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Control of In Vivo Collateral Damage Generated by T Cell Immunity

Govindarajan Thangavelu,* Ronald G. Gill,‡§ Louis Boon,¶ Kristofer K. Ellestad,‖# and Colin C. Anderson*,‡§#

An ongoing dilemma faced during an immune response is generating an effective, often proinflammatory response to eliminate pathogens and/or infected cells while also minimizing collateral damage to adjacent noninfected tissues. The factors limiting bystander cell injury during an Ag-specific immune response in vivo are largely unknown. In this study, using an in vivo model of islet transplants in TCR transgenic mice, we show that both CD4 and CD8 T cells do have the capacity to inflict adjacent tissue damage and that this injury is greatly enhanced in sensitized hosts. CD4 T cell–mediated killing of specific and bystander cells occurred via different mechanisms. Unlike specific target cell killing, CD4-mediated bystander injury required tissue Fas expression and was inhibited with anti–IFN-γ Ab treatment in vivo. Moreover, bystander cell injury was not entirely nonspecific but rather required, in naive recipients, that the MHC allele expressed by the bystanders was self. Importantly, the coinhibitor programmed death-1 plays an important role in restraining bystander cell injury mediated either by defined TCR transgenic T cells or by polyclonal T cell populations. Thus, the differential requirements for specific versus bystander cell injury suggest that there are opportunities for inhibiting immune pathology without compromising Ag-specific immunity in vivo. The Journal of Immunology, 2013, 191: 000–000.

The immune system protects the host by responding against invasive microorganisms. However, the immune response generated is not always highly specific, killing not only target cells but also bystander cells in the vicinity of the specific target cells. Previous studies have documented the ability of CTLs to cause bystander killing in vitro (1–3). Such bystander killing has been shown to be mediated by Fas/Fas ligand (CD95/CD95 ligand) interactions or by TNF (1) and was dependent on the allele of MHC expressed by the “bystander” cells in some settings (2) but not others (4). In transplant models, using a mixture of syngeneic and xenogeneic islets (5, 6) or with allogenic skin grafts (7, 8), results supported the concept of selective killing of specific target cells. In contrast, other skin transplant models have shown significant bystander damage to adjacent syngeneic cells (9), and bystander injury occurred in mixed xenogeneic and syngeneic islets in primed recipients (10). Importantly, it is not yet clear how bystander damage is mediated in vivo and, conversely, what mechanisms restrain this form of collateral tissue injury. Understanding both the expression and regulation of bystander cell injury has important therapeutic implications. On the one hand, promoting bystander cell injury could be beneficial in tumor immunotherapy by preventing the escape of malignant or tumor variants with reduced Ag expression. On the other hand, finding means to limit bystander killing would be beneficial for preventing autoimmunity or attenuating transplant rejection.

Materials and Methods

Mice

Adult C57BL/6 (B6; H-2b) and C3H/HeCr (H-2h) mice were obtained from the National Cancer Institute (Frederick, MD). Adult C57BL/10 (B10; H-2k), B10.BR (H-2k), and B6.MRL-Tg(b2m<sup>−/−</sup>)(lov) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Marilyn Rag2<sup>−/−</sup> mice were generated by crossing B6.129S7-<i>Tnfrsf6</i>lpr<sup>−/−</sup>/J (11), MataHari Rag1<sup>−/−</sup> (12), OT-1 Rag1<sup>−/−</sup>-, B6.129S7-<i>RagFm<sup>Monel</sup></i> (B6 Rag<sup>−/−</sup>)<sup>−/−</sup>, and GFP Rag<sup>−/−</sup> mice were bred at the University of Alberta. GFP Rag<sup>−/−</sup> mice (abbreviated as B6 GFF<sup>−/−</sup>) were developed by crossing B6.129S7-<i>RagFm<sup>Monel</sup></i> and C57BL/6-Tg(UBC-GFP)30Scha/J. B6 mOVA Rag<sup>−/−</sup> mice (abbreviated as B6 OVA<sup>++</sup>) were developed by crossing Act-mOVA transgenic (Tg) (13) and B6 Rag2<sup>−/−</sup> mice. B6 Rag<sup>−/−</sup>-<i>C-H2-Ab<sup>bm12</sup></i> (H-2<sup>bm12</sup>) mice were developed by crossing B6.129S7-<i>RagFm<sup>Monel</sup></i> and B6.C-H2-Ab<sup>bm12</sup> (H-2<sup>bm12</sup>) mice. C57BL/6-Pdcdl1<sup>−/−</sup>-<i>N11</i> (programmed death-1 [PD-1]<sup>−/−</sup>) mice were originally generated by Honjo and colleagues (14). C57BL/6-B and T lymphocyte attenuator (BLTA)<sup>−/−</sup> mice were generated by Prof. Ken Murphy and bred at the University of Alberta. BALB/c (H-2k) mice were from University of Alberta and Charles River Laboratories (Montreal, QC, Canada). Marilyn Rag2<sup>−/−</sup>-<i>PD-1</i> mice lacking PD-1 were generated by crosses with C57BL/6-Pdcdl1<sup>−/−</sup>-<i>N11</i> mice. All procedures followed the guidelines of the Canadian Council on Animal Care.

