Published in final edited form as:

Am J Transplant. 2008 September ; 8(9): 1809–1818. doi:10.1111/j.1600-6143.2008.02335.x.

The roles of CD8 central and effector memory T cell subsets in allograft rejection[1]

Martin H. Oberbarnscheidt[*], **Yue-Harn Ng**[†], and **Geetha Chalasani**[‡,2]

[†]Departments of Medicine (Renal-Electrolyte) and Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261

[‡]Starzl Transplantation Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261

Abstract

The contribution of secondary lymphoid tissue homing central memory T cells (T_{CM}) and peripheral tissue homing effector memory T cells (T_{EM}) to allograft rejection is not known. We tested whether T_{EM} is the principal subset responsible for allograft rejection due to the non-lymphoid location of target antigens. Skin allograft rejection was studied after transferring either CD8 T_{CM} or T_{EM} to wild type mice and to mice that lack secondary lymphoid tissues. We found that CD8 T_{CM} and T_{EM} were equally effective at rejecting allografts in wild type hosts. However, CD8 T_{EM} were significantly better than T_{CM} at rejecting allografts in the absence of secondary lymphoid tissues. CD8 T_{CM} were dependent upon secondary lymphoid tissues more than T_{EM} for optimal differentiation into effectors that migrate into the allograft. Recall of either CD8 T_{CM} or T_{EM} led to accumulation of T_{EM} after allograft rejection. These findings indicate that either CD8 T_{CM} or T_{EM} mediate allograft rejection but T_{EM} have an advantage over T_{CM} in immune surveillance of peripheral tissues, including transplanted organs.

Keywords

T cells; memory; transplantation

Introduction

The alloimmune response is a T-cell dependent process that culminates in the rejection of transplanted organs. The immune repertoire of adult humans contains high frequencies of alloreactive memory T cells that play an important role in allograft rejection (1, 2). The presence of donor-specific memory T cells correlates with acute and chronic rejection in humans and hinders tolerance induction in experimental animals (1, 3–7). Immunosuppressive strategies that inhibit naïve T cells are ineffective against memory T

[1]This work is supported by NIH grant AI059137 (GC) and John Merrill Transplant Research Scholar Award (GC) from the American Society of Nephrology and the American Society of Transplantation. MHO is a recipient of the American Society of Transplantation International Fellowship Award.

[2]Address correspondence and reprint requests to Dr. Geetha Chalasani, University of Pittsburgh School of Medicine, W1554 BST, 200 Lothrop Street, Pittsburgh, PA 15261. chalasanig@dom.pitt.edu.

[*]Present address: Department of Nephrology and Hypertension, University of Schleswig-Holstein, Campus Kiel, 24105 Kiel, Germany

cells because of several inherent advantages over their naïve counterparts such as markedly extended lifespan, lower activation threshold, increased proliferative capacity, rapid secretion of multiple effector cytokines, and homing to both lymphoid and non-lymphoid (peripheral) tissues (8–10).

Two subsets of memory T cells have been described in mice and humans based on their homing patterns and effector function: secondary lymphoid tissue-homing CD62L^{hi} CCR7^{hi} central memory (T_{CM}) T cells with delayed effector function, and peripheral tissue-homing CD62L^{lo} CCR7^{lo} effector memory (T_{EM}) T cells with immediate effector function (11–14). The contribution of CD8 T_{CM} and T_{EM} subsets to protective immunity against bacterial and viral infections is dependent upon the type and location of pathogen. CD8 T_{CM} are superior to T_{EM} at clearing systemic lymphocytic choriomeningitis and vesicular stomatitis virus infections; CD8 T_{CM} are equivalent to T_{EM} at clearing *Listeria monocytogenes* infection; while CD8 T_{CM} are inferior to T_{EM} at clearing Sendai and vaccinia virus infections, in mice (12, 15–18). However, the relative importance of memory T cell subsets in allograft rejection is less clear (7, 19, 20). Understanding which memory T cell subset is the principal mediator in allograft rejection has biomedical significance because agents that inhibit lymphocyte trafficking are potential candidates for use in clinical transplantation (21).

We hypothesized that peripheral tissue-homing T_{EM} is the principal memory subset responsible for allograft rejection since a transplanted organ represents not only a foreign antigen, but also a complex non-lymphoid peripheral tissue harboring antigen presenting cells (APCs) and endothelial cells that can modulate memory T cell behavior. To test this hypothesis, polyclonal alloreactive memory T cells generated in wild type (*wt*) hosts were sorted for CD8 T_{CM} or T_{EM} and transferred into *wt* and splenectomized alymphoplastic (*aly/aly*) recipients of skin allografts. Splenectomized *aly/aly* (*aly/aly-spleen*) mice lack all secondary lymphoid tissues and are therefore suitable for testing the function of transferred CD8 T_{CM} and T_{EM} in non-lymphoid tissues (22). We report that CD8 T_{CM} and T_{EM} were equally effective at mediating allograft rejection in *wt* hosts that have secondary lymphoid tissues. In contrast, CD8 T_{EM} were significantly more effective than T_{CM} at rejecting skin allografts in *aly/aly-spleen* hosts that lack all secondary lymphoid tissues. CD8 T_{EM} infiltration into the allograft was independent of secondary lymphoid tissues whereas T_{CM} were dependent upon secondary lymphoid tissues for optimal differentiation into effectors that migrate into the allograft. These results show that either CD8 T_{CM} or T_{EM} reject allografts effectively and CD8 T_{EM} is the principal subpopulation responsible for immune surveillance of transplanted organs.

Materials and Methods

Mice

C57BL/6 (Thy1.2, H-2^b; hereafter *wt*), B6.PL-*Thy1^a*/Cy (Thy1.1, H-2^b; hereafter Thy1.1) and BALB/c (H-2^d) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *Alymphoplasia* mice (*Map3k14$^{-/-}$*, Thy1.2, H-2^b, hereafter *aly/aly*) were purchased from CLEA (Osaka, Japan) (23). All animals were maintained under SPF conditions and procedures were performed as per IACUC guidelines.

Splenectomy, skin transplantation and co-stimulation blockade

Splenectomy and partial thickness skin transplantation were performed using established techniques (24). Skin grafts were monitored daily and rejection was defined as > 90% graft necrosis. *Wt* recipients of skin allografts were treated after transplantation with CTLA4-Ig (0.25mg, i.p.) and anti-CD40L (MR1) (0.25mg, i.p.) (Bioexpress Inc, MA) (days 0, 2, 4 and 6). Kaplan-Meier survival analysis was used to assess differences in allograft survival and p value of < 0.05 was considered significant.

Generation, isolation, and adoptive transfer of memory T cells

Thy1.1 mice were immunized with 3×10^7 BALB/c splenocytes (i.p.). 6 – 12 weeks later, spleen and lymph node (LN) cells were enriched for T cells by negative selection via MACS (Miltenyi Biotec, Auburn, CA), and sorted for $CD8^+CD44^{high}CD62L^{high}$ (CD8 T_{CM}) and $CD8^+CD44^{high}CD62L^{low}$ (CD8 T_{EM}) populations (> 95% purity) on BD FACS Aria. $1.5 – 2 \times 10^6$ CD8 T_{CM} or $4 – 6 \times 10^5$ CD8 T_{EM} containing similar numbers of alloreactive $IFN\gamma^+$ T cells were adoptively transferred in each experiment.

Cell harvest and enumeration after adoptive transfer

Adoptive hosts were sacrificed either at 8 days after transplantation (effector phase) or at 6 – 10 weeks after allograft rejection (secondary memory phase). Cells from spleen, lymph nodes, blood, liver, lungs, bone marrow and skin grafts were harvested as described (24–26). Harvested cells from tissues were counted and analyzed by flow cytometry after gating on $CD8^+Thy1.1^+$ population. Antigen-specific cells were analyzed by measuring BALB/c-reactive $IFN\gamma^+$ T cells within the harvested $CD8^+Thy1.1^+$ population by flow cytometry after intracellular cytokine staining (24). Statistical analyses was performed using unpaired Student's t test and differences with $p < 0.05$ were considered significant.

Flow cytometry, intracellular cytokine staining and in vivo cytotoxicity

Fluorochrome-tagged antibodies for flow cytometry were purchased from BD Pharmingen (San Diego, CA), eBioscience (San Diego, CA) and R&D systems (Minneapolis, MN). Intracellular IFNγ producing cells were detected after *ex-vivo* stimulation with BALB/c splenocytes ($H\text{-}2^d$) for 6-hrs in the presence of Brefeldin A. *In-vivo* cytotoxicity of CD8 T_{CM} and T_{EM} was assessed in *wt* B6 mice pre-treated with anti-NK1.1 (PK136, 300μg) to deplete NK cells. 3-days later, sorted CD8 T_{CM} (1.9×10^6) or T_{EM} (0.5×10^6) containing similar numbers of BALB/c-reactive $IFN\gamma^+$ T cells were transferred. Equal numbers of CFSE labeled syngeneic $H\text{-}2^b$ (5×10^6, 2μM, B6) and allogeneic $H\text{-}2^d$ (5×10^6, 0.2μM, BALB/c) splenocytes were injected. 12-hrs later, *in-vivo* target cell killing was measured as loss of allogeneic *vs* syngeneic cells in adoptive hosts of T_{CM} or T_{EM} compared to naïve controls using the formula: $100\text{-}[(\%H\text{-}2^d \text{ cells in memory T cell recipients}/\%H\text{-}2^b \text{ cells in memory T cell recipients} \div \%H\text{-}2^d \text{ cells in naïve mice}/\%H\text{-}2^b \text{ cells in naïve mice}) \times 100]$ (27). Flow acquisition was performed on LSRII analyzers (BD Biosciences, San Diego, CA), and data analyzed using Flowjo software (Treestar, Ashland, OR).

