Role of a selecting ligand in shaping the murine γδ-TCR repertoire

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Edited by Arthur Weiss, University of California, San Francisco, San Francisco, CA, and approved January 3, 2018 (received for review October 19, 2017)

Unlike αβ-T lineage cells, where the role of ligand in intrathymic selection is well established, the role of ligand in the development of γδ-T cells remains controversial. Here we provide evidence for the role of a bona fide selecting ligand in shaping the γδ-T cell-receptor (TCR) repertoire. Reactivity of the γδ-TCR with the major histocompatibility complex (MHC) Class Ib ligands, H2-T10/22, is critically dependent upon the EGYEL motif in the complementarity determining region 3 (CDR3) of TCRδ. In the absence of H2-T10/22 ligand, the commitment of H2-T10/22 reactive γδ-T cells to the γδ fate is diminished, and the specification of those γδ committed cells to the IFN-γ or interleukin-17 effector fate is altered. Furthermore, those cells that do adopt the γδ fate and mature exhibit a profound alteration in the γδTCR repertoire, including depletion of the EGYEL motif and reductions in both CDR3δ length and charge. Taken together, these data suggest that ligand plays an important role in shaping the TCR repertoire of γδ-T cells.

γδ-T cells | repertoire selection | γδ-T cell ligand | γδ-T lineage commitment | H2-T

Vertebrates have two major T lymphocyte lineages—αβ and γδ—which play at least partially distinct roles in immune responses. αβ-T cells are key mediators of the adaptive immune response and their T cell-receptor (TCR) complexes recognize peptide ligands in the context of major histocompatibility complex (MHC) Class I and Class II proteins. The role of αβTCR/MHC interactions in regulating αβ-T cell development and αβ-TCR repertoire selection has been well documented (1, 2). In contrast, γδ-T cells act at the interface of the adaptive and innate immune responses and are not classically MHC-restricted, instead recognizing a variety of ligands that do not require proteolytic processing. Importantly, the role of γδTCR ligands in supporting the development of γδ-T cells remains poorly understood and controversial, in part due to the paucity of potential selecting ligands that might be employed to address this question (3–5).

A pair of highly homologous nonclassical MHC Class Ib molecules, H2-T10 and H2-T22, have been demonstrated to be ligands for a subset of γδ-T cells (~0.5% of murine γδ-T cells) (6). These proteins have been validated as γδTCR ligands by virtue of their direct binding to the γδTCR as measured by both surface plasmon resonance and cocrystallization (7). Importantly, their binding to the γδTCR is critically dependent upon the EGYEL motif encoded by complimentarity determining region 3 (CDR3) of TCRδ (8). Experiments intended to determine whether H2-T10/22 serve as selecting ligands that regulate the development of H2-T10/22-reactive γδ-T cell progenitors have failed to settle this issue. Efforts to address this issue have employed an indirect approach to attenuate H2-T10/22 surface expression, β2M deficiency, which is inadequate both because it fails to completely eliminate surface expression and because it fails to directly address the role of H2-T10/22, since β2M deficiency attenuates the surface expression of β2M-dependent structures in addition to H2-T10/22 (9, 10). The use of β2M deficiency to attenuate surface expression of H2-T10/22 has led to conflicting observations regarding the role of H2-T10/22 in the selection of T10/22-reactive γδ-T cells. Experiments utilizing the H2-T10/22-reactive KN6 γδTCR transgenic (Tg) mice have consistently revealed that indirect attenuation of H2-T10/22 surface expression using β2M deficiency impairs adoption of the γδ fate (10, 11). In contrast, similar experiments using a distinct H2-T10/22-reactive γδTCR transgenic model (G8) provided conflicting results, in one case indicating that H2-T10/22 expression promoted positive selection of γδ-T cells (12), while in another it caused their deletion (13). The discrepancies observed in using the G8 γδTCR Tg model may relate to its ~10-fold greater affinity for H2-T10/22 ligand than the KN6 γδTCR (7). An attempt to address the effect of β2M deficiency on the development and γδTCR repertoire of polyclonal, H2-T10/22-reactive γδ-T cells also failed to resolve the issue. This study employed tetramer binding to identify H2-T10/22 γδ-T cells, which is entirely appropriate; however, tetramer binding lacks the sensitivity required to effectively assess alterations in the γδTCR repertoire of H2-T10/22 reactive progenitors, since it reacts with γδTCRs differing in affinity for ligand by as much as 10-fold (i.e., G8 and KN6) (7). Such differences in affinity result are markedly different outcomes of selection of αβ lineage progenitors (7, 14–16), and so may also do so for γδ lineage progenitors. Consequently, direct analysis of the γδTCR repertoire of tetramer-binding cells must be performed as part of any assessment of the role of ligand in γδ-T cell development and shaping of the γδTCR repertoire. Here, we directly address the role of selecting ligand in

Significance

While intrathymic self-ligands have well-characterized roles in shaping the αβ-T cell receptor (TCR) repertoire, the role of self-ligands in shaping the γδ-TCR repertoire remains unknown. Ablation of the only known γδ-T cell selecting ligands, 22/22, impaired the commitment of progenitors to the γδ-T lineage and also profoundly altered the γδ-T cell repertoire, reducing CDR3δ charge and length and depleting the critical EGYEL binding motif. γδ-T cell selecting ligands, therefore, play a crucial role in influencing the repertoire of at least some subsets of γδ-T cells.

