Altered composition of the γδ T cell pool in lymph nodes during ageing enhances tumour growth

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ABSTRACT

How age-associated decline of immune function leads to increased cancer incidence is poorly understood. Here, we have characterized the cellular composition of the γδ T cell pool in peripheral lymph nodes (pLNs) upon ageing. We found that ageing has minimal cell-intrinsic effects on function and global gene expression of γδ T cells, and TCRγδ diversity remained stable. However, ageing altered TCRδ chain usage and clonal structure of γδ T cell subsets. Importantly, IL-17-producing γδ17 T cells dominated the γδ T cell pool of aged mice - mainly due to the selective expansion of Vγ6+ γδ17 T cells and augmented γδ17-polarisation of Vγ4+ T cells. Expansion of the γδ17 T cell compartment was supported by increased Interleukin-7 expression in the T cell zone of old mice. In a Lewis lung cancer model, pro-tumourigenic Vγ6+ γδ17 T cells were exclusively activated in the tumour-draining LN and their infiltration into the tumour correlated with increased tumour size in aged mice. Thus, upon ageing, substantial compositional changes of γδ T cell pool in a dysregulated pLN microenvironment promote tumour growth.
INTRODUCTION

A decline of potent T cell responses during ageing has been linked to increased susceptibility to infection and the drastic rise in cancer incidence observed in elderly mice and humans [1-3]. Three interrelated components of the immune response are affected by immunosenescence: the immune cells themselves, the supporting lymphoid organs and circulating factors that guide responses of immune cells as well as lymphoid organs [2]. In αβ T cells, a restricted TCR repertoire, loss of intrinsic cell functions, compromised priming as well as chronic and low-grade inflammation have been associated with impaired anti-tumour responses [1].

γδ T cells are unconventional T cells that combine adaptive features with rapid innate-like responses to mediate responses to infection, tissue damage, and cancer [4]. In contrast to αβ T cells that acquire cytokine-secreting effector functions upon activation in the periphery, murine γδ T cells acquire their effector potential in the thymus where they differentiate into either IFN-γ-producing (γδ1) or IL-17-producing (γδ17) lineages [5]. It is this pre-activated differentiation state and unique innate-like activities, that enable γδ T cells to rapidly infiltrate into inflammatory sites, such as tumours, in the periphery. Here they modulate the early local microenvironment and subsequent αβ T cell responses by secretion of pro-inflammatory cytokines [6, 7].

The anti-tumour effects of γδ T cells are well-established in various cancer models - mainly due to their extensive cytotoxic capacity and IFN-γ production [8, 9]. However, tumour-promoting roles of the IL-17-producing γδ T cell subsets have emerged [10, 11]. Pro-tumour mechanisms of IL-17 produced by Vγ4+ and Vγδ6+ γδ T cells include the promotion of angiogenesis [12] and recruitment of immunosuppressive cells, such as myeloid-derived suppressor cells (MDSCs) [13, 14] and peritoneal macrophages [15].

Most studies on the functions of γδ T cells have focused on their roles in barrier tissues - mainly skin [16] and gut [17] - and in the tumour mass itself [9]. However, whether γδ T cells, resident in peripheral lymph node (pLN), are important for tumour-specific responses, as they have recently been shown to be for the response to inflammatory stimuli [18-21], remains unclear.

Also, currently unknown is how the γδ T cell pool in peripheral lymphoid tissues changes upon ageing, and how age-related alterations may affect the tumour microenvironment. For the first time, we have characterised the γδ T cell compartment in pLNs during ageing and investigated the functional relevance for regulating anti-tumour immune responses.