Results

Characterization of polyclonal alloreactive CD8 memory T cell subsets

Alloreactive memory T cells were generated in *wt* hosts to study polyclonal CD8 T_{CM} and T_{EM} subsets. Briefly, B6 Thy1.1 mice were immunized with BALB/c splenocytes. 6 – 12 weeks later, spleen and LN cells were harvested and sorted for CD8 memory T cell subsets by gating on $CD8^+CD44^{high}CD62L^{high}$ cells (CD8 T_{CM}) and $CD8^+CD44^{high}CD62L^{low}$ cells (CD8 T_{EM}) (Fig. 1A). Phenotypic analysis showed that CD8 T_{EM} contained a sub-population that is $CD69^{high}$, $1B11^{high}$, $CD27^{low}$ and $CD127^{low}$, suggesting a more activated phenotype and less dependence on co-stimulation and/or cytokines than T_{CM} (Fig. 1B) (17, 28, 29). *Ex-vivo* alloreactive IFNγ production was used to detect antigen-specific cells within the polyclonal CD8 T_{CM} and T_{EM} subsets since antigen-specific MHC-tetramer$^+$ cells in either subset were $IFN\gamma^+$ upon *ex-vivo* recall in viral studies (12). BALB/c-reactive $IFN\gamma^+$ T cells constituted 1.6 ± 0.3% of unfractionated CD8 memory T cells, 1.3 ± 0.3% of CD8 T_{CM}, and 4.4 ± 1.3% of CD8 T_{EM} subset (Figs. 1C–1D). Intracellular TNFα correlated with IFNγ but Granzyme B and IL-2 were not detected above background (data not shown) (12). BALB/c-reactive, $IFN\gamma^+$ cells within CD8 T_{CM} and T_{EM} increased over time upon *ex-vivo* re-stimulation and were approximately 3 – 4 fold more in T_{EM} than T_{CM} subset (Fig. 1E). These findings suggest that CD8 T_{EM} contained 3 – 4 fold more alloreactive memory T cells than T_{CM}.

In-vivo cytotoxicity of CD8 T_{CM} and T_{EM} was assessed after transfer to *wt* adoptive hosts pre-treated with anti-NK1.1 (PK136) antibody to inhibit NK cell mediated killing of allogeneic cells (30). Since CD8 T_{EM} contained 3 – 4 fold more alloreactive $IFN\gamma^+$ cells than T_{CM} (Fig. 1D), 3 – 4 fold more total T_{CM} (1.9×10^6) than T_{EM} (0.5×10^6) were transferred to *wt* hosts to compare similar numbers of antigen-specific cells. Spleen and LN from adoptive hosts were analyzed to assess killing since splenocyte target cells were found mainly in these tissues and not in peripheral tissues (data not shown). BALB/c-cell killing by CD8 T_{EM} was significantly better than T_{CM} in the spleen (27 ± 3% *vs* 15 ± 3%, $p = 0.04$, $n = 3$, Figs. 1F–1G), and comparable to T_{CM} in the LN (21 ± 3% *vs* 18 ± 7%, $p = 0.1$, NS, $n = 3$, Figs. 1F–1G). Therefore, both CD8 T_{CM} and T_{EM} killed allogeneic cells *in-vivo* but T_{EM} were better cytotoxic cells than T_{CM}.

Alloreactive CD8 T_{CM} and T_{EM} were assessed for the expression of chemokine and adhesion receptors that guide their migration and recruitment into inflammatory sites. As reported, CCR7 expression was higher on CD8 T_{CM} than T_{EM} cells (Fig. 1H) (12, 31). CXCR3, CCR5 and LFA-1 were upregulated on both subsets whereas VLA-4 ($\alpha_4\beta_1$) expression was restricted to CD8 T_{EM} (Fig. 1H). These findings suggest that both subsets can migrate in response to chemokines such as MIG, IP-10, I-TAC, MIP-1α, MIP-1β, and RANTES produced at inflammatory sites, including transplanted organs (32, 33). However, following migration, CD8 T_{EM} that express VLA-4 ($\alpha_4\beta_1$) can extravasate readily into allografts (34–38) and hence, might have an advantage over T_{CM} in causing allograft rejection.

Allograft rejection in adoptive hosts of CD8 T_{CM} and T_{EM}

To test which alloreactive CD8 memory T cell subset is more effective at allograft rejection, skin allograft survival was compared in adoptive hosts of CD8 T_{CM} and T_{EM}. Sorted CD8 T_{CM} and T_{EM} (Fig. 1A) were transferred into *wt* recipients of BALB/c-skin allografts. Since CD8 T_{EM} contained 3 – 4 fold more alloreactive IFNγ^+ T cells than T_{CM} (Fig. 1D), 3 – 4 fold more total T_{CM} ($1.5 \times 10^6 – 2 \times 10^6$) than T_{EM} ($0.5 \times 10^6 – 0.6 \times 10^6$) were transferred in each experiment to compare similar numbers of antigen-specific cells. *Wt* hosts of CD8 T_{CM} or T_{EM} rejected skin allografts comparable to sensitized recipients (MST = 15, 15, and 16 days, respectively, $n = 3 – 4$/grp; $p = 0.4$, NS), and not significantly different from naïve mice (MST = 18 days, $n = 4$; $p = 0.06$ and 0.4, respectively, NS) (Fig. 2A). Since endogenous naïve T cells also contributed to observed skin allograft rejection in *wt* hosts, recipients were treated with CTLA4-Ig and anti-CD40L to inhibit naïve T cell activation to better assess rejection mediated by transferred CD8 T_{CM} and T_{EM} (5, 6, 39). Recall of either CD8 T_{CM} or T_{EM} subset in *wt* recipients treated with co-stimulation blockade led to skin allograft rejection that was comparable to sensitized recipients (MST = 14, 16 and 15 days, respectively, $n = 4 – 6$/grp; $p = 0.06$ and 0.5, respectively, NS) and significantly accelerated than naïve recipients (MST = 25 days, $n = 4$; $p = 0.02$) (Fig. 2B). Therefore, in *wt* hosts, both CD8 T_{CM} and T_{EM} subsets were equally effective in mediating allograft rejection.

CD8 T_{CM} have a resting phenotype and do not express VLA-4, suggesting that further differentiation is required for their extravasation and function in non-lymphoid tissues (Figs. 1B and 1H). It is not clear if the preferential location of CD8 T_{CM} within secondary lymphoid tissues suggests dependence on these tissues for differentiation into effectors analogous to naïve T cells (22). To address this question, sorted CD8 T_{CM} ($1.5 \times 10^6 – 2 \times 10^6$) or T_{EM} ($0.4 \times 10^6 – 0.6 \times 10^6$) containing similar numbers of alloreactive IFNγ^+ T cells were transferred to *aly/aly-spleen* mice and BALB/c-skin allograft rejection was tested. Skin allograft rejection in *aly/aly-spleen* recipients of CD8 T_{CM} was significantly delayed than in recipients of T_{EM} (MST = 57 days, $n = 7$ *vs* MST = 36 days, $n = 5$, respectively; $p = 0.008$, Fig. 2C). Allografts in *aly/aly-spleen* mice that did not receive memory T cells survived > 100 days compared to recipients of memory T cells ($n = 4$; $p = 0.0002$, Fig. 2C). These data show that alloreactive CD8 T_{EM} were significantly more effective than T_{CM} in causing allograft rejection in the absence of secondary lymphoid tissues. However, in adoptive hosts of CD8 T_{EM}, skin allograft rejection was accelerated in *wt* than in *aly/aly-spleen* recipients (MST 16 and 36 days, respectively, $n = 5 – 6$/grp; $p = 0.003$) (Figs. 2B–2C) suggesting that secondary lymphoid organs support recall of both subsets.