Author contributions: D.L.W. designed research; S.P.F. and F.C. performed research; F.C., L.K., P.Z., L.T., J.C.Z.-P., and D.J.K. contributed new reagents/analytic tools; S.P.F., F.C., and R.L.D. analyzed data; and S.P.F. and D.L.W. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The data reported in this paper have been deposited in the Sequence Read Archive, https://www.ncbi.nlm.nih.gov/sra (accession no. SRP129848).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1718328115/-/DCSupplemental.

regulating γδ-T cell development by both genetic ablation of the *H2T* locus and by performing next-generation sequencing (NGS) of the TCR genes of H2-T22 tetramer-reactive γδ-T cells to evaluate how the elimination of ligand impacts the γδTCR repertoire.

Results

H2-T10 and H2-T22 are encoded by the *H2t* locus, a 180-Kb locus located on Chromosome 17. To determine how γδTCR ligand regulates γδ-T cell development, we generated mice deficient for H2-T10 and H2-T22. Because of the high sequence homology and close genomic proximity of the *H2-t10* and *H2-t22* genes, we ablated the entire *H2t* locus. Zinc finger nucleases were employed to sequentially insert *lox*P sites immediately 5′ of the *H2-t24* and 3′ of *H2-t3* genes, respectively, following which the entire locus was deleted in the germline using a pan-Cre transgene (Fig. S1*A*). Genomic PCR of the *H2t* locus and quantitative PCR for *H2t10* and *H2t22* mRNA revealed complete elimination of the *H2t* locus (Fig. S1 *B* and *C*). Moreover, there were no gross abnormalities in T cell development in H2T-deficient mice, either in T cell development in the thymus (Fig. S2) or in peripheral T lymphocyte populations (Fig. S3).

The KN6 γδTCR is reactive with H2-T10/22. KN6 Tg progenitors that encounter $T22^{d}$ during intrathymic development undergo commitment to the γδ lineage, as indicated by remaining CD4-CD8- (double negative), down-regulating CD24, and inducing expression of the γδ-lineage commitment marker, CD73 (Fig. 1 *A* and *B*) (10, 17). CD73 expression is induced in γδTCR+ progenitors by encounter with TCR ligand and continues to be expressed following maturation and exit to the periphery (17). Importantly, when the expression of H2-T10/22 is eliminated by genetic ablation of the *H2t* locus in $H2t^{-/-}$ mice, KN6 Tg γδ-T cell progenitors failed to adopt the γδ fate and were instead diverted to the αβ-T cell fate, as assessed by the lack of CD73 induction and by differentiation to the CD4+CD8+ (double-positive or DP) stage (Fig. 1 *A* and *B*). Furthermore, the loss of H2-T10/22 also reduced the number of mature CD73+ γδ-T cells in the spleen (Fig. S4). Therefore, monoclonal, T10/22-reactive KN6 γδ TCR-expressing progenitors depend on the H2-T10/22 ligand to adopt the γδ-T cell fate during development in the thymus.

To determine whether the development of polyclonal, H2-T22-reactive γδ-T cell progenitors was similarly dependent upon the presence of H2-T10/22 for adoption of the γδ fate, we monitored their developmental progression in $H2t^{-/-}$ mice by H2-T22 tetramer staining (Fig. 1 *C* and *D*). The $H2t^{-/-}$ mice were backcrossed to the C57BL/6 background for 10 generations and $H2t^{+/-}$ and $H2t^{-/-}$ littermates were compared to exclude any potential differences due to residual strain background and/or microbiome influences. While development of T22-reactive γδ-T cells was identical in $H2t^{+/-}$ versus $H2t^{+/+}$ mice (Fig. S5), it was markedly altered in $H2t^{-/-}$ mice. Indeed, the proportion and number of immature CD24+ progenitors that had committed to the γδ lineage, as indicated by CD73 induction, was greatly reduced among T22-reactive progenitors (Fig. 1 *C* and *D*). Moreover, the percentage and number of CD24−CD73+ mature γδ-T cells was also reduced in $H2t^{-/-}$ mice, although this did not quite reach statistical significance ($P = 0.07$; Fig. 1 *C* and *D*). The γδTCR complex is silenced when γδTCR-expressing progenitors adopt the αβ fate and differentiate to the DP stage. Consequently, it is not possible to monitor development of T22-reactive γδ progenitors to the DP stage in vivo (10). Thus, to assess whether T22-reactive γδ-T cell progenitors switch to the αβ lineage and develop to the DP stage in the absence of T10/22 ligand, we utilized an in vitro system in which T22-reactive γδ-T cell progenitors were cultured on H2T-deficient fibroblasts expressing the Notch ligand, Delta-like 1. Indeed, when immature T22-reactive γδTCR+ progenitors from H2T-deficient mice were cultured on H2T-deficient fibroblast monolayers, a substantial proportion of the cells was diverted to the DP stage (Fig. S6). The mean absolute number of T22-reactive γδ-T cells in the spleen was also modestly

