bystander β cells were not subject to killing but grew at the interface of immune damage

We next studied noninvasive CD8⁺ T-effector cell activity in target tissue, by using CD8⁺ OT1 transgenic T cells (Hogquist et al., 1994), which are specific toward a model antigen, ovalbumin. Donor islets from both the RIP-mOVA transgenic mice (Kurts et al., 1996), which express ovalbumin in β cells, and the MIP-CFP mice, which lack ovalbumin but have CFP-labeled β cells (Hara et al., 2006), were grafted together in ACE of C57BL/6 (B6) animals. We selected transplanted animals that carried at least one pair of conjoined mOVA⁺CFP⁻ and mOVA⁺CFP⁺ grafts like Siamese twins, and transferred them with antigen-activated CD8⁺GFP⁺ OT1 T-effector cells. This was done to examine antigen-specific killing versus bystander tissue destruction (Tite and Janeway, 1984). The CD8⁺ OT1 T-effector cells selectively destroyed β cells carrying the specific antigen (Fig. 3, A–C).

Strikingly, the bystander RIP-mOVA⁺CFP⁻ islets conjoined to damaged mOVA⁺CFP⁻ islet grafts grew at the interface of immune destruction within days, rather than being subjected to killing (Fig. 3 A). Increase of the islet mass lacking the specific antigen required close juxtaposition with the site of antigen-specific immune responses. RIP-mOVA⁺CFP⁺ graft mass remained constant if it was not immediately adjacent (i.e., isolated) to a graft harboring the specific antigen (Fig. 3, A–C). Importantly, the increased mass of conjoined RIP-mOVA⁺CFP⁺ grafts was not a result of hypertrophy of the β cells, as the nuclear density of the conjoined and isolated islets was unchanged (Fig. S1). Moreover, imaging analyses showed preservation of the three-dimensional (3D) structure of these islets (Videos 2 and 3), precluding the possibility of imaging artifacts associated with islet flattening over time. Because the CFP expression in these islets is driven by the insulin promoter (Hara et al., 2006) and therefore labels differentiated β cells, this direct observation suggests that β cells can regenerate by replication at the site of immune damage, with an extraordinary potential of doubling in days.

Proliferation of bystander β cells in islet grafts under the kidney capsule

To examine β cell replication under inflammatory conditions in a site other than ACE, we performed our experiments using a conventional islet transplantation model. Recapitulating the settings in ACE using islets with or without specific antigens for CD8⁺ OT1 T-effector cells, B6 recipient mice were transplanted under the kidney capsule with islets from either wild-type B6 or RIP-mOVA⁺ transgenic donors, or a mixture of the two. The premixed islets from RIP-mOVA⁺ and wild-type B6 donors, or single group controls, were pelleted by centrifugation before transplantation under kidney capsule. The kidney subcapsular space, although does not allow noninvasive longitudinal analyses of the same graft tissue at a cellular level, enabled the retrieval of a relatively large number of islet grafts for histological analyses.

Figure 3. CD8⁺ T-effector cells damaged antigen-specific target cells but not bystander cells. (A) Longitudinal fluorescence confocal micrographs (z stacks shown as maximum projection) of islet grafts in ACE, acquired noninvasively in vivo. Visualized were infiltration and damage by OVA-specific GFP-labeled CD8⁺OT1 T-effector cells in OVA⁺CFP⁻ islet grafts (gray) versus bystander OVA⁺CFP⁻ islets (blue). Data represent a total of 6 pairs of antigen-specific OVA⁺CFP⁺ and nonspecific OVA⁺CFP⁻ islets conjoined like Siamese twins (left), total 6 separated OVA⁺CFP⁻ islets (right) in the same ACE environment but not conjoined with OVA⁺CFP⁺ grafts, and total 6 separated OVA⁺CFP⁻ islet grafts, from 5 different mice in three experiments. (B) Changes in relative islet mass (volume; mean ± SEM) of OT1 antigen-specific islets (OVA⁺) compared with bystander CFP islets in nonadjacent positions, after CD8⁺ OT1 T-effector cells transfer. (C) Changes in relative mass (volume; mean ± SEM) of bystander islet grafts conjoined with specific islet graft or separated from the specific islet graft in the same ACE. Bars, 100 µm. **, P < 0.01; ***, P < 0.001. See Videos 2 and 3 for 3D islet mass visualization before and after CD8⁺ T-effector cell-mediated damage to the specific OVA⁺ tissue.
After engraftment of islets transplanted under the kidney capsule, the recipient animals were injected with activated CD8+ OT1+ Treg cells, as in the animals bearing ACE grafts (Fig. 3). We then administered BrdU to the animals to label proliferating cells. The CD8+ Treg cells destroyed the RIP-mOVA+ islet grafts. This was confirmed by microscopic examination and insulin-staining of kidney tissue sections at the site of the islet engraftment in animals transplanted with RIP-mOVA+ islet alone (unpublished data). Using tissue sections of the islet grafts from the mice receiving B6 islets alone or a mixture of B6 and RIP-mOVA+ islets, we conducted immunofluorescence staining to detect BrdU incorporation in the nuclei of proliferating B cells. We found that in the group with the mixed engraftment (RIP-mOVA+ and B6 islets) and subsequent destruction of RIP-mOVA+ islets by OT1 CD8+ Treg cells, there was a substantial increase in the proportion of BrdU+ B cells in the remaining B6 islets, compared with the group that was transplanted with B6 islets alone (Fig. 4).