Recall and differentiation of CD8 T_{CM} and T_{EM} in adoptive hosts

To understand why allograft rejection was delayed in *aly/aly-spleen* hosts of CD8 T_{CM}, differentiation of CD8 T_{CM} and T_{EM} into effectors and migration into the allograft was compared in adoptive hosts. Sorted CD8 T_{CM} (2×10^6) or T_{EM} (0.5×10^6) containing similar numbers of alloreactive IFNγ^+ T cells were transferred into *wt* and *aly/aly-spleen* hosts that underwent BALB/c-skin transplantation. 8 days later, cells were harvested from lymphoid and non-lymphoid tissues in adoptive hosts, and analyzed after gating on CD8$^+$Thy1.1$^+$ T cells. A large population of CD8 T_{CM} had acquired a CD49d^{high} CD62L^{low} effector phenotype in *wt* hosts (50 ± 10%, $n = 3$) compared to *aly/aly-spleen* hosts (15 ± 4%,

n = 4) suggesting that differentiation of CD8 T_{CM} into effectors was impaired in the absence of secondary lymphoid tissues (Fig. 3A). CD8 T_{EM} retained a $CD49d^{high}$ $CD62L^{low}$ effector phenotype in harvested tissues of both adoptive hosts (Fig. 3A). BALB/c-reactive $IFN\gamma^+$ T cells derived from CD8 T_{CM} and T_{EM} were found mainly in the $CD49d^{high}$ population (data not shown). At 8 days after skin transplantation, BALB/c-reactive $IFN\gamma^+$ T cells present in the harvested $CD8^+Thy1.1^+$ T cells were found exclusively within the proliferated $CFSE^{low}$ population in adoptive hosts of CD8 T_{CM} and T_{EM} suggesting differentiation into alloreactive effectors (Fig. 3B). Transferred CD8 T_{CM} and T_{EM} contained $CD44^{hi}$ memory phenotype T cells of irrelevant specificities that possibly underwent bystander proliferation in adoptive hosts contributing to $CFSE^{low}$ cells within the $CD8^+Thy1.1^+$ population that were not $IFN\gamma^+$ (Fig. 3B) (40–42). $CFSE^{low}$ $IFN\gamma^+$ effector T cells from CD8 T_{CM} in *wt* constituted a larger population of harvested $CD8^+Thy1.1^+$ cells than in *aly/aly-spleen* hosts (15 ± 5% *vs* 1.5 ± 0.5%, *n* = 3, respectively) compared to $CFSE^{low}$ $IFN\gamma^+$ effectors from T_{EM} (6 ± 3% *vs* 3 ± 2%, *n* = 3, respectively) (Fig. 3B). The greater proliferative advantage of alloreactive CD8 T_{CM} in *wt* hosts contributed to 100-fold more alloreactive $IFN\gamma^+$ effectors from T_{CM} compared to a 4-fold increase in alloreactive effectors from T_{EM} in *wt* than in *aly/aly-spleen* hosts ($p = 0.008$ and $p = 0.06$, NS, respectively, *n* = 3 – 4, Figs. 3C–3D). Alloreactive effector T cells from CD8 T_{CM} compared to T_{EM} were 13-fold more in *wt* hosts ($p = 0.03$, *n* = 3 – 4) and 3-fold less in *aly/aly-spleen* hosts ($p = 0.07$, NS, *n* = 3 – 4) at 8 days (Figs. 3C–3D). Fewer alloreactive $IFN\gamma^+$ effector T cells from CD8 T_{CM} in *aly/aly-spleen* hosts was not due to impaired survival since T_{CM} were recovered from naïve adoptive hosts in comparable numbers (Fig. 3E) (24), and ratio of recovered T_{CM} to T_{EM} (3.5:1) in allograft recipients was similar to cells transferred (Figs. 3F–3G). At 30-days after transplantation, more CD8 T_{CM} in *aly/aly-spleen* hosts had acquired a $CD49d^{high}$ $CD62L^{low}$ effector phenotype and differentiated into 3-fold more alloreactive $IFN\gamma^+$ effector T cells than T_{EM} (Figs. 3H–3I). These findings suggested that differentiation of CD8 T_{CM} into effectors was significantly delayed in the absence of secondary lymphoid tissues.

Migration of alloreactive effectors derived from CD8 T_{CM} and T_{EM} and their infiltration into skin allografts was compared in adoptive hosts. Alloreactive $IFN\gamma^+$ effector T cells from either CD8 T_{CM} or T_{EM} migrated broadly to non-lymphoid tissues and infiltrated into skin allografts to a comparable extent in *wt* hosts (Fig. 4A*;* $p = 0.08$, NS, *n* = 3 – 4, Fig. 4C). Despite greater numbers of total alloreactive effectors from CD8 T_{CM} than T_{EM} in *wt* hosts (Figs. 3B – 3D), alloreactive effectors from T_{EM} were comparable to T_{CM} in skin allografts suggesting that early migration and accumulation of T_{EM} in the allograft contributed to equally effective allograft rejection as T_{CM} (Fig. 2B). In *aly/aly-spleen* allograft recipients, alloreactive $IFN\gamma^+$ effector T cells from CD8 T_{CM} were found mainly in lungs and liver but not in skin allograft, whereas alloreactive effectors derived from T_{EM} were found in lungs, bone marrow and skin allograft at 8 days (Fig. 4B; $p = 0.01$, *n* = 3 – 4, Fig. 4D). Alloreactive $IFN\gamma^+$ effector T cells from both subsets were found in blood, lung, liver, skin allograft (Fig. 4E) and more alloreactive effectors from T_{EM} than T_{CM} had infiltrated skin allografts (Fig. 4E–4F) despite fewer total alloreactive $IFN\gamma^+$ effectors derived from T_{EM} (Fig. 3I) in *aly/aly-spleen* recipients at 30-days. Thus, CD8 T_{EM} infiltrated skin allografts early after transplantation whereas differentiation of T_{CM} into alloreactive effectors that infiltrate the

allograft was delayed in the absence of secondary lymphoid tissues contributing to significantly delayed allograft rejection.

Persistence of CD8 T_{CM} and T_{EM} in adoptive hosts after allograft rejection

Hallmark of memory T cell recall is the generation of secondary memory T cells that mediate better protective immunity (43, 44). To test which alloreactive CD8 memory subset persists as secondary memory T cells after allograft rejection, sorted CD8 T_{CM} (2×10^6) or T_{EM} (0.5×10^6) containing similar numbers of alloreactive memory T cells were transferred to *wt* and *aly/aly-spleen* skin allograft recipients. 6 – 10 weeks after rejection, cells were harvested from lymphoid and non-lymphoid tissues and analyzed after gating on $CD8^+Thy1.1^+$ T cells. BALB/c-reactive $IFN\gamma^+$ T cells within the harvested $CD8^+Thy1.1^+$ population were measured to assess antigen-specific secondary memory T cells derived from T_{CM} and T_{EM}. Alloreactive $IFN\gamma^+$ T cells derived from CD8 T_{CM} and T_{EM} were comparable in harvested tissues of adoptive hosts ($n = 4 – 5$/grp) (Figs. 5A and 5B) suggesting that both subsets persist as secondary memory T cells in either *wt* or *aly/aly-spleen* adoptive hosts after allograft rejection. Recall of CD8 T_{CM} in *wt* hosts led to both $CD62L^{high}$ $CD49d^{low}$ T_{CM} and $CD62L^{low}$ $CD49d^{high}$ T_{EM} that contained alloreactive $IFN\gamma^+$ T cells compared to $CD62L^{low}$ $CD49d^{high}$ alloreactive T_{EM} alone in *aly/aly-spleen* hosts (Figs. 5C and 5D). CD8 T_{EM} persisted as $CD62L^{low}$ $CD49d^{high}$, alloreactive T_{EM} in either *wt* or *aly/aly-spleen* hosts (Figs. 5C and 5D). Thus, CD8 T_{CM} alone differentiated into alloreactive T_{CM} secondary memory after allograft rejection in *wt* and not in *aly/aly-spleen* hosts suggesting that generation of T_{CM} secondary memory requires secondary lymphoid tissues and/or that non-lymphoid tissues promote differentiation of T_{CM} to T_{EM} (29). T_{CM} *vs* T_{EM} phenotype of alloreactive secondary memory T cells was confirmed by assessing their tissue distribution in adoptive hosts. In *wt* hosts, alloreactive $IFN\gamma^+$ T cells from CD8 T_{CM} were found mainly in spleen and LNs than non-lymphoid tissues while those from T_{EM} were found in spleen, bone marrow and liver ($n = 4$) (Fig. 5E). Alloreactive $IFN\gamma^+$ T cells from either CD8 T_{CM} or T_{EM} were comparable in bone marrow, lung and liver tissues in *aly/aly-spleen* hosts ($n = 5$) (Fig. 5F). Thus, either CD8 T_{CM} or T_{EM} persisted as alloreactive secondary memory in lymphoid and non-lymphoid tissues and both subsets led to T_{EM} after allograft rejection that can function independently of secondary lymphoid tissues.

Discussion

We addressed the roles of alloreactive CD8 T_{CM} and T_{EM} subsets in allograft rejection using an *in vivo* polyclonal system that is analogous to physiologic immune responses in the transplant setting. Alloreactive memory T cells were generated in *wt* hosts, sorted into CD8 T_{CM} and T_{EM} subsets and transferred to *wt* or *aly/aly-spleen* hosts that lack secondary lymphoid tissues to compare skin allograft rejection. We find that CD8 T_{CM} and T_{EM} were equally effective at mediating skin allograft rejection in *wt* hosts. However, in hosts that lack secondary lymphoid tissues, CD8 T_{EM} were significantly more effective than T_{CM} in mediating skin allograft rejection. Optimal differentiation of CD8 T_{CM} into effectors that migrate into the allograft was impaired in the absence of secondary lymphoid tissues leading to delayed allograft rejection. Following allograft rejection, either CD8 T_{CM} or T_{EM} can

persist as alloreactive T_{EM} secondary memory that function independently of secondary lymphoid tissues.

Allograft rejection by CD8 T_{CM} and T_{EM} was addressed in an *in-vivo* polyclonal system since (a) alloimmune responses target a diverse array of antigenic epitopes and are polyclonal (45); (b) function of a monoclonal population of cells such as TCR-tg T cells may not be representative of physiologic immune responses; and (c) TCR-tg T cells result in artificially high antigen specific precursor frequencies that skew the generation of T_{CM} *vs* T_{EM} and alter the outcome of immune responses (46–48). Antigen-specific cells present within the polyclonal CD8 T_{CM} and T_{EM} subsets were detected by identifying alloreactive IFNγ^+ T cells. CD8 T_{CM} or T_{EM} containing similar numbers of alloreactive IFNγ^+ cells were transferred to accurately compare function of CD8 T_{CM} *vs* T_{EM} that led to transfer of 3 – 4 fold more total CD8 T_{CM} than T_{EM} in each experiment. Using this approach might have lead to transfer of more alloreactive CD8 T_{CM} than T_{EM} and possibly underestimated the functional advantages of T_{EM} over T_{CM} *in vivo*. Inspite of transferring more total CD8 T_{CM}, T_{EM} were equally effective to T_{CM} in *wt* hosts and better than T_{CM} in *aly/aly-spleen* hosts in rejecting allografts. When total number of transferred CD8 T_{CM} was matched to T_{EM} (0.5×10^6 each), skin allograft survival in *aly/aly-spleen* hosts was prolonged >100 days (data not shown).