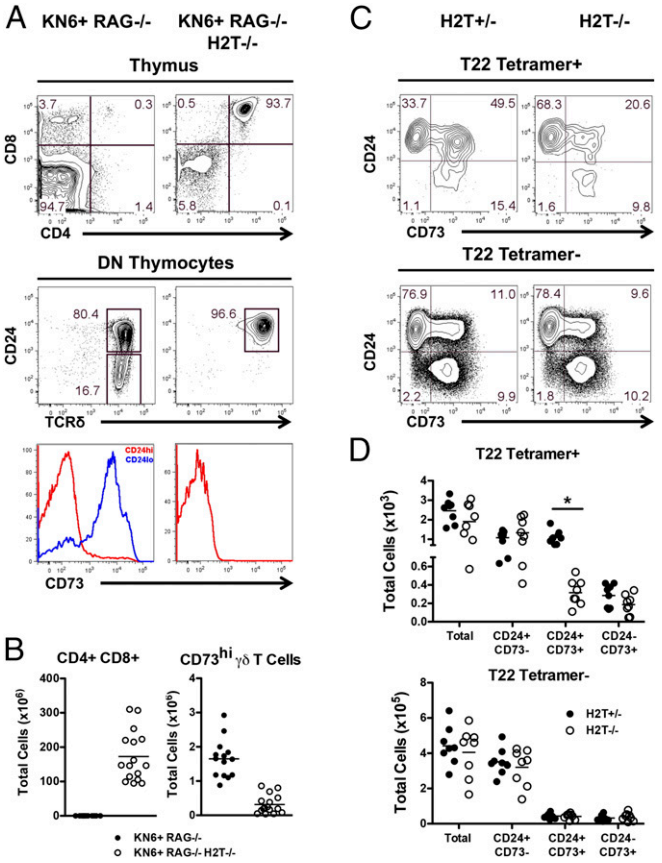

Fig. 1. γδTCR ligand is required for γδ-T cell development. (*A*) Flow cytometric analysis of thymi from KN6+RAG$^{-/-}$ and KN6+RAG$^{-/-}$*H2t*$^{-/-}$ mice that had been backcrossed to the BALB/c background. Total thymocytes were gated on Thy1.2 (CD90.2)+ cells and then analyzed for expression of CD4 and CD8 (*Top*). Gated CD4-CD8- thymocytes were analyzed for expression of TCRδ and CD24 (*Middle*). Gated CD24^{hi}TCRδ+ and CD24^{lo}TCRδ+ cells were analyzed for expression of CD73 (*Bottom*). (*B*) Absolute numbers were calculated for CD4+CD8+ thymocytes (*Left*) and CD73^{hi}TCRδ+ thymocytes (*Right*) electronically gated as in A. Absolute numbers were calculated based on gate frequencies with each dot representing an individual mouse. n = 16 mice per genotype. (*C*) Flow cytometric analysis of thymi from *H2t*$^{+/-}$ and *H2t*$^{-/-}$ mice. Total thymocytes were electronically gated on lineage-(lacking CD45R, CD11c, Gr1, Ter119, TCRβ) CD4−CD8−TCRδ+T22 tetramer+ (*Top*) or T22 tetramer− (*Bottom*) and then analyzed for expression of CD24 and CD73. (*D*) Absolute numbers for the indicated populations were calculated based on gate frequencies with each dot representing an individual mouse n = 8 mice per genotype, *$P < 0.001$, two-tailed Student's t test.

reduced in H2T-deficient mice, but did not reach statistical significance (Fig. S7). In contrast, there were no alterations in the remaining γδ-T cells that are not T22-reactive (Fig. 1 *C* and *D* and Fig. S7), suggesting that the products of the *H2t* locus do not serve as selecting ligands for the majority of γδ-T cell progenitors. However, collectively, these results demonstrate that the H2-T10/22-selecting ligands play an important role in mediating lineage commitment and development of both monoclonal and polyclonal T22-reactive γδ-T cell progenitors.

In addition to undergoing γδ lineage commitment, many γδ-T cells acquire their effector fate during development in the thymus (18). Previous reports have suggested that γδTCR–ligand interactions play a critical role in this process, with TCR–ligand engagement inducing cells to become IFN γ producers, and its absence promoting their development into interleukin-17 (IL-17) producers (16). To determine whether H2T deficiency altered effector fate, we measured IL-17 and IFNγ production by

intracellular staining. H2T deficiency severely attenuated the production of IFNγ by KN6 Tg progenitors while increasing the proportion of IL-17 producers (Fig. 2*A*). Previous reports have revealed that CD122 surface expression marks antigen-experienced γδ-T cells, with $CD122^{hi}$ γδ-T cells preferentially secreting IFNγ, while $CD122^{lo}$ γδ-T cells primarily produce IL-17 (16). Consistent with this, T22 tetramer-binding γδ-T cells from $H2t^{-/-}$ mice were depleted of $CD122^{hi}$ progenitors (Fig. 2*B*), suggesting that these cells would preferentially produce IL-17. Indeed, activation of the IL-17 reporter among T22-reactive γδ-T cell progenitors was increased in H2T-deficient mice, indicating a diversion to the IL-17 producing fate (Fig. 2*C*). In contrast, there were no alterations in CD122 surface expression or the percentage of IL17-producers among the γδ-T cell population that were not T22-reactive (Fig. 2 *B* and *C*). Thus, in addition to inducing commitment to the γδ-T cell lineage, γδTCR ligands also regulate the specification of effector fate, with the presence of ligand facilitating adoption of the IFNγ-producing fate and its absence favoring IL-17 production.