Although it cannot be determined with absolute certainty in the histological analyses whether one particular B6 islet was once in direct contact with one particular RIP-mOVA+ islet after the latter was destroyed, the islets from RIP-mOVA+ and wild-type B6 donors were premixed and pelleted before being implanted in the renal subcapsular space. In addition, we counted 34–36 islets in each group. Thus, increased BrdU staining in the B6 islets in the mixed transplant group, compared with that in the B6 islets alone transplant group, represents at a group level the effect of close physical proximity of the bystander B6 islets with the RIP-mOVA+ islets before the latter was destroyed by OT1 Treg cells. These results, obtained with a platform distinct from the ACE model, corroborated the notion that B cells lacking specific antigens are not subject to bystander killing or damage but replicate in an inflammatory setting, which is consistent with the observations from the imaging analyses of islet grafts in ACE.

**Treg cells interacted with Teff cells through direct cell–cell contact in nonlymphoid target tissue**

Immune effector function at the target tissue is controlled by various mechanisms coordinated by Treg cells (Josefowicz et al., 2012). How exactly Treg cells suppress immune response in vivo is still debated. Initial studies with in vitro transwell culture systems showed that Treg cell suppression was effective only if Treg cells were placed in the same culture chamber with Teff cells and antigen-presenting cells (Takahashi et al., 1998; Thornton and Shevach, 1998). Although subsequent studies showed that Treg cells could inhibit Teff cell activation by modulating antigen-presenting cells (Tadokoro et al., 2006; Onishi et al., 2008; Wing et al., 2008), several in vitro studies also demonstrated that both human and murine Treg cells could directly suppress Teff cells independent of antigen-presenting cells (Ermann et al., 2001; Nakamura et al., 2001; Piccirillo and Shevach, 2001; Baecher-Allan et al., 2006; Hagness et al., 2012; Huang et al., 2012). However, ex vivo and in vivo imaging studies in lymph nodes did not detect stable contact between Treg and Teff cells (Mempel et al., 2006; Tang et al., 2006). This contradiction between in vitro and in vivo studies has left a doubt about the in vivo relevance of contact-dependent Treg suppression. The in vitro trademark activity of Treg cells remains to be reconciled in vivo.

In this study, we examined the pathophysiological relevance of Treg–Teff contact at the effector phase in the target tissue in vivo. We used the NOD.SCID reconstitution model with antigen-specific Treg and Teff cells that are genetically tagged with different fluorescence markers for stable labeling and longitudinal study. Adoptive transfer of T cells to lymphopenic...
animals is followed by homeostatic proliferation and activation of the transferred T cells to fill empty niches in the lymphoid organs (Surh and Sprent, 2008), which could complicate studies of T cell activation and differentiation. However, lymphopenia-associated activation is likely to have minimal impact on our study, as we focused on T cell biology at the final effector phase in the nonlymphoid target tissue, i.e., during the effector phase after activation and differentiation. The lymphopenic reconstitution model is also necessary to avoid undercounting invisible interactions (see Materials and methods) and to generate meaningful measurement of the interactive behavior among T cell subsets.

Indeed, in the T<sub>reg</sub> cell–protected grafts, a majority of T<sub>eff</sub> cells were in direct contact with T<sub>reg</sub> cells (Fig. 5A); they displayed a dynamic and contact-featured choreography. The interaction between the T<sub>reg</sub> and T<sub>eff</sub> cells usually persisted for the entire length of the imaging sessions (≥30 min) and was characterized by reduced motility (Fig. 5, A–E; and Video 4). This direct contact between T<sub>reg</sub> and T<sub>eff</sub> cells was not due to mere crowdedness; in tissue areas that were only sparsely infiltrated, long-lasting contact between T<sub>reg</sub> and T<sub>eff</sub> cells was still evident (Fig. S2).

**Contact interaction between T<sub>reg</sub> and T<sub>eff</sub> cells with or without CD11c<sup>+</sup> DCs**

T<sub>reg</sub> cells can dampen the expression of the co-stimulatory molecules CD80 and CD86 on the surface of DCs, and thus inhibit T cell activation (Shevach, 2008; Wing et al., 2008). Whether the function of T<sub>reg</sub> cells in the target tissue depends on DCs during the effector phase remains unclear. To examine T<sub>reg</sub>–T<sub>eff</sub> interaction in the context of DCs in protected

![Figure 5. Noninvasive intravital visualization of T<sub>reg</sub>–T<sub>eff</sub> cell contact in target tissue.](image-url)