Secondary lymphoid organs supported differentiation of both CD8 T_{CM} and T_{EM} resulting in increased alloreactive effectors and accelerated skin allograft rejection in *wt* compared to *aly/aly-spleen* hosts. It is possible that residual activation of endogenous T and B cells despite co-stimulation blockade also contributed to allograft rejection in *wt* recipients. CD8 T_{CM} and T_{EM} mediated equally effective skin allograft rejection in *wt* hosts whereas T_{EM} functioned significantly better than T_{CM} in *aly/aly-spleen* hosts. Differentiation of CD8 T_{CM} into effectors that upregulated VLA-4 and migrated into the allograft was significantly delayed in the absence of secondary lymphoid tissues. Alloreactive CD8 T_{EM} expressed VLA-4 constitutively that could have contributed to early infiltration into the allograft independent of differentiation in secondary lymphoid tissues. CD8 T_{EM} were also better than T_{CM} in lysis of allogeneic target cells *in-vivo*. These subtle advantages of CD8 T_{EM} over T_{CM} led to more rapid allograft rejection by T_{EM} in hosts that lacked secondary lymphoid tissues but were of lesser significance in *wt* hosts that supported differentiation of both subsets. It is possible that optimal activation of CD8 T_{CM} requires interaction with antigen-bearing professional APCs that is promoted in secondary lymphoid organs (49). Since skin allograft rejection is a lymph-node dependent response (22, 50) and more likely to be dependent on CD8 T_{CM} than T_{EM}, it remains to be determined whether CD8 T_{CM} and T_{EM} respond similarly to other organ allografts (such as heart and kidney) that are less dependent on LN and more dependent on spleen (51–53).

Migration to sites where antigen is located and extravasation into inflamed tissues is essential for immune surveillance (54, 55). Although either CD8 T_{CM} or T_{EM} subset can cause allograft rejection, ability of CD8 T_{EM} to extravasate directly into allografts is an advantage over T_{CM} in promoting allograft rejection when activation requirements are constrained. The findings reported here implicate alloreactive CD8 T_{EM} as the principal subset responsible for immune surveillance of allografts. Similarly, T_{EM} control viruses

residing in peripheral tissues and limit metastatic invasion in colorectal cancer (55–57). Our findings along with these reports emphasize the role of T_{EM} in immune surveillance of non-lymphoid tissues.

Acknowledgements

We are indebted to Dr. Fadi Lakkis for helpful discussions and greatly appreciate critical review of the manuscript by Drs. Robert Hendricks, Jennifer Woodward and Rosemary Hoffman. We thank Lonnette Diggs (Yale University) and Autumn Marlowe (University of Pittsburgh) for technical assistance.

Abbreviations used in this paper

aly	alymphoplasia
T_{CM}	central memory
T_{EM}	effector memory

References

1. Heeger PS, Greenspan NS, Kuhlenschmidt S, Dejelo C, Hricik DE, Schulak JA, et al. Pretransplant frequency of donor-specific, IFN-γ-producing lymphocytes is a manifestation of immunologic memory and correlates with the risk of posttransplant rejection episodes. J Immunol. 1999; 163:2267–2275. [PubMed: 10438971]
2. Akbar AN, Amlot PL, Timms A, Lombardi G, Lechler R, Janossy G. The development of primed/memory CD8+ lymphocytes in vitro and in rejecting kidneys after transplantation. Clin Exp Immunol. 1990; 81:225–231. [PubMed: 1974831]
3. Najafian N, Salama A, Fedoseyeva E, Benichou G, Sayegh M. Enzyme-linked immunosorbent spot assay analysis of peripheral blood lymphocyte reactivity to donor HLA-DR peptides: potential novel assay for prediction of outcomes for renal transplant recipients. J Am Soc Nephrol. 2002; 13:252–259. [PubMed: 11752045]
4. Pearl JP, Parris J, Hale DA, Hoffman SC, Bernstein WB, McCoy KL, et al. Immunocompetent T cells with a memory-like phenotype are the dominant cell type following antibody-mediated T-cell depletion. Am J Transplant. 2005; 5:465–474. [PubMed: 15707400]
5. Valujskikh A, Pantenburg B, Heeger P. Primed allospecific T cells prevent the effects of costimulatory blockade on prolonged cardiac allograft survival in mice. Am J Transplant. 2002; 2:501–509. [PubMed: 12118893]
6. Zhai Y, Meng L, Gao F, Busuttil R, Kupiec-Weglinski J. Allograft rejection by primed/memory CD8+ T cells is CD154 blockade resistant: therapeutic implications for sensitized transplant recipients. J Immunol. 2002; 169:4667–4673. [PubMed: 12370407]
7. Adams A, Williams A, Jones T, Shirasugi N, Durham M, Kaech S, et al. Heterologous immunity provides a potent barrier to transplantation tolerance. J Clin Invest. 2003; 111:1887–1895. [PubMed: 12813024]
8. Kedl RM, Mescher MF. Qualitative differences between naive and memory T cells make a major contribution to the more rapid and efficient memory CD8+ T cell response. J Immunol. 1998; 161:674–683. [PubMed: 9670942]
9. Masopust D, Vezys V, Marzo AL, Lefrancois L. Preferential localization of effector memory cells in nonlymphoid tissue. Science. 2001; 291:2413–2417. [PubMed: 11264538]
10. Gourley TS, Wherry EJ, Masopust D, Ahmed R. Generation and maintenance of immunological memory. Semin Immunol. 2004; 16:323–333. [PubMed: 15528077]
11. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature. 1999; 401:708–712. [PubMed: 10537110]

12. Wherry E, Teichgraber V, Becker T, Masopust D, Kaech S, Antia R, et al. Lineage relationship and protective immunity of memory CD8 T cell subsets. Nat Immunol. 2003; 4:225–234. [PubMed: 12563257]
13. Geginat J, Sallusto F, Lanzavecchia A. Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4+ T cells. J Exp Med. 2001; 194:1711–1719. [PubMed: 11748273]
14. Weninger W, Crowley MA, Manjunath N, von Andrian UH. Migratory properties of naive, effector, and memory CD8+ T cells. J Exp Med. 2001; 194:953–966. [PubMed: 11581317]
15. Klonowski KD, Marzo AL, Williams KJ, Lee S-J, Pham Q-M, Lefrancois L. CD8 T cell recall responses are regulated by the tissue tropism of the memory cell and pathogen. J Immunol. 2006; 177:6738–6746. [PubMed: 17082587]
16. Roberts A, Woodland D. Cutting edge: Effector memory CD8+ T cells play a prominent role in recall responses to secondary viral infection in the lung. J Immunol. 2004; 172:6533–6537. [PubMed: 15153466]
17. Bachmann MF, Wolint P, Schwarz K, Jager P, Oxenius A. Functional properties and lineage relationship of CD8+ T cell subsets identified by expression of IL-7 receptor alpha and CD62L. J Immunol. 2005; 175:4686–4696. [PubMed: 16177116]
18. Moyron-Quiroz JE, Rangel-Moreno J, Hartson L, Kusser K, Tighe MP, Klonowski KD, et al. Persistence and responsiveness of immunologic memory in the absence of secondary lymphoid organs. Immunity. 2006; 25:643–654. [PubMed: 17045819]
19. Zhang Q, Chen Y, Fairchild RL, Heeger PS, Valujskikh A. Lymphoid sequestration of alloreactive memory CD4 T cells promotes cardiac allograft survival. J Immunol. 2006; 176:770–777. [PubMed: 16393960]
20. Tang AL, Bingaman AW, Kadavil EA, Leeser DB, Farber DL. Generation and functional capacity of polyclonal alloantigen-specific memory CD4 T cells. Am J Transplant. 2006; 6:1275–1284. [PubMed: 16686752]
21. Yabu JM, Vincenti F. Novel immunosuppression: small molecules and biologics. Semin Nephrol. 2007; 27:479–486. [PubMed: 17616278]
22. Lakkis FG, Arakelov A, Konieczny BT, Inoue Y. Immunologic 'ignorance' of vascularized organ transplants in the absence of secondary lymphoid tissue. Nat Med. 2000; 6:686–688. [PubMed: 10835686]
23. Miyawaki S, Nakamura Y, Suzuka H, Koba M, Yasumizu R, Ikehara S, et al. A new mutation *aly*, that induces a generalized lack of lymph nodes accompanied by immunodeficiency in mice. Eur J Immunol. 1994; 24:429–434. [PubMed: 8299692]
24. Obhrai J, Oberbarnscheidt M, Hand T, Diggs L, Chalasani G, Lakkis F. Effector T cell differentiation and memory T cell maintenance outside secondary lymphoid organs. J Immunol. 2006; 176:4051–4058. [PubMed: 16547240]
25. Chervenick PA, Boggs DR, Marsh JC, Cartwright GE, Wintrobe MM. Quantitative studies of blood and bone marrow neutrophils in normal mice. Am J Physiol. 1968; 215:353–360. [PubMed: 5665168]
26. Nasr IW, Reel M, Oberbarnscheidt MH, Mounzer RH, Baddoura FK, Ruddle NH, et al. Tertiary lymphoid tissues generate effector and memory T cells that lead to allograft rejection. Am J Transplant. 2007; 7:1071–1079. [PubMed: 17359505]
27. Barber DL, Wherry EJ, Ahmed R. Cutting edge: rapid in vivo killing by memory CD8 T cells. J Immunol. 2003; 171:27–31. [PubMed: 12816979]
28. Baars PA, Sierro S, Arens R, Tesselaar K, Hooibrink B, Klenerman P, et al. Properties of murine (CD8+)CD27- T cells. Eur J Immunol. 2005; 35:3131–3141. [PubMed: 16220536]
29. Marzo AL, Yagita H, Lefrancois L. Cutting edge: migration to nonlymphoid tissues results in functional conversion of central to effector memory CD8 T cells. J Immunol. 2007; 179:36–40. [PubMed: 17579018]
30. Brehm MA, Daniels KA, Ortaldo JR, Welsh RM. Rapid conversion of effector mechanisms from NK to T cells during virus-induced lysis of allogeneic implants in vivo. J Immunol. 2005; 174:6663–6671. [PubMed: 15905505]