While H2T deficiency clearly impaired γδ lineage commitment and influenced effector fate, the development of T22-reactive γδ-T cells was not completely blocked, raising the question of how T22-reactive progenitors were able to develop in the absence of nominal ligand. One possibility is that these progenitors cross-react with and are undergoing selection on a ligand other than H2-T10/22. If this were the case, the γδTCR repertoire would be expected to differ from that selected by H2-T10/22. To assess this possibility, we isolated T22-reactive immature CD24+CD73+ and mature CD24−CD73+ γδ-T cells from $H2t^{+/-}$ and $H2t^{-/-}$ mice and performed NGS of the TCRγ and TCRδ chains (19). We observed no alterations of Vγ usage, CDR3γ length, or CDR3γ charge in T22-reactive γδ-T lineage progenitors that develop in the presence or absence of ligand (Fig. 3 *A*–*C*). Furthermore, restriction of the TCRγ repertoire using a Vγ2 Tg did not significantly alter development, as T22-reactive Vγ2 Tg progenitors exhibited an impairment of both commitment to the γδ fate (CD24+CD73+) and maturation (CD24−CD73+), as observed in non-Tg tetramer-binding progenitors (Fig. 3 *D* and *E*). Collectively, these results suggest that the TCRγ chain does not play a critical role in influencing T10/22 binding, consistent with a previous report implicating CDR3δ as the critical determinant of T22 reactivity (7).

In contrast to the TCRγ chain, H2T deficiency had a striking impact on the TCRδ repertoire of T22-reactive CD24+CD73+ immature and CD24−CD73+ mature γδ-T cell progenitors (Fig. 4). Specifically, the CDR3δ sequences of T22-reactive CD24−CD73+ mature γδ-T cells from $H2t^{-/-}$ mice were two amino acids shorter than CD24−CD73+ mature γδ-T cells from $H2t^{+/-}$ mice (Fig. 4*A*). Moreover, while the CDR3δ sequences from T22-reactive CD24+CD73+ and CD24−CD73+ progenitors isolated from $H2t^{+/-}$ mice exhibited a negative charge, this was altered by H2T deficiency. Indeed, the CDR3δ sequences of progenitors from H2T-deficient mice was altered in that the charge in immature CD24+CD73+ progenitors was more neutral, while that in mature CD24−CD73+ γδ-T cell progenitors was actually slightly positive (Fig. 4*B*). Finally, previous reports identified the EGYEL motif encoded by the Dδ2 element as being crucial for binding of the TCRδ chain to H2-T10/22 (8). In agreement, we found that ∼40% of the CDR3δ sequences isolated from T22-reactive CD24+CD73+ immature and CD24−CD73+ mature γδ-T cell progenitors that developed in the presence of ligand contained the EGYEL motif (Fig. 4*C*). However, T22-reactive γδ-T cell progenitors that developed in the absence of ligand had substantial reductions in the percentage of CDR3δ sequences containing the EGYEL motif, with only ∼30% of CD24+CD73+ immature CDR3δ sequences having this motif, which was further reduced to less than 10% among CD24−CD73+ mature γδ-T cells. It is important to note that the majority of T22-reactive, committed, and mature γδ-T cells developing in H2T-expressing mice express a TCRδ subunit that lacks the EGYEL motif, suggesting that additional motifs are capable of supporting T22 reactivity. Taken together, our analysis reveals that the absence of H2-T10/22–selecting ligands results in a distinct γδTCR repertoire that is depleted of the consensus-binding motif, which indicates that γδ-TCR–ligand interactions in the thymus have a profound impact on the γδ-TCR repertoire.

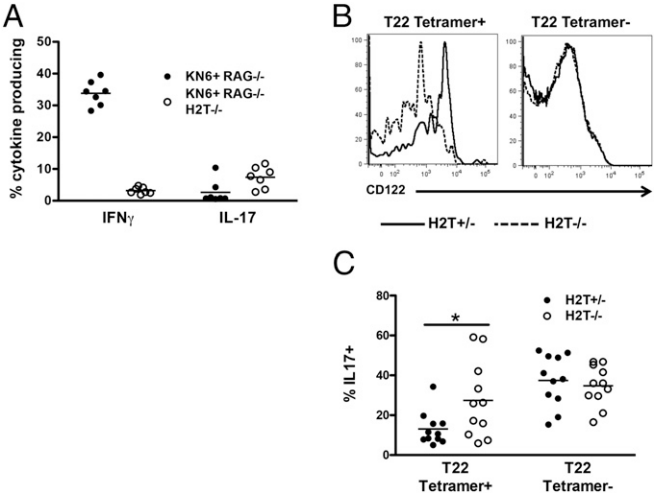

Fig. 2. γδTCR ligand influences γδ-T cell-effector fate. (*A*) CD4−CD8− thymocytes were isolated from KN6+$RAG^{-/-}$ and KN6+$RAG^{-/-}$$H2t^{-/-}$ mice and stimulated with PMA (100 ng/mL) and ionomycin (1 μg/mL) in the presence of Brefeldin A (10 μg/mL) for 4 h at 37 °C. Intracellular flow cytometric analysis was performed for IFN-γ and interleukin-17. Each dot represents an individual mouse. $n = 7$ mice per genotype. (*B*) Thymocytes from $H2t^{+/-}$ and $H2t^{-/-}$ mice were gated on lineage-(lacking B220, CD11c, Gr1, Ter119, TCRβ) CD4−CD8−TCRδ+ T22 tetramer+ and T22 tetramer− cells and analyzed for CD122 expression. $n = 8$ mice per genotype. (*C*) Thymocytes from $H2t^{+/-}$ IL17-GFP+ and $H2t^{-/-}$ IL17-GFP+ mice were gated on lineage-(lacking CD45R, CD11c, Gr1, Ter119, TCRβ) CD4−CD8−TCRδ+$CD24^{lo}$T22 tetramer+ and T22 tetramer− cells and analyzed for expression of GFP, as a surrogate for IL17 production. $n = 11$ mice per genotype, $^*P < 0.05$, two-tailed Student's *t* test.