(A) Snapshots from representative time-lapse recordings demonstrating stable, long-lasting interaction between CD4<sup>+</sup> T<sub>reg</sub>–T<sub>eff</sub> interactions within the islet tissue. The majority of interactions lasted for the entire length of the recordings (≥30 min; see Video 4). Absolute (B) and relative (C) interaction time between the CD4<sup>+</sup>BDC2.5 T<sub>eff</sub> and T<sub>reg</sub> cells (n = 50 cell pairs; mean ± SEM). Relative interaction time is calculated by dividing absolute interaction time with total imaging session length. Of note, many of the interactions were already established at the beginning of imaging; hence, actual interaction times are likely longer than those measured during the in vivo imaging timeframe which is limited by the animals’ tolerance of general anesthesia. Data points represent one T<sub>reg</sub>–T<sub>eff</sub> pair and lines represent the mean ± SEM. The interaction index (D) and velocity (E) of both T<sub>reg</sub> and T<sub>eff</sub> cells in a mode of contact interaction or not (n = 55–100 cells; mean ± SEM). Data represent three experiments with 6 mice. ***, P < 0.001. Bar, 30 µm.
target tissues, we injected fluorescence-conjugated anti-CD11c antibodies to visualize DCs, in addition to GFP- and CFP-labeled T<sub>reg</sub> and T<sub>eff</sub> cells, respectively (Fig. 6, A and B). CD11c<sup>+</sup> DCs could be detected in the islet graft, mostly at the periphery (Fig. 6 B). Consequently, the majority of T<sub>reg</sub>–T<sub>eff</sub> cell interactions within the graft occurred in the absence of DCs, and it was also the most abundant among the various types of interactions of T<sub>reg</sub>–T<sub>eff</sub> and/or CD11c<sup>+</sup> DCs. Clusters of the three types of cells, T<sub>reg</sub>–T<sub>eff</sub>–DC, could be detected but at a much lower frequency than that of T<sub>reg</sub>–T<sub>eff</sub> cell interaction without DCs. T<sub>eff</sub>–DC or T<sub>reg</sub>–DC interactions could be found at minor frequencies (Fig. 6 C). The interactions between T<sub>reg</sub>–T<sub>eff</sub> cells were also stable, with or without CD11c<sup>+</sup> DCs (Fig. 6, D and E). The interactions between CD11c<sup>+</sup> cells and T<sub>eff</sub> or T<sub>reg</sub> cells, although occurring in only a minor proportion of the T cells, were also mainly long lasting, with T<sub>reg</sub>–CD11c<sup>+</sup> cells interactions being somewhat less stable (Fig. 6, D and E). Overall, these results show that direct contact-based interactions between T<sub>reg</sub> and T<sub>eff</sub> cells persisted with or without CD11c<sup>+</sup> DCs, which could reflect distinct subsets of T cells or distinct stages of the T cell function in the target tissues. The functional relevance of the different types of interactions has already been documented in vitro (Takahashi et al., 1998; Thornton and Shevach, 1998; Ermann et al., 2001; Nakamura et al., 2001; Piccirillo and Shevach, 2001; Baecher-Allan et al., 2006; Tadokoro et al., 2006; Onishi et al., 2008; Wing et al., 2008; Hagness et al., 2012; Huang et al., 2012). Our noninvasive in vivo imaging studies shows that those direct interactions do exist in vivo in target tissue. Further studies are needed to determine which interactions are most relevant in what settings for which types of functions.

**T<sub>reg</sub> cells persistently interacted with T<sub>eff</sub> cells even when outnumbered by T<sub>eff</sub> cells in damaged target tissues**

Next, we examined the behavior of T<sub>reg</sub> cells in a setting of failed immune regulation. We found that most T<sub>reg</sub> cells at the site of extensive tissue damage were still persistently interacting with T<sub>eff</sub> cells, with durations (interaction time) comparable to those in the protected tissues (Fig. 7, A and B). However, T<sub>reg</sub> cells were largely outnumbered by T<sub>eff</sub> cells; as a result, most T<sub>eff</sub> cells were without T<sub>reg</sub> cell interactions (Fig. 7, C and D). Thus, regardless of success or failure in protecting the target tissue, T<sub>reg</sub> cells persistently interacted with T<sub>eff</sub> cells, but an imbalance in the numbers of T<sub>reg</sub> versus T<sub>eff</sub> cells characterized the outcome, i.e., immune damage versus protection.