31. Unsoeld H, Krautwald S, Voehringer D, Kunzendorf U, Pircher H. CCR7+ and CCR7− memory T cells do not differ in immediate effector cell function. J Immunol. 2002; 169:638–641. [PubMed: 12097363]
32. El-Sawy T, Fahmy N, Fairchild R. Chemokines: directing leukocyte infiltration into allografts. Curr Opin Immunol. 2002; 14:562–568. [PubMed: 12183154]
33. Carvalho-Gasper M, Stephen Billing J, Spriewald BM, Wood KJ. Chemokine gene expression during allograft rejection: Comparison of two quantitative PCR techniques. J Immunol Methods. 2005; 301:41–52. [PubMed: 16018884]
34. Bevilacqua MP. Endothelial-Leukocyte Adhesion Molecules. Annu Rev Immunol. 1993; 11:767–804. [PubMed: 8476577]
35. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell. 1994; 76:301–314. [PubMed: 7507411]
36. Mobley JL, Dailey MO. Regulation of adhesion molecule expression by CD8 T cells in vivo. I. Differential regulation of gp90MEL-14 (LECAM-1), Pgp-1, LFA-1, and VLA-4 alpha during the differentiation of cytotoxic T lymphocytes induced by allografts. J Immunol. 1992; 148:2348–2356. [PubMed: 1373165]
37. Stegall MD, Dean PG, Ninova D, Cohen AJ, Shepard GM, Gup C, et al. α4 integrin in iselt allograft rejection. Transplantation. 2001; 71:1549–1555. [PubMed: 11435963]
38. Christensen JP, Andersson EC, Scheynius A, Marker O, Thomsen A. Alpha 4 integrin directs virus-activated CD8+ T cells to sites of infection. J Immunol. 1995; 154:5293–5301. [PubMed: 7537304]
39. Larsen C, Elwood E, Alexander D, Ritchie S, Hendrix R, Tucker-Burden C, et al. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. Nature. 1996; 381:434–438. [PubMed: 8632801]
40. Judge A, Zhang X, Fujii H, Surh C, Sprent J. Interleukin 15 controls both proliferation and survival of a subset of memory-phenotype CD8+ T cells. J Exp Med. 2002; 196:935–946. [PubMed: 12370255]
41. Tough DF, Zhang X, Sprent J. An IFN-gamma-dependent pathway controls stimulation of memory phenotype CD8+ T cell turnover in vivo by IL-12, IL-18, and IFN-gamma. J Immunol. 2001; 166:6007–6011. [PubMed: 11342616]
42. Eberl G, Brawand P, Macdonald HR. Selective bystander proliferation of memory CD4+ and CD8+ T cells upon NK T or T cell activation. J Immunol. 2000; 165:4305–4311. [PubMed: 11035065]
43. Masopust D, Ha SJ, Vezys V, Ahmed R. Stimulation history dictates memory CD8 T cell phenotype: implications for prime-boost vaccination. J Immunol. 2006; 177:831–839. [PubMed: 16818737]
44. Jabbari A, Harty JT. Secondary memory CD8+ T cells are more protective but slower to acquire a central-memory phenotype. J Exp Med. 2006; 203:919–932. [PubMed: 16567385]
45. Sherman LA, Chattopadhyay S. The molecular basis of allorecognition. Annu Rev Immunol. 1993; 11:385–402. [PubMed: 8476567]
46. Hataye J, Moon JJ, Khoruts A, Reilly C, Jenkins MK. Naive and memory CD4+ T cell survival controlled by clonal abundance. Science. 2006; 312:114–116. [PubMed: 16513943]
47. Marzo AL, Klonowski KD, Le Bon A, Borrow P, Tough DF, Lefrancois L. Initial T cell frequency dictates memory CD8+ T cell lineage commitment. Nat Immunol. 2005; 6:793–799. [PubMed: 16025119]
48. Ford ML, Koehn BH, Wagener ME, Jiang W, Gangappa S, Pearson TC, et al. Antigen-specific precursor frequency impacts T cell proliferation, differentiation, and requirement for costimulation. J Exp Med. 2007; 204:299–309. [PubMed: 17261633]
49. Zammit DJ, Cauley LS, Pham QM, Lefrancois L. Dendritic cells maximize the memory CD8 T cell response to infection. Immunity. 2005; 22:561–570. [PubMed: 15894274]
50. Barker CF, Billingham RE. The role of afferent lymphatics in the rejection of skin homografts. J Exp Med. 1968; 128:197–221. [PubMed: 4873840]
51. Medawar PB. The homograft reaction. Proc Roy Soc (London), Ser B. 1957; 149:145–148. [PubMed: 13614379]

52. Vetto RM, Lawson RK. The role of vascular endothelium in the afferent pathway as suggested by the alymphatic renal homotransplant. Transplantation. 1967; 5:1537–1539.

53. Larsen CP, Morris PJ, Austyn JM. Migration of dendritic leukocytes from cardiac allografts into host spleens. J Exp Med. 1990; 171:307–314. [PubMed: 2404081]

54. Stock AT, Jones CM, Heath WR, Carbone FR. Cutting edge: central memory T cells do not show accelerated proliferation or tissue infiltration in response to localized herpes simplex virus-1 infection. J Immunol. 2006; 177:1411–1415. [PubMed: 16849445]

55. Bachmann MF, Wolint P, Schwarz K, Oxenius A. Recall proliferation potential of memory CD8+ T cells and antiviral protection. J Immunol. 2005; 175:4677–4685. [PubMed: 16177115]

56. Pagès F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R, et al. Effector memory T cells, early metastasis, and survival in colorectal cancer. N Engl J Med. 2005; 353:2654–2666. [PubMed: 16371631]

57. Wüthrich C, Kesari S, Kim W-K, Williams K, Gelman R, Elmeric D, et al. Characterization of lymphocytic infiltrates in progressive multifocal leukoencephalopathy: Co-localization of CD8 T cells with JCV-infected glial cells. Neurology. 2006; 68:985–990.