We find that, upon ageing, the γδ T cell pool in pLNs becomes entirely biased towards the γδ17 lineage, whilst γδ1 T cell numbers are significantly reduced. We establish that this striking γδ17 bias is due to a substantial accumulation of Vγδ6+ γδ17 T cells and, in part, an increased γδ17 polarisation of Vγ4+ and Vγ2/3/7 T cell subsets in old mice. The γδ17 lineage bias is supported by increased IL-7 production in the pLNs of old mice, which provides a selective niche for the expansion of γδ17 T cells. Interestingly, γδ TCR diversity is not affected, but TCRδ chain usage and clonal substructure is altered upon ageing. Upon tumour challenge, Vγ6+ γδ17 T cells become activated in pLNs, migrate into the tumour and create a pro-tumour microenvironment that enhances tumour growth in the aged mice.
These results demonstrate that the γδ T cell pool in pLNs is essential for shaping the balance of pro- and anti-tumour immune responses. Bias towards the pro-tumourigenic γδ17 lineage during ageing thus may be a crucial contributor to age-related increase in tumour incidence.

RESULTS

γδ17 T cells constitute the majority of the γδ T cell pool in peripheral lymph nodes of old mice

To determine the effect of ageing on size and composition of the γδ T cell pool, we analysed inguinal and axillary lymph nodes (here termed peripheral lymph nodes, pLNs) from young (3 months old) and old (>21 months old) C57BL/6 mice. Upon ageing the proportion of γδ T cells amongst all CD3+ T cells in pLNs was increased 2-fold (Fig. 1A). The absolute number of γδ T cells in pLNs was slightly decreased (Fig. 1B) as a consequence of the smaller pLN size in old animals. However, when normalized to the same size, γδ T cells are 2 times more abundant in pLNs from aged mice (Fig. 5D, left panel). The maturation status, assessed by the characteristic lack of CD24 expression by mature γδ T cells, was similar between the two populations (Suppl. Fig. 1A). Thus, mature γδ T cells are enriched in the pLNs of old mice.

In the thymus, commitment of γδ T cells towards γδ1 and γδ17 lineages can be distinguished by expression of CD44 and CD45RB [22]. We first confirmed that this phenotypic segregation of γδ1 (CD44+ CD45RB+) and γδ17 (CD44hi CD45RB-ve) T cells is also observed in pLNs (Suppl. Fig. 1B). Upon stimulation with PMA/ionomycin, CD44hi CD45RB-ve cells produce IL-17 but not IFN-γ, whereas CD44+ CD45RB+ cells produce IFN-γ and not IL-17. CD44-ve CD45RB+ cells - an intermediate cell population undergoing differentiation towards the γδ1 lineage - produce only limited IFN-γ upon stimulation, and CD44-ve CD45RB-ve progenitor cells produce neither IL-17 nor IFN-γ. Consistent with previous reports [5, 10, 22-26], CD44hi CD45RB-ve γδ17 T cells were IL-7Rhi CCR6+ IL-23Rhi CD27-ve CD62L-ve, while CD44+ CD45RB+ γδ1 T cells showed an IL-7Rlo CCR6-ve IL-23Rlo CD27hi CD62Lhi phenotype (Suppl. Fig. 1C). We next defined the contribution of γδ1 and γδ17 lineages to the γδ T cell pool in pLNs. We found that γδ1 T cells and γδ1 precursor (γδ1int) cells constitute greater than 80% of the γδ T population in pLNs of young mice whereas γδ17 T cells represented only 15% (Fig. 1C). Strikingly, this bias was reversed in old mice: γδ1 T cells are diminished and the γδ17 T cell population increases to 60-80% of total γδ T cells (Fig. 1C). pLNs from middle-aged (12 months old) animals showed an intermediate phenotype, suggesting that loss of γδ1 and gain of γδ17 T cells occurs gradually upon ageing (Suppl. Fig. 2A). We further confirmed the age-specific γδ1/γδ17 lineage redistribution using CD27 as an additional marker to separate γδ1 (CD27+) and γδ17 (CD27-ve) T cells, again observing increased proportion of γδ17 T cells (CD27-ve) in pLNs of aged mice [27] (Suppl. Fig. 2B). γδ17 T cells resembled highly activated T cells (CD44hi CD62L-ve), as previously reported [24]. Interestingly, γδ1 T cells had a central memory-like phenotype (CD44int CD62L+) and γδ1int T cells showed a naïve-like phenotype (CD44-ve CD62L-) (Suppl. Fig. 2C, D). Taken together, upon ageing the γδ T cell population undergoes a dramatic redistribution favouring the γδ17 T cell lineage.
Previous studies established that a high fat diet leading to obesity can result in a similar γδ17 phenotype in mice [28, 29]. The mice analysed in this study were fed a standard diet, but some old mice were obese. Importantly, both obese and lean old mice presented with a γδ17 bias and we observed no correlation between obesity and γδ17 phenotype. Moreover, our analyses of lean, middle-aged (12-month old) mice that displayed an intermediate γδ17 phenotype in pLNs point towards a gradual accumulation of the phenotype with age.