The imbalance of T<sub>eff</sub> versus T<sub>reg</sub> cells in the target tissue developed in some animals but not others even though they were injected with the same type of T<sub>reg</sub> and T<sub>eff</sub> cell mixture in the same batch of experiments. We studied the kinetics of the imbalance, taking advantage of our noninvasive platform to image both T<sub>reg</sub> and T<sub>eff</sub> cell populations in the same islet grafts longitudinally. The grafts were analyzed at two time points: days 10–12, when all animals were free of diabetes but had an onset of infiltration of both T<sub>reg</sub> and T<sub>eff</sub> cells (without substantial damage of the grafts); and days 17–20 when some animals suffered from new-onset diabetes (the islet damaged...
T cell behavior was analyzed in the graft tissue that was being destroyed due to failed immune regulation or protected by successful T\textsubscript{reg} cell regulation. Protected islet grafts had at least 80% of original islet mass, whereas damaged islet graft had 20% or less. (A) Representative fluorescence micrographs (3D rendering) showing protected (left) and damaged (right) islet grafts (gray; visualized by laser backscatter) in NOD-SCID mice reconstituted with GFP-labeled CD4\textsuperscript{+} BDC2.5 T\textsubscript{reg} cells and CFP-labeled CD4\textsuperscript{+} BDC2.5 T\textsubscript{eff} cells. (B) T\textsubscript{reg}–T\textsubscript{eff} interaction time in protected versus damaged grafts (mean ± SEM). (C) Ratios of T\textsubscript{reg}–T\textsubscript{eff} cell pairs within protected versus damaged grafts. The T\textsubscript{reg}–T\textsubscript{eff} cell pairs outside of the target graft tissue, in the iris in the same ACE, were analyzed as controls for the target tissue (mean ± SEM). (D) Interaction index (ratio of T\textsubscript{eff} numbers in the graft tissue, in the iris in the same ACE, were analyzed as controls for the target tissue (mean ± SEM). Imaging experiments in A–D were done on days 18–25 after T cell transfer. [E–G] T\textsubscript{eff} and T\textsubscript{reg} cell numbers in the target tissue at the initial phase of T cell infiltration (days 10–12, all animals were free of diabetes) and a later stage (days 17–20) when some animals developed diabetes with near complete (>80%) damage of the islet grafts. (E) T\textsubscript{eff} cell numbers in protected versus damaged grafts (mean ± SEM). Imaging experiments in A–D were done on days 18–25 after T cell transfer. [E–G] T\textsubscript{eff} and T\textsubscript{reg} cell numbers in the target tissue at the initial phase of T cell infiltration (days 10–12, all animals were free of diabetes) and a later stage (days 17–20) when some animals developed diabetes with near complete (>80%) damage of the islet grafts. (F) T\textsubscript{reg} cell numbers and T\textsubscript{reg}/T\textsubscript{eff} ratios (G) in the target tissue were analyzed in the same graft before and after extensive damage occurred (if it occurred). Results represent 3 experiments in a total of 4–6 mice in each group (the protected vs. damaged groups). Each data point represents one islet. Unpaired Student’s t tests were performed in B and D and one-way ANOVAs with Tukey’s post-hoc analyses were performed in C and E–G; *, P < 0.05; **, P < 0.01; ***, P < 0.001. Bar, 50 µm.

**Figure 7.** T\textsubscript{reg} cells persistently interacted with T\textsubscript{eff} cells in damaged tissue, despite an imbalance in T\textsubscript{reg} versus T\textsubscript{eff} numbers. CD4\textsuperscript{+} T cell behavior was analyzed in the graft tissue that was being destroyed due to failed immune regulation or protected by successful T\textsubscript{reg} cell regulation. Protected islet grafts had at least 80% of original islet mass, whereas damaged islet graft had 20% or less. (A) Representative fluorescence micrographs (3D rendering) showing protected (left) and damaged (right) islet grafts (gray; visualized by laser backscatter) in NOD-SCID mice reconstituted with GFP-labeled CD4\textsuperscript{+} BDC2.5 T\textsubscript{reg} cells and CFP-labeled CD4\textsuperscript{+} BDC2.5 T\textsubscript{eff} cells. (B) T\textsubscript{reg}–T\textsubscript{eff} interaction time in protected versus damaged grafts (mean ± SEM). (C) Ratios of T\textsubscript{reg}–T\textsubscript{eff} cell pairs within protected versus damaged grafts. The T\textsubscript{reg}–T\textsubscript{eff} cell pairs outside of the target graft tissue, in the iris in the same ACE, were analyzed as controls for the target tissue (mean ± SEM). (D) Interaction index (ratio of T\textsubscript{eff} numbers in the graft tissue, in the iris in the same ACE, were analyzed as controls for the target tissue (mean ± SEM). Imaging experiments in A–D were done on days 18–25 after T cell transfer. [E–G] T\textsubscript{eff} and T\textsubscript{reg} cell numbers in the target tissue at the initial phase of T cell infiltration (days 10–12, all animals were free of diabetes) and a later stage (days 17–20) when some animals developed diabetes with near complete (>80%) damage of the islet grafts. (E) T\textsubcript{eff} cell numbers in protected versus damaged grafts (mean ± SEM). Imaging experiments in A–D were done on days 18–25 after T cell transfer. [E–G] T\textsubscript{eff} and T\textsubscript{reg} cell numbers in the target tissue at the initial phase of T cell infiltration (days 10–12, all animals were free of diabetes) and a later stage (days 17–20) when some animals developed diabetes with near complete (>80%) damage of the islet grafts. (F) T\textsubscript{reg} cell numbers and T\textsubscript{reg}/T\textsubscript{eff} ratios (G) in the target tissue were analyzed in the same graft before and after extensive damage occurred (if it occurred). Results represent 3 experiments in a total of 4–6 mice in each group (the protected vs. damaged groups). Each data point represents one islet. Unpaired Student’s t tests were performed in B and D and one-way ANOVAs with Tukey’s post-hoc analyses were performed in C and E–G; *, P < 0.05; **, P < 0.01; ***, P < 0.001. Bar, 50 µm.
A role of CTLA4 in T$_{reg}$–T$_{eff}$ cell interaction, likely through motility regulation