Diabetes induction and islet transplantation

Recipient mice were administered streptozotocin (Sigma-Aldrich, Mississauga, ON, Canada) at 185–190 mg/kg to induce diabetes. Recipients were considered diabetic after two consecutive blood glucose levels readings of >20 mmol/L using a OneTouch Ultra glucometer (LifeScan Canada, Burnaby, BC, Canada). Diabetic recipients were transplanted with mixed islets (800) from two different donor types in equal numbers (400 each); controls received 400 target or bystanders islets alone. Bystander killing was defined as consecutive blood glucose measurements of >15
mmol/l. Male or OVA+/+ islet grafts and female or OVA+ islet grafts were used as targets and bystanders in anti–histocompatibility Y chromosome Ag (H-Y) or anti-OVA TCR Tg mice, respectively. Targets from GFP Rag2/2 mice were used in anti-H-Y TCR Tg recipients to allow rejection of target islets to be monitored. In the case of B6 wild-type (WT) or PD-12/2 recipients, BALB/c allografts were used as targets, whereas respective syngeneic islet grafts (WT or PD-1−/−) were used as bystanders.

Histology and immunofluorescence

Graft-bearing kidneys were harvested and processed in either paraffin or embedded in OCT, blocked with goat serum, and stained with DAPI, anti-insulin (Dako), and anti-GFP (Santa Cruz Biotechnology), or anti-insulin and H&E. Secondary Abs were labeled with Alexa Fluor 488 and Alexa Fluor 594 (Invitrogen). Sections without primary Abs were used as negative controls. Slides were visualized on a compound fluorescent microscope (Axioplan; Carl Zeiss).

Immunization and IFN-γ neutralization

Where indicated, anti-H-Y or OT-1 TCR Tg recipients were immunized with 2 × 106 live male or OVA+ splenocytes, respectively, 1 wk before transplantation. WT or PD-1−/− recipients were immunized with 4 × 106 allogeneic (BALB/c) live splenocytes 2 wk before transplantation. Where indicated, Marilyn recipients were given neutralizing anti-IFN-γ (XMG 1.2) Ab (0.5 mg/mouse) every 5 d from day 3 to 33 after transplantation.

FIGURE 1. Experimental systems used in studies of bystander injury using mixed islet grafts. (A) TCR Tg models to study bystander killing by naive CD4 or CD8 T cells. Islets that express the cognate Ag for the host T cells are referred to as targets, whereas islets that do not express the cognate Ag are bystanders. (B) By changing the donor of bystander islets, the roles of bystander MHC haplotype and Fas were examined. The roles of IFN-γ, prior Ag-specific priming, and host PD-1 expression were also tested. (C) Model to assess the ability of polyclonal T cells to cause bystander injury and the potential regulation of this process by donor-specific priming and coinhibitors (PD-1 and BTLA).