To determine the functional consequence of increased γδ17 T cells in pLNs of old mice, we assessed cytokine production upon in vitro stimulation with PMA/ţonomycin. While on average 10% of γδ T cells from young mice produced IL-17, the proportion of IL-17-producing γδ T cells increased to 50% in old mice. In contrast, over 20% of γδ T cells produced IFN-γ in young mice, and this decreased to below 10% of γδ T cells in old mice (Fig. 1D). Notably, the absolute levels of IL-17 and IFN-γ production by individual activated cells were similar between young and old γδ T cells (Suppl. Fig. 2E), indicating that, once activated, cytokine production capacity of γδ T cells is maintained during ageing. Despite γδ T cells representing only 1-2% of total T lymphocytes in pLNs, they constitute approximately half of the IL-17-producing cells upon stimulation (Fig. 1E). Taken together, we conclude that the prevalent IFN-γ response by γδ T cells in young mice becomes skewed towards an IL-17-dominated response during ageing.

**Composition of γδ T cell subsets in the pLN pool changes during ageing**

Based on their TCRγ chain usage, γδ T cells can be classified into different subsets, each with distinct tissue distribution and degree of plasticity with regard to differentiation towards the γδ1 and γδ17 lineage during thymic development or in the periphery (Fig. 2A) [5, 30]. We sought to uncover the nature of the γδ17 bias observed in pLNs of old mice. Using the strategy described in Fig. 2B, we discriminated γδ T cell subsets (Heilig and Tonegawa nomenclature) [31] according to their lineage commitment. Consistent with previous reports [10, 30], Vγ1+ and Vγ4+ T cells were the major γδ T cell subsets in pLNs of young mice (Fig. 2C). By contrast, in pLNs of old mice, the Vγ1+ T cell pool contracted 2-fold, and strikingly the Vγ6+ T cell pool, which was barely detectable in young mice, expanded more than 10-fold. The Vγ4+ T cell pool was also slightly smaller in pLNs of old mice (Fig. 2C).

Vγ1+ T cells were predominantly committed to the γδ1 lineage in young and old mice, whereas Vγ2/3/7 and Vγ4+ T cells gave rise to both γδ1 and γδ17 T cells (Fig. 2D). Although γδ1 T cells constitute the majority of the Vγ2/3/7 and Vγ4+ T cell pool in the pLNs of young mice, γδ17 cells were considerably enriched in the Vγ2/3/7 (Fig. 2D, upper right panel) and Vγ4+ (Fig. 2D, lower left panel) T cell pool in pLNs of old mice. Vγ6+ T cells are invariant and exclusively committed to the γδ17 lineage in both young and old mice (Fig. 2D, lower right panel).

Thus, enrichment of γδ17 lineage-committed Vγ6+ T cells and changes in lineage commitment of Vγ4+ and Vγ2/3/7 T cells underpin the increase of γδ17 T cells in pLNs during ageing.