The function of T$_{reg}$ cells depends on CTLA4 (Wing et al., 2008), which also regulates T$_{eff}$ cell function (Tefft et al., 2006). We tested here the role of CTLA4 in maintaining T$_{reg}$–T$_{eff}$ cell interaction by administering anti-CTLA4-antibody blockade after stable T$_{reg}$–T$_{eff}$ cell interactions and T$_{reg}$–cell protection were established. The anti-CTLA4 treatment under this condition did not cause diabetes (data not shown). It increased T$_{eff}$ cell numbers in the target tissue, more so than in T$_{reg}$ cell numbers (Fig. 9, A and B), and resulted in decreased T$_{reg}$/T$_{eff}$ ratios (Fig. 9, C). The treatment did not immediately disrupt the interaction between the CD4$^+$ T$_{reg}$–T$_{eff}$ pairs. However, the proportion of interacting T$_{reg}$–T$_{eff}$ pairs declined over time after CTLA4 blockade (Fig. 9, D), and their interaction time was shortened (Fig. 9, E and F). Although CTLA4 blockade led to increased motility of CD4$^+$ BDC2.5 T$_{eff}$ and T$_{reg}$ cells in T$_{reg}$–cell–protected grafts, it decreased the motility of CD8$^+$ OT1 T$_{eff}$ cells. Moreover, in a model wherein CTLA4 in CD8$^+$ OT1 T$_{eff}$ cells were modulated with RNAi (Chen et al., 2006; Miska et al., 2012), CTLA4 reduction decreased motility of T$_{eff}$ cells, suggesting an intrinsic effect of CTLA4 in T$_{eff}$ cell motility (Fig. 10 and Videos 5 and 6). Collectively, these results suggest that CTLA4 might influence T$_{reg}$–T$_{eff}$ interaction through motility regulation, but the exact effect depends on the nature of the immune settings and cell types.

DISCUSSION

Control of immune damage at the effector phase is a crucial and perhaps the most realistic therapeutic target in clinical intervention of immune–mediated diseases (Chatenoud, 2011). Improvement of therapeutic interventions will require in–depth understanding of the immune cell behavior in target tissues and of the reaction of target tissue cells in response to insult. The current study suggests that the contact–dependent mode of immune cell interaction in the target tissue is a critical part of pathophysiology at the effector phase of immune responses, and immune tolerance induction may be facilitated by promoting intimacy between pathogenic and protective immune cells. In this regard, it is highly relevant that tissue...
tools should enable further studies to investigate such mechanisms in vivo.

It is important to make the distinction between our findings of stable Treg–Teff contact interaction in target tissue and those in previous reports on lack of direct Treg–Teff contact in lymph nodes (Mempel et al., 2006; Tang et al., 2006). Treg cells play a major role in peripheral immune tolerance, likely through a variety of mechanisms (Tisch and Wang, 2008; Shevach, 2011; Josefowicz et al., 2012). However, key features of Treg cell biology in vivo remain to be clarified, including whether Treg cells interact with T eff cells through direct contact. Two groups independently reported that Treg cells could not suppress in vitro proliferation responses of T eff cells if they were placed in a different chamber in a trans-well culture system (Takahashi et al., 1998; Thornton and Shevach, 1998).

Although this could be attributed to an effect of Treg cells on antigen-presenting cells (Tadokoro et al., 2006; Onishi et al., 2008; Wing et al., 2008), robust evidence has also been presented for antigen-specificity, as opposed to bystander killing (Tite and Janeway, 1984), shapes tissue fate in the effector phase.

With the tools currently available for longitudinal imaging of antigen-specific T cells in target tissues, we uncovered some basic behaviors of different lineages of T cells during the effector phase. CD8+ T cells are well known for contact-dependent killing. CD4+ T cells, on the other hand, are better known as various helper subsets, although increasing attention has been put on their potential cytotoxicity function. Although our current models do not allow us to compare the biology of CD4+ and CD8+ T cells in an ideally analogous setting, our studies provide in vivo evidence for contact-based killing of target cells by both. The observation adds to efforts to understand the behaviors of these two distinct lineages of T cells at various stages of their activation, differentiation and functioning (Mandl et al., 2012). Our results, however, do not exclude indirect mechanisms of target killing by CD4+ T eff cells, and development of new tools should enable further studies to investigate such mechanisms in vivo.