FIGURE 2. CD4 bystander killing in vivo: MHC allele requirements in naive and primed recipients. (A) Diabetic female Marilyn hosts were transplanted with either targets alone (B6 Rag−/− male islets), bystanders alone (female islets, or (B) mixed islets of targets and bystanders. Marilyn hosts were naive (A, B) or previously primed (C, immunized to H-Y). Bystander islets were from female B6 (H-2b; denoted simply as bystanders) or either C3H or B10.BR (H-2k bystanders) mice. For naive hosts that received H-2k bystanders (n = 8), bystander islets were C3H (n = 4) or B10.BR (n = 4). H-2k bystander islets in primed hosts (C) were from C3H. (D) Assessment of target islet survival by immunofluorescence immediately after rejection or ∼100 d after transplantation in animals that remained normoglycemic. Top, Presence of mixed male B6 GFP−/− target islets (yellow; GFP+ and insulin+) and GFP2 female bystander islets (red) from control Rag−/− recipients that are unable to reject the islets (n = 3). Bottom, Representative (n = 4) image of islet grafts from Marilyn recipients that were transplanted with male B6 GFP−/− targets and female MHC-mismatched (B10.BR; GFP−) bystander islets. Original magnification ×400. Data presented in (A) and (B) were compiled from five separate experiments. Data in (C) are from one experiment with the indicated number of animals. Data presented in (D) are representative of two separate experiments.
Statistical analysis

Statistical analyses employed Prism 4 (GraphPad Software) and the log-rank test to compare survival curves. A p value <0.05 was considered statistically significant.

Results

Naive CD4 T cells mediate bystander injury in vivo that is restricted to self-MHC of bystanders

To define the potential for CD4 versus CD8 T cells to cause bystander killing in vivo we used an islet transplantation model in monoclonal TCR Tg mice (for experimental outline, see Fig. 1). Marilyn mice (anti-H-Y CD4+ T cells) were used to test the capacity of CD4 T cells to induce bystander killing. Consistent with the results from our previous study (15), naive female Marilyn recipients rapidly rejected target Ag-expressing (H-Y) male islets (referred to as targets) but uniformly accepted non-Ag–expressing female islets (Fig. 2A). By mixing specific (male) target and bystander (female) islets, we tested whether recipient CD4 T cells could injure the adjacent female bystander islets in the course of rejecting the specific male target islets. Interestingly, most of the animals with mixed islets injured both the male and the adjacent female islets as reflected by a return to hyperglycemia, indicating bystander damage to the female islets (hyperglycemia in Fig. 2B; see histology in Fig. 4C, left). Having demonstrated that CD4-mediated bystander killing can occur in vivo, we investigated the factors that influence the capacity for bystander killing. First, to determine a role for cell MHC expression in bystander cell injury, we tested islets expressing different MHC haplotypes (non-self) for their relative susceptibility to bystander injury. Interestingly, bystander killing was substantially reduced (p = 0.0059) when there was a change in the MHC haplotype of the bystander cells to H-2k (Fig. 2B; see histology in Fig. 4D, left). The MHC-mismatched bystanders had increased resistance to rejection independent of the strain background (C3H or B10.BR), indicating that it was the MHC haplotype and not the influence of other background genes that impacted vulnerability to bystander killing. To test whether the lack of rejection of bystanders might have been due to some unanticipated ability of bystanders to inhibit the rejection of the specific islet targets, we used male B6 GFP+/+ target islets to allow histologic assessment of the specific target (male) versus the bystander islet tissue. Results demonstrated that there was a selective preservation of the H-2k (GFP+) bystander islets but elimination of the specific male GFP+/+ target islets, indicating that the MHC-unrelated islets could resist damage during rejection of the male graft (Fig. 2D). In contrast to these findings using naive recipients, sensitized recipients (previously primed to the target H-Y Ag) rejected bystander islets independent of the MHC haplotype of the bystanders (naive versus primed with H-2k bystanders, p = 0.0004; Fig. 2C; see histology in Fig. 4D, 4E, left). These data suggest that a previously primed CD4 T cell response has a greater capacity for bystander killing with less specificity for surface MHC expression. Taken together, results suggest that bystander tissue injury is not entirely nonspecific but rather shows a degree of self-MHC restriction. Moreover, bystander tissue injury in sensitized hosts is more vigorous and less selective and does not discriminate between MHC alleles on the adjacent tissue.