To determine whether the biased γδ17 phenotype we observe in aged mice is specific to the pLNs, we investigated the γδ T cell pool in the spleen, another secondary lymphoid organ. In the spleen we found a small decrease in γδ T cell number and the proportion of γδ T cells in total CD3+ T lymphocytes (Suppl. Fig. 3A, B), while the maturation of γδ T cells was unaffected by ageing (Suppl. Fig. 3C). Vγ6+ T cells were also enriched, albeit to a lesser degree, thus leading to a γδ17 biased cell pool in the spleen of old mice (Suppl. Fig. 3D, E). The decline of
the splenic Vγ1+ T cell pool during ageing was less severe compared with pLNs (Suppl. Fig. 3E).

**Ageing has minimal impact on the transcriptome of γδ T cells**

In order to determine the mechanism underlying the γδ17 bias in old pLNs, we carried out transcriptome analysis to compare purified Vγ6+ γδ17, Vγ4+ γδ17, Vγ4+ γδ1, and Vγ1+ γδ1 T cells from young and old pLNs (gating strategy provided in Suppl. Fig. 4). We confirmed purity of sorted populations by analysis of characteristic transcription factors, surface markers, cytokines, chemokines and receptors as well as effector molecules, reported to delineate respective γδ1 and γδ17 T cell subsets (Fig. 3A and Suppl. Fig. 5). Overall, when compared with γδ1 T cells (Vγ1+ and Vγ4+), γδ17 T cells (Vγ4+ and Vγ6+) showed higher expression of Cd44 and lower expression of Ptprc, which are both surface markers used for the segregation of γδ1 and γδ17 T cells by FACS sorting [22]. Consistent with previous reports, Vγ4+ and Vγ6+ γδ17 T cells expressed Ccr2, Ccr6, Il17r and Il23r at higher level and down-regulated expression of Cd27 and Sell [20, 23, 24, 26, 27, 32, 33]. Master transcription factors were highly expressed in the respective lineage: Rorc, Sox13, Mal and Zbtb16 in γδ17 T cells and Eomes, Tbx21 and Id3 in γδ1 T cells [18, 26, 34-41]. In homeostasis, γδ1 T cells expressed higher levels of Ifng and γδ17 T cells sporadically expressed Il17a. Interestingly, Vγ4+ and Vγ6+ γδ17 T cells expressed high levels of TCR complex component CD3ε [42] and of Tcrg-C3 and Tcrg-C1, respectively. By contrast, cytotoxic molecules and NK receptors were highly expressed in Vγ1+ and Vγ4+ γδ1 T cells (Fig. 3A and Suppl. Fig. 5).

Principal component analysis (PCA) revealed distinct separation between γδ1 and γδ17 lineages in PC1 but γδ T cells expressing different Vγ chains were not separated in PC2 (Fig. 3B). Notably, γδ T cells from young animals showed higher variance in the γδ1 lineage and cells from old mice showed higher variance in the γδ17 lineage along PC2 (Fig. 3B). Nevertheless, direct comparison of each γδ T cell subset from young and old mice identified only a small number of differentially expressed genes in Vγ4+ γδ1 and γδ17 subsets (Fig 3C). No changes between young and old mice were detected in Vγ6+ γδ17 and Vγ1+ γδ1 T cells.

Since no major functional or transcriptomic changes were detected between young and old γδ T cell subsets we investigated whether the increase of γδ17 T cells in old mice could be due to (i) a change in TCR repertoire, and/or (ii) changes in the microenvironment of the pLN upon ageing.