Figure 9. The effect of CTLA4 modulation on Treg–Teff cell interaction. NOD-SCID mice carrying islet grafts in ACE were injected with CD4+ BDC2.5 T eff and Treg cell mixture. After Treg cell protection in the tissue was established (~30 d after T cell transfer), mice were treated with either PBS control or anti-CTLA4 monoclonal antibody (20 µg/g body weight, n = 3 different mice in each group). Notably, the intravital imaging platform enabled us to non-invasively image the same tissue spot in the same animal longitudinally, as in these experiments and throughout the study. Therefore, the pretreatment measurements also serve as internal controls for posttreatment measurements. (A) T eff cell numbers and (B) Treg cells numbers in the target tissues (mean ± SEM). (C) Intra-islet graft Treg/T eff cell ratio over time after anti-CTLA4 antibody blockade (n = 10–11 islet grafts per group per time points; mean ± SEM). (D) Interaction index, calculated as the ratio of T eff cells with or without Treg cell interaction, after anti-CTLA4 or control treatment (mean ± SEM). Arrows in A–D indicated injection of anti-CTLA4 antibodies. (E and F) The duration of Treg–Teff cell interactions after CTLA4 blockade, in actual imaging time (E) and relative to the length of whole imaging session (F; n = 24–40 cell pairs; mean ± SEM). One-way ANOVA across all time points in all groups did not yield statistical significance, likely because of the large variations among individual animals and time points in each group in these longitudinal imaging experiments with live animals; however, there was significant difference among pretreatment controls and posttreatment measurements within the anti-CTLA4 treatment group. *, P < 0.05, one way ANOVA was performed with a Tukey’s multiple comparison’s post-hoc analyses, compared with both pretreatment measurement of the same animal or control-treated animals. **, P < 0.01; ***, P < 0.001.
The effect of CTLA4 on T cell motility may depend on the type of T cells and their settings. (A) The velocity of CD8+ OT1 T_{eff} cells (n = 3 mice, 40 cells, mean ± SEM) in ACE islet grafts in B6 mice is compared with that of CD8+ BDC2.5 T cells in ACE islet grafts in NOD. SCID mice. For CD4+ BDC2.5 T cell analyses, the CD4+ T_{eff} cells (n = 5 mice, 110 cells; mean ± SEM) and CD4+ BDC2.5 T_{reg} cells (n = 5 mice, 101 cells; mean ± SEM) were from animals injected either with T_{eff} and T_{reg} cell mixture (the second and third groups) or from CD4+ BDC2.5 T_{eff} cells only (fourth group, n = 3 mice and 107 cells). Each dot represents one cell and the lines in plots represent mean ± SEM. (B and C) Transient change of intra-graft CD8+ BDC2.5 T cell velocities after anti-CTLA4 treatment (n = 30–90 cells per group for each time point; mean ± SEM). (D and E) The effect of CTLA4 modulation on displacement (D) and velocity (E), respectively, on intra-graft CD8+ OT1 T_{eff} cells. Animals were treated with anti-CTLA4 antibodies (n = 3 mice) or PBS or hamster Ig control (n = 4 mice), or they carried a CTLA4 RNAi transgene (n = 3 mice). Data represent two experiments and are from analyses of 40 cells (mean ± SEM) in each group. Each dot in E represents one cell. **, P < 0.01; ***, P < 0.001. See Videos 5 and 6.
nor did we find a disruptive effect on $T_{reg}$–$T_{eff}$ cell interaction after injecting anti-CD80 or anti-CD86 antibodies directly into ACE (unpublished data). However, our observation in this regard is preliminary in scope and limited to the target tissue, and thus does not invalidate the hypothesis that CTLA4 expressed by $T_{reg}$ cells may bind to CD80/CD86 on $T_{eff}$ cells to facilitate direct cellular interactions (Paust et al., 2004). On the other hand, the data from our studies suggest that CTLA4 might affect $T_{reg}$–$T_{eff}$ cell interaction through other mechanisms. A study with a conditional knockout of CTLA4 showed that $T_{reg}$ cells require CTLA4 for functioning in vivo (Wing et al., 2008). We showed here that CTLA4 played a role in regulating $T_{reg}$–$T_{eff}$ cell interaction in target tissue. This role may be related to motility regulation of both $T_{reg}$ and $T_{eff}$ cells. Of note, the role of CTLA4 in T cell motility control has been debated. Schneider et al. (2006) reported that CTLA4 enhanced the motility of T cells and thus reversed the stop signal originated from engaging the TCR, an effect that may preferentially impact $T_{eff}$ cells over $T_{reg}$ cells (Lu et al., 2012). This evidence suggested that manipulating CTLA4-based motility control could lead to therapeutic advance. Indeed, a recent study found that anti–CTLA4 antibody treatment inhibited CD8$^+$ T cell motility and promoted antitumor immunity (Ruocco et al., 2012). However, another study by Fife et al. (2009) showed that anti–CTLA4 treatment did not alter the motility of autoimmune CD4$^+$ BDC2.5 T cells in draining lymph nodes. Our imaging analyses of CD4$^+$ and CD8$^+$ T cells during the effector phase within target tissue indicate that the exact effect of CTLA4 on T cell motility may vary in different T cell subsets and may be influenced by distinct circumstance of cellular interactions.

In our experiments, CTLA4 blockade caused only modest changes on cellular interaction. It did not substantially break tolerance under the conditions we tested. The small effect on $T_{reg}$–$T_{eff}$ cell interaction could be contributed by altered motility controls, although it is still a challenge to determine the cause and effect relationship in such in vivo settings. It remains to be determined how CTLA4 blockade led to an increase in $T_{eff}$ cells, rather than $T_{reg}$ cells, in the target tissue. The resulting imbalance of $T_{reg}$/$T_{eff}$ ratios, however, did not seem likely to account for the changes in durations of $T_{reg}$–$T_{eff}$ cell interactions, as reduced $T_{reg}$/$T_{eff}$ ratios did not lead to reduced $T_{reg}$–$T_{eff}$ interaction time in the other settings of our studies. Although these results suggest novel facets of CTLA4 function beyond the scope of this study (Han et al., 2012), they may also reconcile the debate on whether CTLA4 function beyond the scope of this study (Han et al., 2009), NOD.CTLA4 shRNA (CTLA4KD7; Chen et al., 2006), NOD.PL4 (Chen et al., 2006), OT1 (Hogquist et al., 1994), RIP-mOVA (Kurts et al., 1996), MIP-CFP (Hara et al., 2006) and CAG-CFP (Hadjantonakis et al., 2002) transgenic lines were described previously. The CTLA4shRNA and PL4 lines were backcrossed onto B6 background for >10 generations (Miska et al., 2012). The CTLA4shRNA transgene caused 2–3-fold reduction in CTLA4 expression. The stability of the RNAi effect in the transgenic lines on different genetic background has been established (Chen et al., 2006; Miska et al., 2012). FIR (Foxp3-IRIS-RFP knock-in) mice (Wan and Flavell, 2005) were backcrossed onto the NOD background for 10 generation to create the NOD.Foxp3$^{3Rk}$ line. CAG-CFP obtained on the C57BL/6 background, were backcrossed onto the NOD genetic background for 10 generations, and crossed with NOD.BDC2.5 and NOD.Foxp3$^{3Rk}$. All animals were maintained in a specific pathogen–free barrier facility at the University of Miami, and the studies are approved by the Institutional Animal Care and Use Committee at the University of Miami.