Differential control of target and bystander injury

Mechanistically, in vitro studies had suggested that the Fas/Fas ligand pathway may be required to induce bystander killing (1) and that such killing is a property of newly synthesized Fas ligand (16). The Fas pathway is also an important mechanism of CD8-mediated islet killing when perforin is deficient (17). As suggested by previous in vitro studies of bystander target killing (2), we tested whether Fas is required in bystander killing in vivo using Fas-deficient (lpr) bystander islets. Unlike WT (Fig. 2B) or PD-1-deficient (Fig. 3A) bystander islet grafts, lpr islets were resistant to bystander injury in vivo (Fig 3A; WT versus lpr bystanders, p = 0.021). In contrast, the target male lpr islets were rejected by Marilyn T cells (Fig. 3A, p = 0.01), indicating that target and bystander tissue injury had distinct requirements. Bystander and target injury could be further distinguished by the dependence on IFN-γ. Treatment with anti–IFN-γ mAb blocked bystander but not target islet injury (Fig. 3A, p = 0.014), consistent with the ability of IFN-γ to upregulate Fas (18). Taken together, Fas, IFN-γ, and the appropriate (self) MHC haplotype expression all contribute to the generation of bystander killing during a CD4 T cell response in vivo.

We then turned to potential mechanisms that restrain the degree of bystander tissue injury by determining whether the coinnhibitory molecule PD-1 plays a role in limiting this process. Coinhibitory molecules such as CTLA-4, PD-1, and BTLA have been shown to be involved in the maintenance of self-tolerance (19, 20). We considered PD-1 as a strong candidate to negatively regulate the capacity for bystander killing because of its function as a coinhibitory receptor and the wide distribution of one of its ligands, PD-ligand 1, which is expressed in both lymphoid and parenchymal tissues, including islets (21). To test for a role for host PD-1 in limiting bystander islet injury, we crossed the Marilyn TCR Tg to the Pdcd-1−/− background (14). We surmised that conditions in
which bystander injury are limited (e.g., MHC mismatched bystanders; Fig. 2B) would have a greater potential to reveal negative regulatory mechanisms. Interestingly, CD4 T cells of PD-1–deficient Marilyn mice had a greater capacity for bystander killing, as they were capable of rejecting MHC class II–disparate B6 Rag-/- C-H2-Ab1 bm12 bystander islets, unlike the response from WT Marilyn CD4 T cells (Fig. 3B, p = 0.01). Thus, PD-1 does appear to play a role in restraining the degree of bystander islet injury in vivo.

Capacity of CD8 T cells to cause bystander injury in vivo

In contrast to CD4 T cells, CD8 T cells in naive monoclonal CD8 TCR Tg mice failed to induce overt bystander killing (Fig. 4A) despite their ability to rapidly reject target islets (2–3 d). By using two different CD8 TCR Tg models (MataHari anti–H-Y/Db and OT-I anti-OVA/Kb), results showed that target islets were rejected very rapidly (2–3 d after transplant), but the adjacent bystander islets were not acutely destroyed (targets versus targets plus bystanders: OT-I, p = 0.0038; MataHari, p = 0.0003). That is, recipients of mixed target and bystander islets did not achieve levels of hyperglycemia defined as overt rejection (>15 mmol/l), and insulin-positive cells clearly remained (Fig. 4C, 4D, right). However, some of these recipients of mixed islets had transiently increased blood glucose (Supplemental Fig. 1), suggesting a degree of bystander injury. We therefore determined whether prior Ag-specific priming could augment the bystander injury mediated by CD8 T cells. Robust bystander killing was indeed observed in sensitized CD8 TCR Tg mice (Fig. 4A, lower panels; bystanders versus targets plus bystanders, p = 0.0016, OT-I and MataHari combined). Bystanders were rejected in primed recipients whether they expressed self or allogeneic MHC (targets plus bystanders versus targets plus H-2k bystanders, p = 0.456). The rejected islets had mononuclear infiltration and an absence or severe reduction in the number of insulin-positive cells (Fig. 4E, right). In contrast to the protracted time course of bystander killing by CD4 T cells (rejection in 2–3 wk; Fig. 2), the extremely rapid target and bystander killing by CD8 T cells (rejection in 2–3 d; Fig. 4) suggested that primed CD8 T cells could simply be preventing initial engraftment (e.g., neovascularization) of the islets. To examine this possibility, we allowed the islets to engraft prior to encounter with specific T cells. Diabetic immune-deficient B6 Rag-/- recipients were grafted with target B6 OVA+/+ islets alone or together with B6 OVA- bystander islets 10 d prior to transfer of CD8 T cells from previously OVA-primed OT-I mice. The transferred OT-I T cells killed the target islets alone (n = 3, days 7, 10, 11) whereas all recipients of mixed targets plus bystanders (n = 3) remained normoglycemic until termination of the experiment at 100 d after T cell reconstitution. These data suggested that the capacity of CD8 T cells to kill bystander islets depended on preventing engraftment of the bystanders. Also consistent with the concept that the CD8 cells prevent engraftment, in a preliminary study we found that Fas expression by bystanders was not required.