**Ageing alters Vδ chain usage and clonal substructure but not global TCR diversity**

The αβ TCR repertoire has been shown to decline with age [43]. We asked whether TCR diversity of γδ T cells from pLNs also changes upon ageing in the variant Vγ4+ and Vγ1+ subsets using invariant Vγ6+ as a control. From the RNA-Seq data (paired-end, 125bp sequencing) of purified γδ T cell subsets, we reconstructed CDR3 sequences using MiXCR in the RNA-Seq mode [44, 45]. We confirmed the ability of MiXCR to reconstruct the correct Vγ chains for each of the γδ T cell subsets (Suppl. Fig. 6). For further downstream analyses, we selected the γδ T cell subset-specific Vγ chains. Focusing on variant Vγ1+ γδ1, Vγ4+ γδ1 and Vγ4+ γδ17 cells, we found surprisingly no significant difference in γδ TCR diversity between young and old animals, as indicated by very similar Inverse Simpson Indexes (Fig. 4A).
Next, we assessed the recombination of TCRδ chains and observed distinct preferences in the choice of Vδ segments amongst the different subsets (Fig. 4B). In young mice, TCRδ chains utilised by Vγ1+ γδ1 T cells contained mainly rearrangements with Vδ6 (~40%) and Vδ1 (~30%) segments, followed by Vδ7 (~10%), Vδ5 (~10%), Vδ2 (~7%), and Vδ12 (~3%), consistent with a previous study [46]. In old mice, the use of Vδ6 increased slightly to ~50% and the use of Vδ1 decreased marginally to ~25% compared with young mice (Fig. 4B, upper-left panel). In young mice, the majority of TCRδ chains from the Vγ4+ γδ1 T cell samples contained a Vδ7 segment (~75%), followed by Vδ5 (~10%), Vδ6 (~8%), and Vδ2 (~7%) again similar to observations in a previous study [46] (Fig. 4B, upper-right panel). In old mice, the preferential use of Vδ7 by Vγ4+ γδ1 T cells was slightly reduced to ~70% (Fig. 4B, upper-right panel). Strikingly, ageing showed a profound impact on the Vδ segment usage for TCRδ recombination of Vγ4+ γδ17 T cells (Fig. 4B, lower-left panel). Vγ4+ γδ17 T cells in young mice preferentially utilised Vδ5 (~50%), Vδ2 (~30%), and Vδ7 segments (~10%), for the recombination of their TCRδ chain. In old mice the preference among Vδ5 and Vδ2 segments was reversed compared with young mice: Vδ2 (~60%) was preferentially used followed by Vδ5 (~30%) (Fig. 4B, lower-left panel). As reported, invariant Vγ6+ T cells from young and old mice used only the Vδ4 segment for the assembly of their γδ TCR [47] (Fig. 4B, lower-right panel). The encoded CDR3 amino acid sequence (CGSDIGGSSWDTRQMFF) is the same to the Vδ1 chain that was previously observed to be paired with Vγ6 chain by others [42, 48, 49].

We interrogated the profound changes in TCRδ chain preference observed in Vγ4+ γδ17 T cells by investigating the clonality in Vδ2 and Vδ5 sequences (Fig. 4C). Looking at the 10 most frequent Vδ2 clones per individual mouse, we found 6 clones expanded (>1%) in old mice representing from >1 to up to 33% of the entire repertoire in the individual mouse. Half of the clonal expansions were private (occurring in 1 out of 4 old mice) while the other half occurred in 2-3 out of the 4 mice. When we looked at the Vδ5 sequences we also found one expanding clone in 1 out of 4 old mice representing up to 18% of the repertoire. Furthermore, we identified two CDR3 clones from separate mice with different nucleotide sequences both giving rise to an emerging Vδ2+ clone with CALMERDIGGIRATDKLV amino acid sequence (Fig. 4C). Most interestingly, we detected the canonical ASGYIGGIRATDKLV (Vγ4Jγ1/Vδ5Dδ2Jδ1) clone [46] in all individuals, and found that this dominant clone in young mice decreases over 50% in 3 out of 4 old mice.

Thus, although organismal ageing did not impact on global γδ TCR diversity, it affected Vδ gene segment usage, led to both private and non-private clonal expansions and a collapse of the recently discovered, dominant invariant ASGYIGGIRATDKLV clone in Vγ4+ γδ17 T cells.