Discussion

The role of ligand stimulation of αβTCR-expressing progenitors in the thymus and its impact on selection of the αβTCR repertoire has been extensively studied. While the importance of these interactions for the development of αβ-lineage T cells is unquestioned, the role of γδ-TCR ligands in the development of γδ-T cells remains a highly controversial issue that is difficult to address because of the paucity of known γδ-TCR–selecting ligands and the models with which to assess their importance. In this study, we have genetically ablated the only validated γδ-TCR–selecting ligands, *H2-t10* and *H2-t22*, and have assessed the effect of their absence on both progenitor fate and the γδTCR repertoire of T22-binding progenitors. Importantly, the total elimination of H2-T10/22 blocks the commitment of monoclonal γδ-TCR Tg progenitors to the γδ lineage and diverts them to the αβ-T cell fate. H2T deficiency also perturbs the development of polyclonal T22-binding γδ-T cell progenitors. Indeed, their commitment to the γδ fate is impaired and those progenitors that are selected and mature in the absence of H2-T10/22 display a substantially altered TCR repertoire that contains CDR3δ sequences that are shorter, less negatively charged, and are depleted of the consensus binding motif, EGYEL. Taken together, these data suggest that γδ-TCR–selecting ligands play an important role in the intrathymic selection of at least some subsets of γδ-T cell progenitors.

The role of γδ-TCR ligands in promoting γδ-T cell progenitors to adopt the γδ-T cell fate remains controversial. Studies analyzing the gene expression of $CD24^{high}$ immature and $CD24^{low}$