**Materials and methods**

Mouse models. Lines of transgenic mouse models were crossed or backcrossed to generate the necessary combinations of specific T cells and target tissue with distinct fluorescence reporters. Detailed descriptions of their genetic makeup, antigen-specificity, and fluorescence properties were provided in Table S1. NOD.BDC2.5 (Katz et al., 1993, NOD.Foxp3$^{3Rk}$ (Feuerer et al., 2009), NOD.CTLA4 shRNA (CTLA4KD7; Chen et al., 2006), NOD.PL4 (Chen et al., 2006), OT1 (Hogquist et al., 1994), RIP-mOVA (Kurts et al., 1996), MIP-CFP (Hara et al., 2006) and CAG-CFP (Hadjantonakis et al., 2002) transgenic lines were described previously. The CTLA4shRNA and PL4 lines were backcrossed onto B6 background for >10 generations (Miska et al., 2012). The CTLA4shRNA transgene caused 2–3-fold reduction in CTLA4 expression. The stability of the RNAi effect in the transgenic lines on different genetic background has been established (Chen et al., 2006; Miska et al., 2012). FIR (Foxp3-IRIS-RFP knock-in) mice (Wan and Flavell, 2005) were backcrossed onto the NOD background for 10 generation to create the NOD.Foxp3$^{3Rk}$ line. CAG-CFP obtained on the C57BL/6 background, were backcrossed onto the NOD genetic background for 10 generations, and crossed with NOD.BDC2.5 and NOD.Foxp3$^{3Rk}$. All animals were maintained in a specific pathogen–free barrier facility at the University of Miami, and the studies are approved by the Institutional Animal Care and Use Committee at the University of Miami.
Intraocular injection of fluorescence-tagged antibodies for in situ imaging. The responses of bystander islet cells in an inflammatory setting were also examined at a transplantation site that is different from ACE, which is the standard transplantation site for experimental studies of islet grafts in rodent recipients. It has been an invaluable research tool for decades (Ricordi et al., 1987). It allows transplantation of islets in a well-confined location that can then be retrieved for histopathological or molecular analyses of a relatively large number of islets. Islets were transplanted under the kidney capsule of B6 mice by the Diabetes Research Institute Preclinical Cell Processing and Translational Models Core facility following standard procedures (Berney et al., 2001; Falco et al., 2012). In brief, pancreatic islets were isolated from either wild-type B6 or RIP-mOVA transgenic donors. B6 islets, RIP-mOVA islets, or a mixture of B6 islets and RIP-mOVA islets were prepared into individual aliquots for each transplant recipient. They were then handpicked with a Hamilton syringe and transferred into a polyethylene tube (PE50; BD; inside diameter 0.58 mm; outside diameter, 0.965 mm) that was kinked at one end, and then pelleted in the kinked tubing by centrifugation at 1,000 rpm for 2 min, to pack them together. The pelleting step was done before the transplantation procedure.

After induction of general anesthesia (isoflurane 2%/oxygen mix, to effect), a left flank incision was performed and the left kidney exteriorized and exposed. Under a dissection microscope and using microsurgical forceps, a small breach was performed on the capsule at the caudal pole of the kidney through which the tubing containing the pelleted islets was gently inserted and pushed toward the opposite (cranial) pole. Islets were gently released in
the renal subcapsular space under visual microscopic inspection. Next, the tubing was gently removed and the breach on the capsule cauterized to prevent back flow. The kidney was repositioned in the abdominal cavity, and the muscular and cutaneous layer sutured. The graft-bearing mice were rested for \( \sim 7 \) wk after transplantation to ensure engraftment, and then adoptively transferred with activated OT1 CD8\(^+\) T cells by intravenous injection. 2 d later, BrdU was injected at a concentration of 10mg/kg every 12 h. On day 10, kidneys were removed, fixed in 4% PFA overnight, followed by immersing in 30% sucrose overnight, and then embedded in OCT. Sections were cut and stained for BrdU incorporation with biotinylated antibodies against BrdU, using an in situ BrdU detection kit (BD) designed for histology use. We modified the secondary staining procedure for immunofluorescence staining. Although isotype controls were not included in the kit, we established the specificity of the BrdU staining procedure with spleen tissue sections from animals with or without BrdU injection treatment. To expose BrdU epitopes in the nuclear DNA, a covered plastic coplin (filled with 50 ml of diluted BD Retrievagen buffer) was preheated in a water bath at 95–97°C. After 30 min, the tissue slides were quickly placed into a preheated jar, and incubated for another 30 min. The tissue slides in the closed jar were then removed and left at ambient temperature for one hour. Primary anti-insulin antibodies (polyclonal guinea pig anti-insulin; DAKO; titration, 1:1,000) and the biotinylated anti-BrdU antibodies were incubated overnight with the tissue sections at 4°C, and then washed according to manufacturer’s instructions. Secondary staining was done with Alexa Fluor 488–conjugated streptavidin (Life Technologies; titration, 1:500) and Alexa Fluor 647–conjugated donkey anti–guinea pig F(ab)\(_2\) (Jackson ImmunoResearch Laboratories; titration, 1:500). Sections were counterstained with DAPI and mounted for fluorescence microscopy. Images were acquired with a Leica inverted TCS SP-5 broadband confocal microscope (using Leica 40×/1.25–0.75NA HCX PL APO lens for oil immersion).