**Increased IL-7 in the LN microenvironment during ageing correlates with accumulation of γδ17 T cells**

To determine whether the microenvironment affects the γδ17 bias, we interrogated the expression of cytokines associated with activation and homeostatic maintenance of γδ17 T cells in primary and secondary lymphoid organs. We determined the mRNA expression levels of IL-1β and IL-23, which promote polarisation of γδ17 T cells in peripheral tissues [26, 32, 50], as well as IL-2, IL-15, and IL-7, which are involved in maintenance of γδ T cells [51-53], in whole pLNs (Fig. 5A) of old and young mice. Expression of IL-1β, IL-23, and IL-15 was not
significantly different between young and old pLNs. IL-2 mRNA expression was low but slightly upregulated in old pLNs. Most strikingly, IL-7 mRNA was highly expressed in the pLNs of both young and old mice and its levels were 3-4 folds upregulated in old mice (Fig. 5A). Interestingly, IL-7 has been reported to preferentially promote the expansion of IL-17-producing CD27ve γδ T cell in the pLNs upon TCR stimulation [24]. As previously reported [22, 25], we found that the expression of IL-7 receptor-α (CD127) is over 2-fold higher in γδ17 compared with γδ1 T cells (Fig. 3A, 5B and Suppl. Fig. 1C).

IL-7 is constitutively secreted by stromal fibroblastic reticular cells in the T cell zone [54] and by lymphatic endothelial cells [55]. To determine whether IL-7 secreting stroma cells generate a supportive niche for Vγ6+ γδ17 T cells during ageing, we used RNAscope® to interrogate the spatial relationship between Vγ6+ γδ17 cells and IL-7-producing cells. In young and old mice γδ T cells were mainly localised in the T cell zone (Fig. 5C). Despite clear involution of pLNs in aged mice, the density of γδ T cells in T cell zone was highly increased (Fig. 5C, D). Consistent with flow cytometric analysis (Fig. 2B), we showed that the proportion of Vγ6+ γδ17 T cells in the γδ T cell pool is dramatically enriched in aged mice, suggesting the selective accumulation of this unique γδ T cell subset (Fig. 5E).

In pLNs, IL-7 expression was mainly restricted to T cell zone where the expression was ~6 fold higher compared with the follicle. Importantly, we found that the T cell zone in the old pLNs contained ~5-fold more IL-7 mRNA compared with young pLNs (Fig. 5F). In both old and young mice, γδ T cells localised to the T cell zone of the pLNs, with only a few cells found in the periphery of follicles (Fig. 5G). Strikingly, all γδ T cells were localised in close proximity to IL-7 mRNA expressing cells (on average <20µm) and this distance was reduced to 10µm for Vγ6+ T cells in old pLN (Fig. 5H).

Taken together, we show that IL-7 production in the T cell zone of pLNs is highly increased upon ageing and correlates with the expansion of γδ17 T cells, especially Vγ6+ T cells, resulting in a skewed peripheral γδ T cell pool that might favour pro-inflammatory immune responses.

**γδ17 T cell bias affects tumour development in aged mice**

γδ T cells have important and well-established anti-tumour roles due to cytotoxic function and IFN-γ secretion of γδ1 T cells. By contrast, γδ17 T cells have been shown to mediate pro-tumour activities [9]. We hypothesized that LN-resident γδ T cells can be activated upon tumour challenge, migrate into the tumour mass and impact on the tumour microenvironment.

First, we tested whether LN-resident γδ T cells can infiltrate into tumours using the 3LL-A9 syngeneic Lewis lung cancer model. We blocked T cell egress from LNs by administering FTY720 to mice [19, 21]. As expected FTY720 treatment reduced the number of CD4+ and CD8+ T cells in the tumour (Suppl. Fig. 7A, B). Strikingly, also the number of γδ T cells was greatly reduced to below 20% upon FTY720 treatment compared with control animals (Fig. 6A). Taken together, we show for the first time that LN-resident γδ T cells can contribute significantly to the γδ T cell pool in tumours.