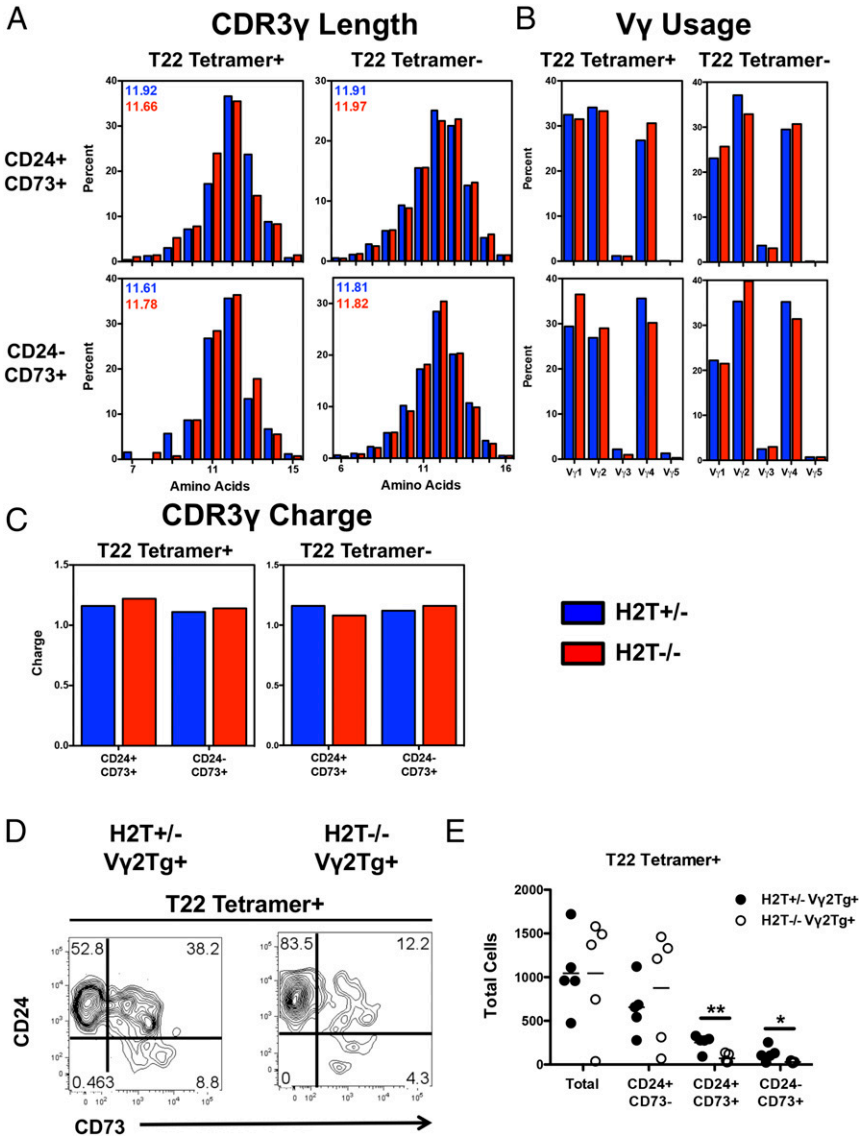

Fig. 3. The TCRγ repertoire is not perceptibly altered by the absence of ligand. NGS of the CDR3γ sequences was performed in T22 tetramer+ and T22 tetramer− CD24+CD73+ and CD24−CD73+ cells, as defined in Fig. 1*C*, to determine (*A*) CDR3γ length, (*B*) Vγ usage, and (*C*) CDR3γ charge. Data are representative of at least two independent experiments. (*D*) Flow cytometric analysis of thymi from $H2t^{+/-}$Vγ2Tg+ and $H2t^{-/-}$Vγ2Tg+ mice. Total thymocytes were electronically gated on lineage-(lacking CD45R, CD11c, Gr1, Ter119, TCRβ) CD4−CD8−TCRδ+ T22 tetramer+ and then analyzed for expression of CD24 and CD73. (*E*) Absolute numbers for the indicated populations were calculated based on gate frequencies with each dot representing an individual mouse (n = 5 mice per genotype). $^{*}P < 0.05$, $^{**}P < 0.01$, two-tailed Student's t test.

mature γδ-T cell subsets have suggested that γδ lineage commitment and adoption of effector fates is TCR independent and predetermined, because immature Vγ subsets expressed transcriptional signatures linked to effector fate (20). However, our studies identified CD73 as a TCR-inducible surface marker that bisects the $CD24^{high}$ immature γδ-T cell subset, and marks γδ lineage commitment (17). Subdividing immature γδ-T cell progenitors using CD73 revealed that these transcriptional signatures are substantially remodeled in association with TCR-mediated induction of CD73 and adoption of the γδ fate, suggesting that TCR signaling plays an important role (17, 21, 22). Moreover, the expression of CD73 on a substantial fraction (25–30%) of γδ-T cell progenitors in the thymus, and on the majority of γδ-T cells in the periphery, indirectly supports the notion that ligand engagement influences the development of a large fraction of γδ-T cells.

A previous attempt to directly address the role of ligand in γδ-T cell development also focused on the T22-reactive subset of γδ-T cells. H2-T10/22 expression was indirectly removed from the cell surface through β2M deficiency, which did not block the development of T22-reactive γδ-T cells, but did alter their effector fate, impairing the development of IFNγ producing γδ-T cells while favoring adoption of the IL-17 producing fate (16). Consequently, the authors concluded that ligand influences effector fate but not γδ-T cell development or the γδTCR repertoire. We also observe the same diversion in effector fate, away from IFNγ production and toward IL-17 production; however, our findings regarding the role of ligand in γδ-T cell development support a different conclusion. We find that H2T deficiency completely arrests the development of monoclonal T22-reactive γδ-TCR Tg progenitors. Moreover, while T22-tetramer-reactive polyclonal progenitors are not reduced in number by H2T deficiency, they do exhibit alterations in development. Their adoption of the γδ fate, as measured by CD73 induction, was impaired and while their maturation was not completely blocked, those γδ-T cell progenitors that did mature exhibited a profoundly altered γδ-TCR repertoire. This was not detected in the previous study because the authors employed tetramer to identify T22-reactive cells, but did not directly assess the effect on the γδ-TCR repertoire. We found that H2T deficiency caused profound differences in CDR3δ, but not in the TCRγ or CDR3γ usage, consistent with a previous report indicating that T22 reactivity is primarily supported by particular CDR3δ sequences encoded by Dδ2 (7). The CDR3δ sequences of T22-reactive γδ-T cell progenitors developing in the absence of ligand were shorter in length, less negatively charged, and depleted of the Dδ2-encoded EGYEL motif. It should be noted that ∼60% of T22-reactive γδ-T cell progenitors that developed to maturity in the