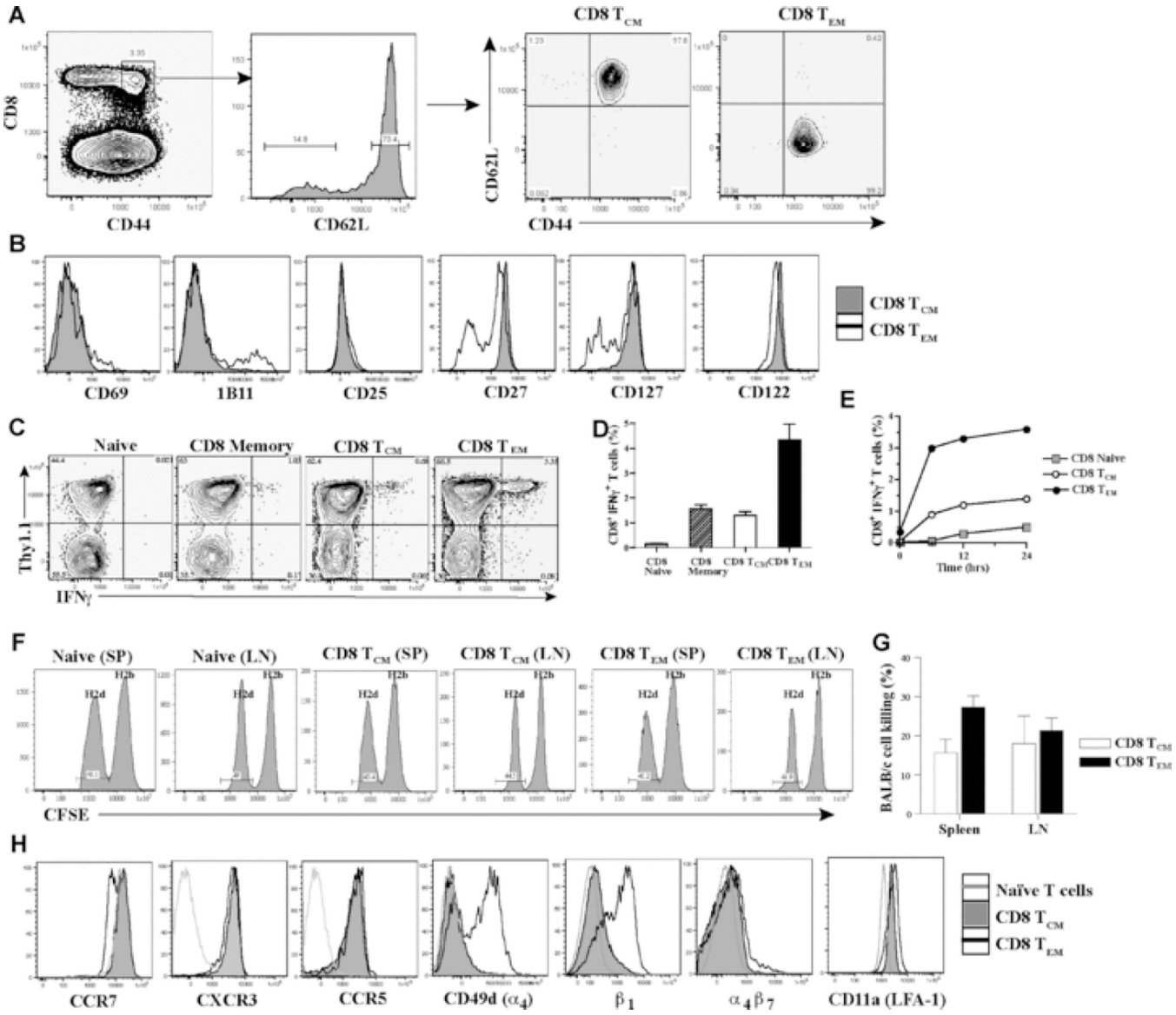

Fig. 1. Characterization of polyclonal alloreactive CD8 memory T cell subsets

Thy1.1 mice were immunized with BALB/c splenocytes (3×10^7, i.p.) and spleen and LN cells were harvested 6 – 12 weeks later to sort for CD8 T_{CM} and T_{EM}. **(A)** Sorting of CD8 memory T cell subsets. CD8 memory T cell subsets were sorted after gating on $CD8^+CD44^{high}CD62L^{high}$ cells (CD8 T_{CM}) and $CD8^+CD44^{high}CD62L^{low}$ cells (CD8 T_{EM}). Sorted cells were tested for purity prior to adoptive transfer. **(B)** Phenotype of CD8 T_{CM} and T_{EM}. Expression of CD69, 1B11, CD25, CD27, CD127 and CD122 on CD8 T_{CM} and T_{EM} is shown (representative FACS plots of 5 – 6 experiments). **(C)** *Ex-vivo* IFNγ production by CD8 T_{CM} and T_{EM}. Sorted CD8 T_{CM} and T_{EM} were assessed for IFNγ production by intracellular cytokine staining after 5-hr *ex-vivo* stimulation with BALB/c splenocytes. IFNγ production within the $Thy1.1^+$ population is shown after gating on $CD8^+$ T cells. Naïve T cells from unimmunized Thy1.1 mice (Naïve) and unfractionated sorted $CD8^+$ $CD44^{high}$ memory T cells (CD8 memory) from immunized Thy1.1 mice that contained both

CD62L^{high} and CD62L^{low} cells were used as controls. Representative FACS plots of 4 – 5 experiments are shown. **(D)** Quantitation of alloreactive IFNγ^+ T cells within CD8 T_{CM} and T_{EM} subsets after 5-hr *ex-vivo* stimulation with BALB/c splenocytes. Percent of IFNγ^+ T cells within naïve, unfractionated CD8 memory (memory) and sorted CD8 T_{CM} and T_{EM} populations. Mean ± SD of 4 – 5 experiments. **(E)** Alloreactive IFNγ^+ T cells within CD8 T_{CM} and T_{EM} subsets upon *ex-vivo* stimulation with BALB/c splenocytes over time. Percent of IFNγ^+ T cells within naïve, sorted CD8 T_{CM} and T_{EM} populations after *ex-vivo* stimulation with BALB/c splenocytes at 6hrs, 12hrs and 24hrs. **(F)** *In-vivo* cytotoxicity of alloreactive CD8 T_{CM} and T_{EM} subsets in *wt* adoptive hosts. *Wt* mice were treated with anti-NK1.1 (PK136, 300μg, i.p.) to deplete NK cells. 3-days later, sorted CD8 T_{CM} (1.9×10^6) or CD8 T_{EM} (5×10^5) containing similar numbers of BALB/c-reactive IFNγ^+ T cells were transferred to *wt* adoptive hosts followed by injection of equal numbers of CFSE labeled H-2^b (5×10^6, 2μM B6, syngeneic) and H-2^d (5×10^6, 0.2μM, BALB/c, allogeneic) splenocytes on the same day. 12-hrs later, *in vivo* killing of target cells was measured in harvested spleen and LN cells as loss of H-2^d target cells compared to loss of H-2^b syngeneic cells in adoptive hosts of memory T cells (T_{CM} or T_{EM}) *versus* naïve B6 control mice (as described in Materials and Methods) (representative FACS plots of $n = 3$ mice/grp are shown). **(G)** Quantitation of *in-vivo* specific lysis of allogeneic cells by alloreactive CD8 T_{CM} and T_{EM} subsets in *wt* adoptive hosts. Percent of BALB/c-cell lysis in harvested spleen and LNs from *wt* adoptive hosts of CD8 T_{CM} or T_{EM}. Mean ± SD of $n = 3$ mice/grp. **(H)** Expression of chemokine and adhesion receptors on CD8 T_{CM} and T_{EM}. Expression of CCR7, CXCR3, CCR5, CD49d (α_4-integrin), β_1-integrin, $\alpha_4\beta_7$ and CD11a (LFA-1) on CD8 T_{CM} and T_{EM} subsets shown in comparison to CD8$^+$ CD44^{low} naïve T cells (representative FACS plots of 3 – 4 experiments).

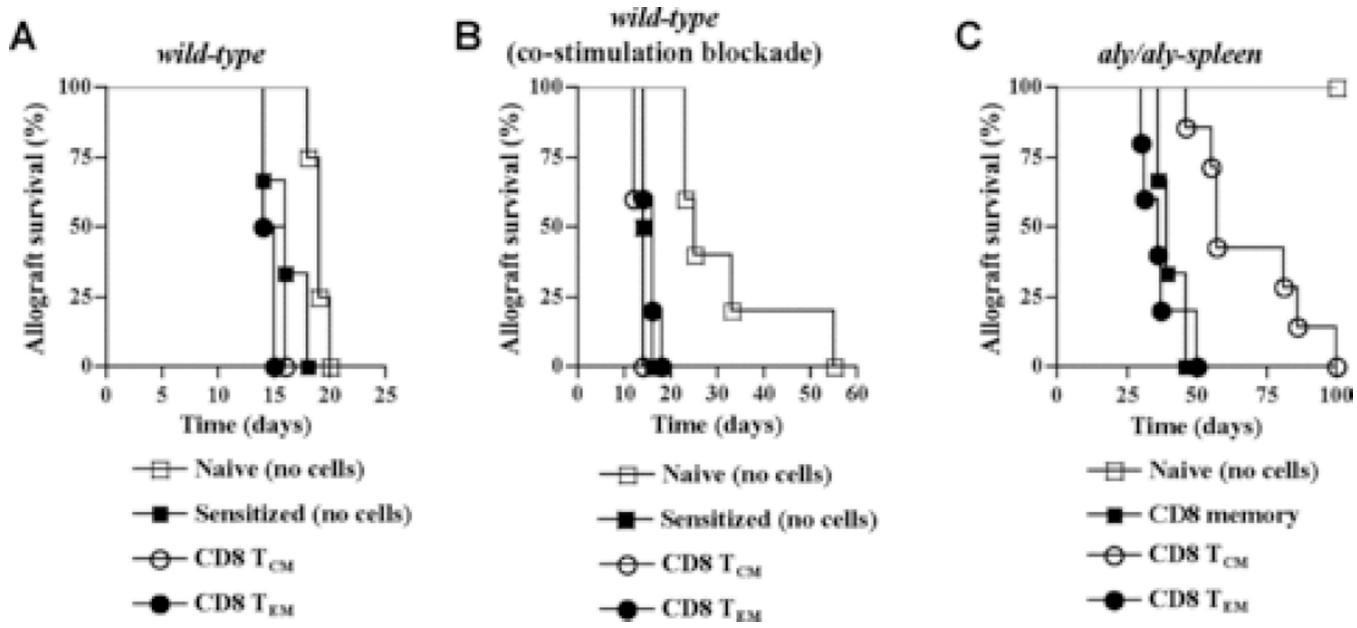

Fig. 2. Allograft rejection in adoptive hosts of CD8 T_{CM} and T_{EM}

Sorted CD8 T_{CM} ($1.5 \times 10^6 – 2 \times 10^6$) or T_{EM} ($0.4 \times 10^6 – 0.6 \times 10^6$) containing similar numbers of BALB/c-reactive IFNγ^+ T cells were transferred into *wt* and *aly/aly-spleen* adoptive hosts followed by BALB/c-skin transplantation 2-days later. **(A)** Skin allograft rejection mediated by CD8 T_{CM} and T_{EM} in *wt* hosts. BALB/c-skin allograft survival was tested in *wt* recipients of CD8 T_{CM} or T_{EM} and compared to unimmunized naïve mice (Naïve, no cells) and sensitized mice (Sensitized, no cells) harboring endogenous memory T cells that had rejected BALB/c-skin allografts 8 – 12 weeks earlier ($n = 3 – 4$ mice/grp). **(B)** Skin allograft rejection mediated by CD8 T_{CM} and T_{EM} in *wt* hosts treated with co-stimulation blockade. *Wt* recipients of CD8 T_{CM} or CD8 T_{EM}, unimmunized naïve mice (Naïve, no cells) and sensitized mice (Sensitized, no cells) were treated with CTLA4-Ig and anti-CD40L, 0.25mg each (i.p.), on day 0, 2, 4 and 6 after BALB/c-skin transplantation and allograft survival was compared ($n = 4 – 6$ mice/grp). **(C)** Skin allograft rejection mediated by CD8 T_{CM} and T_{EM} in *aly/aly-spleen* hosts. *Aly/aly-spleen* recipients of CD8 T_{CM} or CD8 T_{EM} or unfractionated CD8 memory T cells (1×10^6) (CD8 memory) and *aly/aly-spleen* naïve mice without memory T cell transfer (Naïve, no cells) underwent BALB/c-skin transplantation and allograft survival was compared ($n = 4 – 7$ mice/grp).