Next, we asked whether the γδ17-biased γδ T cell pool in old mice can affect the tumour response. Strikingly, we found that 3LL-A9 tumours developed faster in old mice (Fig. 6C). γδ
T cell infiltration into the tumour was similar in young and old mice (Suppl. Fig. 8A, B), but the balance between γδ1 and γδ17 T cells was altered: while tumours from young mice maintained a substantial proportion of anti-tumour γδ1 T cells, over 90% of the γδ T cell pool in tumours of aged mice were tumour-promoting γδ17 T cells (Fig. 6D). The tumour-infiltrating γδ T cell pool in young mice was heterogenous, containing Vγ1+, Vγ2/3/7, Vγ4+, and Vγ6+ T cells. In contrast, the tumour-infiltrating γδ T cell pool in old mice consisted mainly of Vγ6+ T cells (>80%) (Fig. 6E). Importantly, the proportion of Vγ6+ T cells in total tumour-infiltrating γδ T cell pool correlated with tumour size (Fig. 6F). Skin-resident Vγ5+ T cells [56] were absent from the subcutaneous tumours of old and young mice. In the tumour, lineage bias of subsets was very different from the homeostasis observed in the pLNs. In the tumour microenvironment, progenitor and γδ1+int populations are lost, Vγ1+ and surprisingly Vγ4+ T cells were γδ1 and only Vγ2/3/7 and Vγ6+ T cells were γδ17-committed (Suppl. Fig. 8C).

We then asked which cells were activated and/or exhausted (by their PD1 and Tim-3 expression) in the tumour microenvironment to determine the involvement of different subsets in the anti-tumour response, (Fig. 6G, H and Suppl. Fig. 8D). Approximately 50% of γδ T cells in tumours of young mice and 70% of γδ T cells in tumours of old mice were highly activated and exhausted (PD1+, Tim-3+) (Fig. 6G). Interestingly, only γδ17 T cells showed high levels of activation (Suppl. Fig. 8D) while γδ1-committed Vγ1+ and Vγ4+ T cells were not activated (Fig. 6H). The majority of tumour-infiltrating Vγ2/3/7 T cells in both young and old mice were single-positive for Tim-3+. Most intriguingly, only tumour-infiltrating Vγ6+ T cells were highly activated/exhausted with high expression levels of PD-1 and Tim-3 (Fig. 6H).

In the tumour-draining LN, the overall lineage commitment and subset composition of γδ T cells were similar to the steady state in young and old mice (Suppl. Fig. 9A, B). Only the γδ17 bias within Vγ2/3/7 and Vγ4+ T cell subsets during ageing was not observed in the tumour-draining LN of old mice (Suppl. Fig. 9D). At the point of analysis, no activation of Vγ1+, Vγ2/3/7 and Vγ4+ T cells was detected (Suppl. Fig. 9D). Importantly, only Vγ6+ T cells expressed Tim-3 and PD-1 in old and young mice (Suppl. Fig. 9C). No upregulation of Tim-3 or PD-1 by Vγ6+ T cells was observed in the non-tumour-draining LN in tumour bearing mice. In addition, Vγ6+ T cells from pLNs of unchallenged mice did not express Tim-3 at steady state or upon short-term in vitro stimulation with PMA/lonomycin (data not shown). These results suggest that Vγ6+ T cells become activated in the tumour-draining LN. Interestingly, the low number of Tim-3 single positive Vγ2/3/7 γδ17 T cells observed in the tumour cannot be found in the dLN, indicating a LN-independent origin of activation for this subset. These results were confirmed by the use of another syngeneic Lewis lung tumour model (Suppl. Fig. 10).

Taken together, we show that Vγ6+ γδ T cells become selectively activated in the draining LN, migrate into the tumour and represent the majority of the tumour-resident γδ T cells in old mice. Furthermore, the biased γδ17 T cell pool in pLNs of old mice correlates with a pro-tumour microenvironment and enhanced tumour progression.

**DISCUSSION**

How γδ T cells change upon organismal ageing has not been extensively explored. We have conducted the first comprehensive study of the murine γδ T cell compartment in pLNs during
ageing. Remarkably, we found that - upon ageing - the γδ17 lineage dominates the γδ T cell pool at the expense of γδ1 T cells. The striking γδ17 bias with age is due, predominantly, to accumulation of Vγ6+ T cells, and in part by increased γδ17 bias of Vγ4+ and Vγ2/3/7 T cell subsets.