FIGURE 4. Capacity of naive and primed CD4 and CD8 T cells for bystander killing. (A) Naive (top) OT-I or MataHari mice received target islets alone (B6 OVA+/+ for OT-I; B6 male for MataHari), target islets plus bystander islets (bystanders were either B6 female, n = 5; B10.BR, n = 3; or C3H, n = 7; all OVA-), or bystander islets alone (female B6, n = 3; or C3H, n = 2; all OVA-). Data were compiled from a minimum of two separate experiments for each group. Previously primed (bottom) OT-I or MataHari mice received target islets (B6 OVA+/+ for OT-I or B6 male for MataHari) plus bystander islets (bystanders were B6 female OVA-), target islets plus H-2k bystander islets (bystanders were B10.BR OVA-), or bystander islets alone (female B6 OVA-). Data were compiled from three experiments. The numbers for OT-I and MataHari mice, respectively, are depicted. ND, not done. (B–E) Representative (of two separate experiments) H&E- and anti-insulin (brown)–stained islet grafts from different groups of CD4 (Marilyn, left) and CD8 TCR (OT-I, right) Tg recipients. Original magnification ×100. Grafts were harvested 100 d after transplant in normoglycemic hosts (bystanders not rejected) or shortly after rejection in mice that became hyperglycemic.
for bystander rejection by CD8 cells. Primed OT-I recipients rejected both B6 OVA \textsuperscript{1-22} islets and lpr bystander islets (n = 2, days 2, 3).

**Control of bystander injury mediated by polyclonal T cells**

The above studies used TCR Tg models with an extremely high frequency of responding T cells and were useful to define the potential for CD4 and CD8 T cells to generate bystander killing. To apply these concepts to a more physiological setting, we next tested the capacity for bystander killing by a polyclonal T cell repertoire of WT mice. Diabetic WT recipients were transplanted with mixed islets from allogeneic (targets) and syngeneic (bystanders) donors. There was little overt bystander killing detectable in naive or primed WT recipients (Fig. 5A, right, 5B, 5C, left); blood glucose levels tended to be higher in primed recipients but did not reach the threshold of rejection. Thus, WT animals show a greater ability to restrain bystander islet killing than in the TCR Tg models described above. Because our data in TCR Tg mice suggested that coinhibitory molecules may limit bystander killing in vivo, we determined whether PD-1 and/or BTLA coinhibitory molecules played a role in preventing bystander tissue injury during islet allograft rejection. In naive PD-1 \textsuperscript{−/−} recipients (Fig. 5A, left), the blood glucose levels tended to be higher in response to the mixed allogeneic/syngeneic graft, suggesting a degree of bystander killing in naive PD-1−/− deficient recipients. However, overt bystander killing was readily observed in most of the primed PD-1−/− mice (Fig. 5B, 5C, right). Primed BTLA−/− mice did not show such bystander killing (graft survival >100 d in four of four recipients; p = 0.048 compared with PD-1−/−). Thus, a normal polyclonal T cell response can lead to collateral tissue injury, particularly in sensitized recipients, and PD-1 serves to limit this damage and so increases the specificity of the resulting immune response.