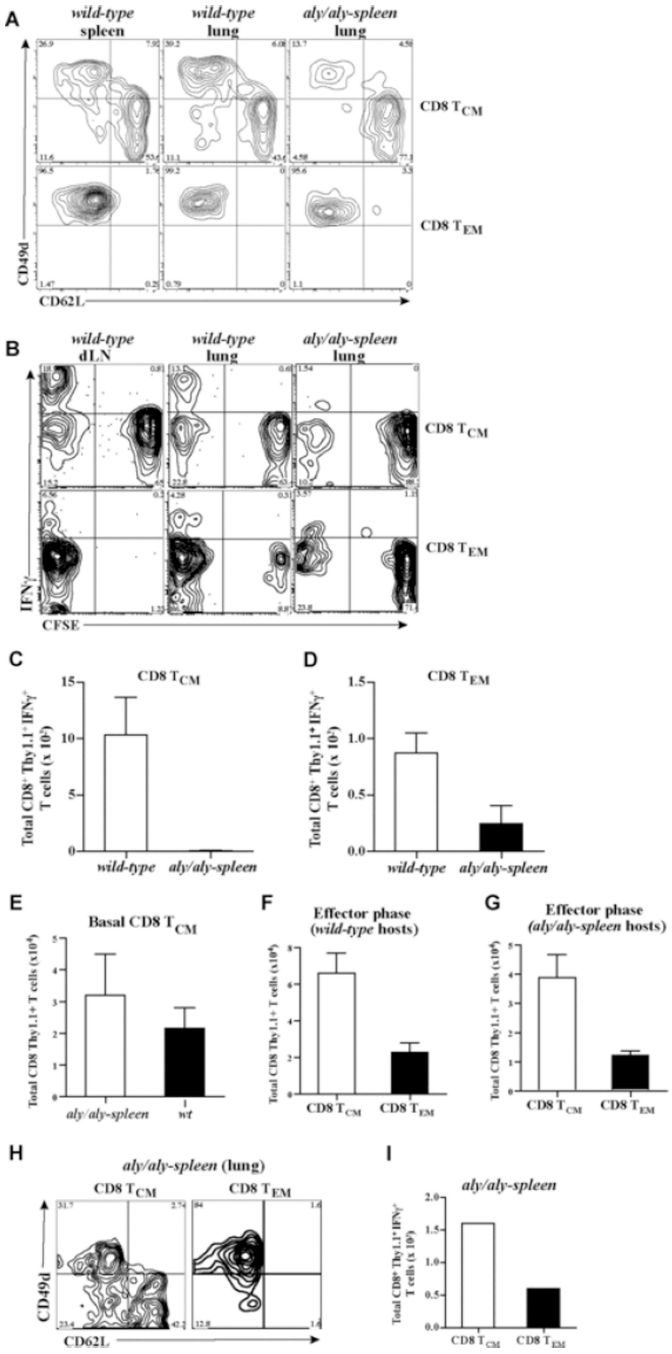

Fig. 3. Differentiation of CD8 T_{CM} and T_{EM} in adoptive hosts

Sorted CD8 T_{CM} (2×10^6) or T_{EM} (0.5×10^6) containing similar numbers of BALB/c-reactive IFNγ^+ T cells were transferred to *wt* and *aly/aly-spleen* mice followed by BALB/c-skin transplantation 2-days later. CD8 T_{CM} and T_{EM} were labeled with 2μM CFSE prior to adoptive transfer to assess proliferation in adoptive hosts at 8 days after BALB/c-skin transplantation. Differentiation and proliferation of CD8 T_{CM} and T_{EM} was assessed after skin transplantation. Cells were harvested from liver (LV), lungs (LG), bone marrow (BM), blood and skin allograft (SK) in both *wt* and *aly/aly-spleen* hosts. Spleen (SP), draining

lymph node (dLN) and non-draining LN cells were also harvested in *wt* hosts. **(A)** Phenotype of activated CD8 T_{CM} and T_{EM} in *wt* and *aly/aly-spleen* hosts at 8-days. Expression of CD49d and CD62L is shown after gating on $CD8^+Thy1.1^+$ population within the harvested cells from spleen and lungs in *wt* mice, and lungs in *aly/aly-spleen* mice. Harvested cells from LN, BM and liver tissues were similar in phenotype (data not shown). Representative FACS plots of 3 – 4 experiments are shown. **(B)** Proliferation of alloreactive CD8 T_{CM} and T_{EM} after recall in adoptive hosts at 8-days. BALB/c-reactive $IFN\gamma^+$ T cells within the harvested cells from all tissues were assessed by intracellular cytokine staining and analyzed after gating on $CD8^+Thy1.1^+$ population. CFSE dilution and IFNγ are shown after gating on $CD8^+Thy1.1^+$ T cells. Representative FACS plots of 3 experiments are shown. **(C – D)** Quantitation of BALB/c-reactive $IFN\gamma^+$ T cells derived from CD8 T_{CM} (C) and CD8 T_{EM} (D) in *wt* and *aly/aly-spleen* adoptive hosts at 8-days after BALB/c-skin transplantation. BALB/c-reactive $IFN\gamma^+$ T cells within the harvested cells from all tissues were assessed by intracellular cytokine staining and enumerated after gating on $CD8^+Thy1.1^+$ population. Mean ± SD of 3 – 4 mice/grp. **(E – G)** Quantitation of $CD8^+Thy1.1^+$ cells from harvested tissues in adoptive hosts of CD8 T_{CM} at 8 days after transfer (E) or at 8 days after transplantation in adoptive hosts of CD8 T_{CM} and T_{EM} (F – G) (Mean ± SD of 3 – 4 mice/grp). **(H)** Phenotype of activated CD8 T_{CM} and T_{EM} in *aly/aly-spleen* hosts at 30-days after BALB/c-skin transplantation (similar to 3A). **(I)** Quantitation of BALB/c-reactive $IFN\gamma^+$ T cells derived from CD8 T_{CM} and T_{EM} in *aly/aly-spleen* adoptive hosts at 30-days after BALB/c-skin transplantation (similar to 3C-D).