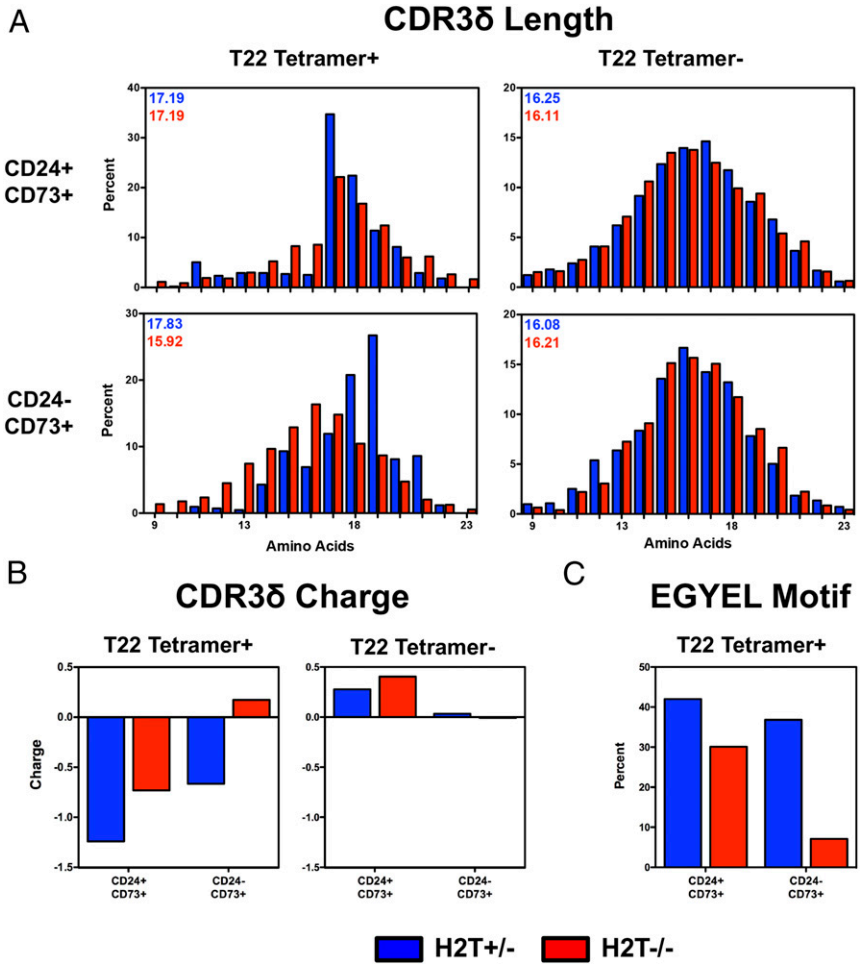

Fig. 4. Alterations in TCRδ repertoire in the absence of γδTCR-selecting ligand. NGS of the CDR3δ sequences was performed on T22 tetramer+ and T22 tetramer− CD24+CD73+ and CD24−CD73+ cells, as defined in Fig. 1*C*, to determine (*A*) CDR3δ length, (*B*) CDR3δ charge, and (*C*) presence of the EGYEL motif within the CDR3δ. Data are representative of at least two independent experiments.

presence of H2T ligand, and ∼90% that matured in its absence, lacked the EGYEL motif, and yet were T22 reactive. This makes two important points. First, structures other than the Dδ2-encoded EGYEL motif can support T22 reactivity. Second, as is true for αβ-lineage T cells, γδ-T cells that are reactive to one ligand (T22) can be selected on another. Taken together, these data provide support for the perspective that ligand does influence the resulting γδ-TCR repertoire of at least some γδ-T cell subsets.

Our results clearly suggest that ligand influences the repertoire; however, the manner in which it influences the repertoire remains to be defined. By analogy to selection of αβ-T cell progenitors, ligand could either positively or negatively influence selection, depending on the nature of the TCR–ligand interaction. γδ-TCR–selecting ligands that positively influence selection of γδ-T cells would be predicted to lead to an enrichment or preservation of consensus motifs that contribute to binding while those negatively influencing selection of γδ-T cells would be expected to be depleted by ligand engagement. Our analysis reveals that H2T deficiency led to a loss of the consensus EGYEL binding motif, consistent with the former possibility that EGYEL-dependent ligand interactions have a positive influence and promote the development of T22-reactive γδ progenitors. Similarly, butyrophilin-like molecules, including Skint1, have also been shown to have a positive role in promoting the development of particular Vγ subsets of γδ-T cells, although it remains unclear whether these molecules are bona fide ligands, or cofactors (23, 24). While these are examples of ligand interactions that promote development, there are others that attenuate development or redirect developing γδ-T cells to alternative fates. Indeed, KN6 Tg progenitors are positively selected on low-affinity H2-T10/22^{d} ligand but negatively selected on H2-T10/22^{b}, which is reported to bind the G8 T10/22-reactive γδTCR with ∼10-fold higher affinity (7, 25). It should be noted that not all of the KN6 progenitors exposed to high-affinity T10^{b} ligand are deleted (25). Indeed, those KN6 γδ-T cell progenitors that survive and mature in the presence of high-affinity T10/22^{b} ligand become PLZF-expressing innate-type γδ cells (26). This indicates that the affinity for ligand, and the consequent differences in TCR signaling, can lead to alternate developmental outcomes, as has been recently observed by manipulating the signaling capacity of γδ lineage progenitors (27, 28). The T22-reactive cells that develop in the absence of T10/22 ligand exhibited reduced representation of the EGYEL motif, as well as CDR3δ sequences that were less negatively charged and reduced in length. These changes in CDR3δ might reduce the affinity of the resulting γδTCRs for T10/22 ligand, as is true for the KN6 γδTCR, which has a shorter CDR3δ and 10-fold lower affinity for T22 than does the T10/22-reactive G8 γδTCR (7). Nevertheless, it is not possible to predict how the absence of ligand has affected the affinity of T22-reactive γδTCRs in the resulting repertoire. Addressing this question will require isolation of the γδTCRs selected in the presence and absence of T10/22 and then assessing their affinity for ligand and their ability to support γδ-T cell development. The outcome of this effort would provide insight into the longstanding and controversial question of the role of intrathymic ligands in γδ selection.

Materials and Methods

Mice. All mice were maintained in Fox Chase Cancer Center's Association for Assessment and Accreditation of Laboratory Animal Care accredited animal

colony and analyzed between 6 and 8 wk of age. KN6 γδ-TCR Tg mice were previously described (10). IL17-GFP reporter mice were purchased from Jackson Laboratory. The *H2t* locus was ablated by employing zinc-finger nuclease (ZFN)-based mutagenesis. ZFN targeting the murine genome just 5′ of *H2t24 and 3′* of *H2t3* was used to introduce double-stranded DNA breaks which were repaired by homologous recombination with either a LoxP containing 100 bp oligonucleotide (*H2t24)* or a LoxP containing 1.3 kb double-stranded donor substrate. The LoxP sites were knocked in sequentially with the 5′ site first, followed by the site at the 3′ end of the *H2t* locus. Successful mutagenesis was detected using Cell nuclease and verified by genomic sequencing. Subsequently, the LoxP-flanked *H2t* locus was excised in the germline using CMV-Cre. The resulting $H2t^{-/-}$ mice were backcrossed to C57BL/6 mice for 10 generations, crossed to the KN6 γδ-TCR Tg on a BALB/c background, and crossed to the IL17-GFP reporter mice. All experiments were approved by the Fox Chase Cancer Center Institutional Animal Care and Use Committee.