**Image analysis.** Image analyses were performed using the Velocity software (version 6; Perkin Elmer) as previously described (Abdulreda et al., 2011). Images were denoised and contrast-enhanced equally for consistant analyses. Quantitative analyses of cellular movement and \( T_{\text{reg}}-T_{\text{eff}} \) cell interaction dynamics were performed automatically in the Velocity with user feedback on drift-corrected 3D time-lapse recordings. Drift correction was performed in Velocity based on proprietary algorithms. T cell counting and movement tracking were performed automatically by the software and dynamic parameters (e.g., velocity, displacement) were derived from time-lapse recordings. \( T_{\text{reg}}-T_{\text{eff}} \) cell interaction time and interaction index were calculated manually. The interaction time between the \( T_{\text{reg}}-T_{\text{eff}} \) cell interacting pairs was calculated manually using the time stamps embedded in each image frame in a time-lapse series. The interaction index is calculated by dividing the number of \( T_{\text{reg}} \) cells interacting with \( T_{\text{eff}} \) cells by the number of \( T_{\text{eff}} \) cells not interacting with \( T_{\text{reg}} \) cells, \( b \) (s) (mixture) was measured by the software, as previously described (Abdulreda et al., 2011), based on volume detected by either laser backscatter or CFP fluorescence.

Annexin V labeling in islet grafts in ACE was quantified with z-stack images that were acquired \( \sim 10–15 \) min after injection of APC-labeled Annexin V directly into ACE. Using proprietary algorithms in Velocity, we then measured in the 3D images the amount of overlap in the volume the Annexin V–positive stain with either that of \( \beta \) cells (visualized by CFP or backscatter) or CFP/GFP-labeled \( T_{\text{reg}} \) cells (Rodriguez-Diaz et al., 2011). Automatic selection, optimized with user feedback, based on fluorescence of Annexin V–positive cells, \( \beta \) cells, and \( T \) cells and volume measurements were performed automatically by the software. The overlap (volume) in Annexin V stain with either that of \( \beta \) or T cells was also derived by the software, and was expressed as a fraction of the total volume of the \( \beta \) and T cells in each islet. Similarly, the number of graft-infiltrating \( T_{\text{reg}} \) cells was automatically measured based on fluorescence intensity (Abdulreda et al., 2011). Islet cell mass was measured using Velocity software as previously described (Abdulreda et al., 2011), based on islet volume detected either by laser backscatter (reflection) or CFP fluorescence. For example, in Fig. 3, the volume of CFP-labeled \( \beta \) cells was measured based on CFP fluorescence which is in this case restricted to the bystander islets in this case. In brief, a proprietary detection algorithm built into the Velocity software was used to detect CFP signal based on fluorescence intensity. The detection threshold was set with user feedback to restrict the selection to the CFP-labeled \( \beta \) cells. Once the selection was made, the volume was derived automatically by the software. Longitudinal analyses on the same individual islets were performed using the same approach, and numerical values of islet volumes were expressed as means \( \pm \) SEM at the different time points under the different conditions.

**Statistical analysis.** Unpaired Student’s \( t \) test was used to compare two samples. For multiple group comparisons, one-way ANOVA tests were performed followed up by Tukey’s post-hoc multiple comparisons test. \( P \leq 0.05 \) was considered significant. Asterisks indicate significance (*, \( P < 0.05; **, P < 0.01; *** , P < 0.001 \)).

**Online supplemental material.** Fig. S1 relates to Fig. 3 and shows digital estimation of cell nuclear density in islet grafts imaged over time in the living animal. Fig. S2 relates to Fig. 5 and shows that the long-lasting contact between \( T_{\text{reg}}-T_{\text{eff}} \) in the target tissue occurred even in areas with sparse infiltration of T cells. Table S1 lists the transgenic mouse models genetically tagged with antigen-specific T cell receptor, fluorescence reporters, and lineage markers for the imaging studies. Video 1 (corresponds to Fig. 1) shows direct interaction between antigen-specific CD4\(^+\) T cells with target \( \beta \)-cells in pancreatic islet grafts in ACE. Videos 2 and 3 (corresponds to Fig. 3) shows antigen-specific CD8\(^+\) OT1 T cell–mediated destruction of OVA\(^+\) islets and concomitant growth of juxtaposed bystander OVA\(^+\) islets. Video 4 (corresponds to Fig. 5) shows stable long-lasting interaction between \( T_{\text{reg}} \) and \( T_{\text{eff}} \) cells in the target tissue. Video 5 and 6 (corresponds to Fig. 10) shows CTLA4 blockade reduces CD8\(^+\) T cell mobility in target tissue. Online supplemental material is available at [http://www.jem.org/cgi/content/full/jem.20130785/DC1](http://www.jem.org/cgi/content/full/jem.20130785/DC1).

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