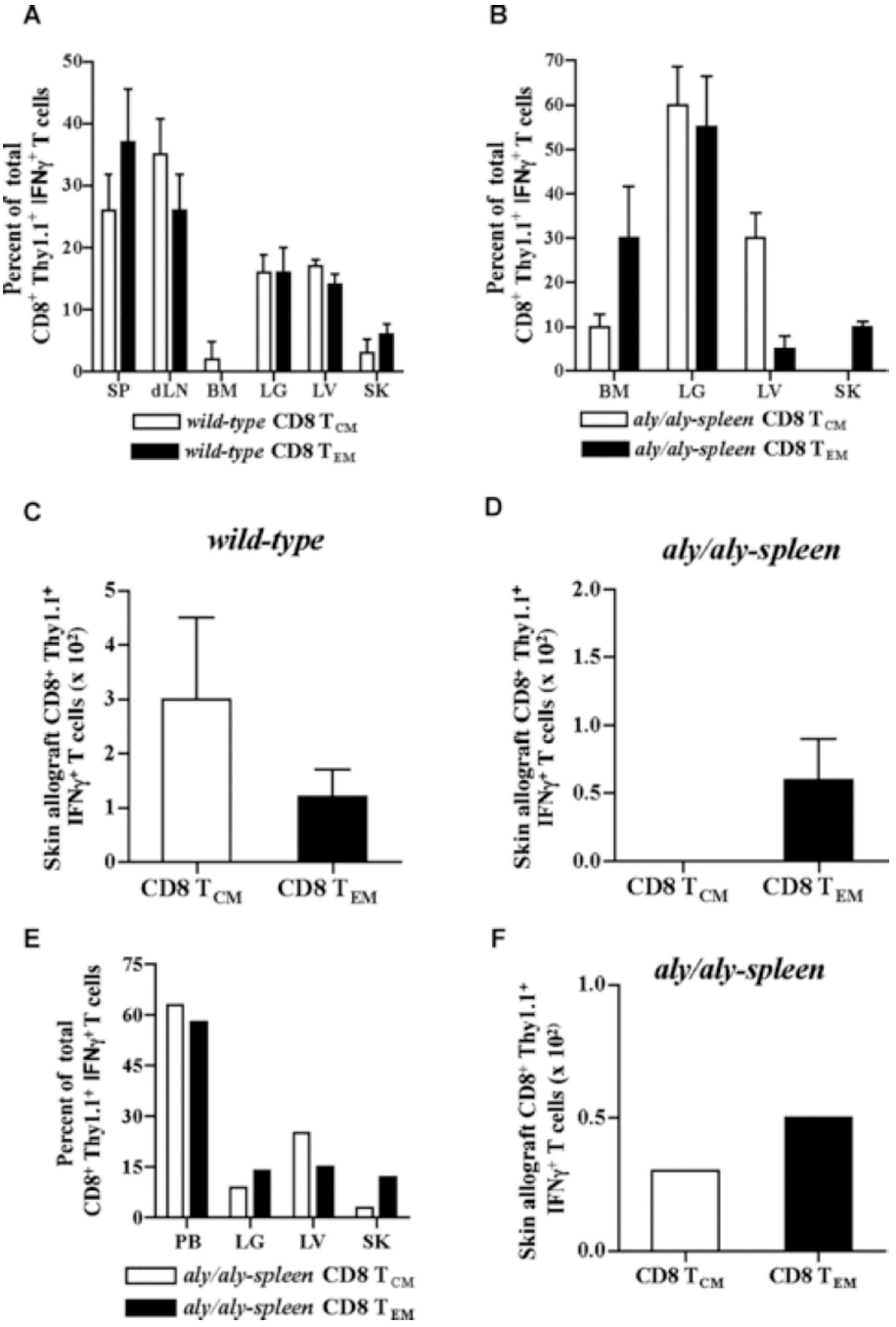

Fig. 4. Migration of alloreactive effectors in adoptive hosts of CD8 T_{CM} and T_{EM}

Sorted CD8 T_{CM} (2×10^6) or T_{EM} (0.5×10^6) containing similar numbers of BALB/c-reactive IFNγ^+ T cells were transferred to *wt* and *aly/aly-spleen* mice followed by BALB/c-skin transplantation 2-days later. Alloreactive effectors from CD8 T_{CM} and T_{EM} were assessed after BALB/c-skin transplantation. Cells were harvested from liver (LV), lungs (LG), bone marrow (BM), blood and skin allograft (SK) in both *wt* and *aly/aly-spleen* hosts. Spleen (SP), draining lymph node (dLN) and non-draining LN cells were also harvested in *wt* hosts. BALB/c-reactive IFNγ^+ T cells within the harvested cells from all tissues were

assessed by intracellular cytokine staining after gating on $CD8^+Thy1.1^+$ population to identify alloreactive effectors derived from CD8 T_{CM} and T_{EM}. **(A – B)** Tissue distribution of alloreactive effector T cells generated from transferred CD8 T_{CM} and T_{EM} in *wt* (A) and *aly/aly-spleen* (B) hosts. $CD8^+Thy1.1^+$ $IFN\gamma^+$ T cells harvested from each tissue is shown as % of total $CD8^+Thy1.1^+$ $IFN\gamma^+$ T cells harvested from all tissues in that recipient (Mean ± SD of 3 – 4 mice/grp). $CD8^+$ $Thy1.1^+$ $IFN\gamma^+$ T cells harvested from blood and non-draining LN were < 1% of total and are not shown. **(C – D)** Quantitation of BALB/c-reactive $IFN\gamma^+$ T cells within the harvested $CD8^+$ $Thy1.1^+$ population from skin allografts in either *wt* (C) or *aly/aly-spleen* (D) adoptive hosts of CD8 T_{CM} and T_{EM} (Mean ± SD of 3 – 4 mice/grp). **(E)** Tissue distribution of alloreactive effector T cells generated from transferred CD8 T_{CM} and T_{EM} in *aly/aly-spleen* hosts at 30-days after BALB/c-skin transplantation (similar to 4B). **(F)** Quantitation of BALB/c-reactive $IFN\gamma^+$ T cells within the harvested $CD8^+$ $Thy1.1^+$ population from skin allografts in *aly/aly-spleen* adoptive hosts of CD8 T_{CM} and T_{EM} at 30-days after BALB/c-skin transplantation.

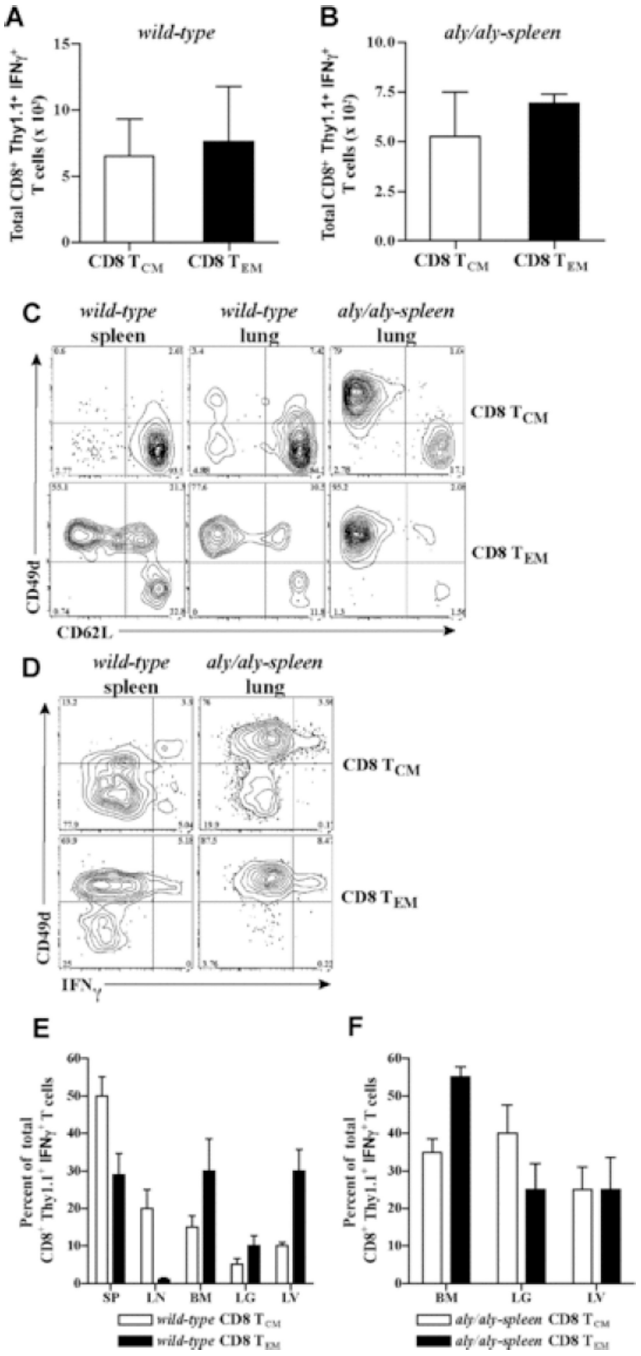

Fig. 5. Persistence of CD8 T_{CM} and T_{EM} in adoptive hosts after allograft rejection

Sorted CD8 T_{CM} (2×10^6) or T_{EM} (0.5×10^6) containing similar numbers of BALB/c-reactive IFNγ^+ T cells were transferred to *wt* and *aly/aly-spleen* mice followed by BALB/c-skin transplantation 2-days later. BALB/c-reactive IFNγ^+ T cells derived from transferred CD8 T_{CM} or CD8 T_{EM} were assessed in adoptive hosts 6 – 10 weeks after allograft rejection. Cells were harvested from liver (LV), lungs (LG), bone marrow (BM) and blood in both *wt* and *aly/aly-spleen* hosts. Spleen (SP) and LN cells were also harvested in *wt* hosts. **(A – B)** Quantitation of alloreactive secondary memory T cells generated from CD8 T_{CM}

and T_{EM} in *wt* (A) and *aly/aly-spleen* (B) adoptive hosts. BALB/c-reactive IFNγ^+ T cells within the harvested cells from all tissues were assessed by intracellular cytokine staining and enumerated after gating on CD8$^+$Thy1.1$^+$ population (Mean ± SD of 4 – 5 mice/grp). **(C – D)** Phenotype of secondary memory T cells derived from transferred CD8 T_{CM} or T_{EM} in *wt* and *aly/aly-spleen* hosts. Expression of CD49d and CD62L is shown after gating on CD8$^+$Thy1.1$^+$ population within the harvested cells from spleen and lungs in *wt* mice, and lungs in *aly/aly-spleen* mice (C). Expression of CD49d and IFNγ is shown after gating on CD8$^+$Thy1.1$^+$ population within the harvested cells from spleen in *wt* mice and lungs in *aly/aly-spleen* mice (D). Harvested cells from LNs, BM and liver tissues were comparable in phenotype (data not shown). Representative FACS plots of 4 – 5 experiments are shown. **(E – F)** Tissue distribution of BALB/c-reactive IFNγ^+ T cells derived from transferred CD8 T_{CM} or T_{EM} in *wt* (E) and *aly/aly-spleen* (F) hosts. CD8$^+$Thy1.1$^+$ IFNγ^+ T cells harvested from each tissue is shown as % of total CD8$^+$Thy1.1$^+$ IFNγ^+ T cells harvested from all tissues in that recipient (Mean ± SD of 4 – 5 mice/grp). CD8$^+$Thy1.1$^+$ IFNγ^+ T cells harvested from blood were < 1% of total and are not shown.