Flow Cytometry. Flow cytometry was performed on single-cell suspensions from thymus and spleen. Cells were isolated and stained with the following antibodies: anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD24 (M1/69), anti-CD25 (PC61), anti-CD44 (IM7), anti-CD73 (TY/11.8), anti-Thy1.2 (53-2.1), anti-TCRδ (GL3), anti-B220 (RA3-6B2), anti-CD11c (N418), anti-Gr1 (RB6-8C5), anti-Ter119 (TER-119), anti-TCRβ (H57-597), anti-IL17A (TC11-18H10.1), and anti-IFNγ (XMG1.2). The antibodies were purchased from eBioscience, BD Bioscience, or BioLegend. Intracellular flow cytometry for cytokine production was performed as previously described (14). Data were analyzed on LSR II (BD PharMingen). Dead cells were excluded from analyses using propidium iodide. FlowJo Software (Treestar) was used to analyze data.

H2-T22 Tetramer. H2-T22 tetramers were produced in fly cells transfected with the cDNA encoding the extracellular domain of T22 extended at the C terminus by a biotinylation sequence (GGIFEAMKMELRD) and a hexa-hisitidine tag; S2 cells were cotransfected with a BirA cDNA to allow direct biotinylation at the time of protein induction. Recombinant proteins were purified by affinity chromatography on Ni-NTA beads followed by anion-exchange chromatography. Biotinylation was measured by streptavidin-Sepharose pull-down and proteins were aliquoted and frozen at −80 °C. H2-T22 monomers were incubated with streptavidin-PE (Invitrogen) to generate H2-T22 tetramer-PE. To stain for H2-T22 tetramer cells, total thymocytes were depleted with anti-CD4 (GK1.5) and anti-CD8 (2.43.H11) hybridoma supernatant and Bio-Mag anti-goat rat IgG magnetic beads (Qiagen).

TCR Repertoire Analysis. Cell populations were purified by flow cytometry using an FACSAria II (BD Biosciences). RNA was isolated using RNeasy MicroKit (Qiagen) per manufacturer's instructions. NGS of the TCRγ and TCRδ chains was performed at iRepertoire. Analysis of CDR3γ and CDR3δ sequencing was performed in R. CDR3γ and CDR3δ charge was determined using the Peptides package in R (29).

Quantitative Real-Time PCR. RNA was purified using the RNeasy MiniPrep Kit (Qiagen) per manufacturer's instructions and converted to cDNA using the SuperScript II kit (Invitrogen) with oligo $dT_{(12–18)}$ primers (Invitrogen). Expression of indicated genes was measured by real-time PCR using stock TaqMan primer/probe sets on an ABI Prism 7700 Real Time PCR machine (Applied Biosystems). Analysis was performed in triplicate, normalized by *Gapdh*, and converted into fold difference. Primer and probes were from Applied Biosystems: *Gapdh*, Mm99999915_g1 and *H2t10*, Mm00456692_g1.

In Vitro Cell Culture. Mouse ear fibroblast lines (mFib) from $H2t^{+/-}$ and $H2t^{-/-}$ mice were generated as previously described (30, 31). Briefly, ears from $H2t^{+/-}$ and $H2t^{-/-}$ mice were diced into small pieces and treated with 1,000 U/mL collagenase for 25 min at 37 °C. Ear pieces were centrifuged, washed with HBSS, and treated with 0.3% trypsin for 60 min at 37 °C. Isolated fibroblasts were cultured in DMEM + 10% FCS + 1% MEM nonessential amino acids + 1% penicillin/streptomycin (Gibco). mFib lines were subsequently transduced with a pMIY-DL1 retrovirus to generate $H2t^{+/-}$ mFib-DL1 and $H2t^{-/-}$ mFib-DL1 lines. Lineage-(lacking CD45R, CD11c, Gr1, Ter119, TCRβ) CD4−CD8− TCRδ+ T22 tetramer+CD24+CD73− and CD24+CD73+ cells were isolated from $H2t^{-/-}$ mice by electronic sorting and cultured on $H2t^{+/-}$ mFib-DL1 or $H2t^{-/-}$ mFib-DL1 for 4 d at 37 °C in the presence of Flt3L (5 ng/mL) and IL7 (5 ng/mL). On day 4, cells were harvested and analyzed for Thy1.2, CD4, and CD8 surface expression by flow cytometry.

ACKNOWLEDGMENTS. We gratefully acknowledge the assistance of the following core facilities of the Fox Chase Cancer Center: Cell Culture, DNA Sequencing, Flow Cytometry, Laboratory Animal and Transgenic Mouse. D.L.W. was supported by NIH Grants P01 AI102853 and Cancer Center Support Grant P30CA006927, an appropriation from the Commonwealth of Pennsylvania, and the Bishop Fund. S.P.F. was supported by NIH Grants T32CA009035 and F32AI120541. F.C. was supported by NIH Grants T32CA009035 and F32AI098241. J.C.Z.-P. is a Canada Research Chair in Developmental Immunology, and supported by the Canadian Institutes of Health Research (MOP-42387) and Krembil Foundation. D.J.K. was supported by Cancer Center Support Grant P30CA006927.

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