**Discussion**

Early studies suggested that the effector mechanism of the immune response is highly cell-specific (7, 8), a view that is still highly accepted. We demonstrate that T cell responses do have the intrinsic capacity to trigger considerable damage to adjacent tissues in the setting of a high frequency T cell (TCR Tg) response or in a previously primed polyclonal population. Importantly, PD-1 plays a role in restraining the degree of such bystander cell/tissue injury. Consistent with in vitro studies (3, 22), we found that in vivo CD4-mediated bystander killing was dependent on Fas expression. Our data do not rule out possible contributions by other pathways (e.g., perforin). In our TCR Tg model the T cells recognized minor-H Ags in self-MHC. However, the ability of polyclonal T cells to mediate bystander damage triggered by a fully MHC-mismatched islet graft suggested that T cells directly recognizing allogeneic MHC are also able to mediate bystander damage. An examination of bystander damage using allogeneic MHC-specific TCR Tg cells to further test this possibility is warranted.

In the TCR Tg models much of the collateral tissue injury was driven by CD4 T cells, and the primary response of CD8 T cells did not contribute pronounced bystander killing in our in vivo assays. Our data do not exclude the possibility that CD8 T cells cause some bystander killing even in naive recipients when the specific and bystander targets are in very close proximity. Because each islet is a structure on the order of 1000 cells, only the outermost cells of a given bystander islet can be in very close proximity to a target islet. As such, our model can only detect bystander killing when it is occurring to such a high degree that even some of the innermost cells of the bystander islet are destroyed. Our system of mixing entire islets together is therefore likely to be less sensitive than models that employ grafts from allogenic donors, in which target and bystander cells are generally in intimate proximity. Even a small degree of bystander killing (e.g., one to three cells deep) may help prevent transmission of virus to adjacent uninfected cells (23) and reduce escape of malignant cells. The trend toward increased blood sugars in primed WT recipients of mixed islets (Fig. 5) opens the possibility that low-level bystander killing might be detrimental in the long-term for graft function. Furthermore, our studies may also have underestimated the capacity for bystander killing by CD8 T cells, as the CD8 T cells in the TCR Tg mice would not have received help from CD4 T cells that occurs within a normal T cell repertoire. The finding that CD8 T cells could prevent engraftment, including that of bystanders, is an important caveat when considering the potential role for bystander cell “killing” in the setting of transplantation. It is possible that early vigorous T cell reactivity can lead to impaired initial tissue engraftment, leading to generalized graft failure rather than mediating the injury of just the adjacent bystander cells. This is an important distinction because the results suggest that CD8 T cells may have relatively limited intrinsic capacity to trigger significant...
Fas-dependent injury to bystander islets, in distinction from the capacity of CD4 T cells to trigger this type of collateral cell injury. Consistent with true collateral damage rather than prevention of engraftment by CD4 T cells, CD4 TCR Tg recipients usually took 2–3 wk to reject the islets even though engraftment/revascularization of islets is normally evident within the first week after transplantation (24). This conclusion is also supported by a recent study showing CD8 T cells but not CD4 T cells have a substantial role in preventing islet engrafment (25).

It is intriguing that the bystander injury observed in this study is not compatible with an entirely nonspecific form of tissue injury. The finding that the MHC haplotype of the bystander target plays a role in the vulnerability to injury strongly suggests that there is some Ag-specific component to this response. Because TCRs are intrinsically biased to self-MHC recognition owing to positive selection, it is conceivable that specific T cells show some degree reactivity to self-MHC molecules (26), especially in the setting of tissue inflammation. Alternatively, in some cases adjacent self-type cells could conceivably acquire Ags from specific targets and represent these Ags to specific effector T cells in the context of self-MHC and serve as more specific targets. Currently, it is not clear how the MHC of the bystander tissue contributes to such injury; this will be an important subject of future studies.

The reasons for increased bystander killing in sensitized PD-1−/− mice may be due to the enhanced priming, cytokine (e.g., IFN-γ), or cytolytic responses of T cells. PD-1 inhibition of TCR signaling (27) may promote bystander cell survival by being more effective within bystander islets where the TCR stimulus would naturally be less than within target islets. That PD-1 is required to limit the collateral damage during an immune response has important implications for understanding the mechanism of action of new clinical approaches in cancer immunotherapy targeting PD-1 (28), where enhanced collateral damage may be a necessary component of treatment efficacy. Future studies on the role of other factors that can limit or prevent bystander killing are warranted. Given their increased ability to suppress weakly stimulated responses (29), regulatory T cells can be anticipated to also have a major role in limiting collateral damage and thereby enhance specificity of the immune response.

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Disclosures
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