The transcriptome of γδ T cells from young and old mice showed only minimal differences in gene expression. We found that Ly6C is upregulated in Vγ4+ γδ17 T cells from aged mice. Previous work has determined that cross-linking of Ly6C induces LFA clustering/adhesion thereby supporting the homing of naive and central memory CD8+ T cells to the lymph node [57, 58]. Whether this is a contributing factor enabling Vγ4+ γδ17 T cells to accumulate in the pLNs requires further investigation. In addition, old γδ T cells secrete the same level of IFN-γ and IL-17 as their young counterparts. Similar results have been observed for naïve CD4+ T cells at transcriptional and functional level [59]. Our results thus support the notion that γδ T cells are intrinsically unaffected by ageing and instead the age-related γδ17 bias is a function of the altered environment, for instance, as we have shown, in the pLNs.

For the first time we localised γδ T cells, especially Vγ6+ T cells, in pLNs of old mice and found that the majority of murine γδ T cells reside in the T cell zone. γδ T cells were also sporadically observed in subcapsular and medullary sinuses as previously described [60, 61] but at low level. While peripheral γδ1 T cells are mainly replenished through thymic output, γδ17 T cells are maintained persistently in the periphery [62]. Importantly, we discovered that IL-7 is highly expressed in the aged pLNs and the number of γδ T cells correlated with the amount of IL-7. γδ T cells are found in close proximity to IL-7 (on average <20μm in young and <10μm in old mice) indicating that IL-7 producing cells are creating a niche in which IL-7Rα hi Vγ6+ γδ17 T cells can be maintained in old mice.

We next characterised the γδ TCR repertoire of variant subsets in young and old mice in order to elucidate changes that lead to a reduced γδ1 T cell pool and expansions in γδ17 cells. In contrast to previous work demonstrating collapse of TCR diversity in αβ T cells upon ageing [43], the TCR diversity does not collapse in γδ T cells of aged mice. TCR diversity was higher in the γδ1 subset compared with γδ17. Interestingly, Vδ chain usage was altered especially in variable Vγ4+ γδ17 T cells upon ageing. Analysis of the CDR3 regions of the altered Vδ chains revealed private and semi-private clonal expansions within the Vδ2 and Vδ5 repertoire. Interestingly, we observed that the innate Vγ4Jγ1/Vδ5Dδ2Jδ1 (ASGYIGGIRATDKLV) clone in the Vδ5 repertoire [46] declined in 3 out of 4 old animals analysed. Taken together, we have shown that consequences of ageing on the γδ TCR repertoire are: (i) altered δ chain usage (ii) clonal expansions of γδ17 clones perhaps indicating the appearance of age-related antigens; (iii) the loss of a recently described invariant, innate clone indicating loss of a specific γδ T cell reactivity upon ageing.

The role of the LN-resident γδ T cell pool in cancer has not been explored. In tumours, γδ T cells are the main source of IFN-γ at the early stage of tumour development in young mice [6]. We asked whether the acquired γδ17-bias in pLNs during ageing would impact on the early tumour microenvironment. Using a Lewis lung carcinoma model and blocking egress of T cells from LNs using FTY720, we found that γδ T cells egressing from pLNs are constituting the majority of the γδ T cell pool in the tumour. Importantly, Vγ6+ T cells but not any other γδ subset become activated in the tumour-draining LN. Due to the high numbers of these pro-
tumourigenic cells in the pLNs, the tumour microenvironment becomes highly tumour-promoting and tumours progress faster in old mice. Interestingly, γδ17-committed Vγ4+ and Vγ2/3/7 T cells are not activated upon tumour challenge, indicating that only the invariant Vγ6 TCR can recognise tumour-associated antigens or other signals, at least in the 3LL-A9 model.

Ageing is associated with chronic inflammation resulting from systemically increased pro-inflammatory cytokines. This predisposition to inflammatory responses can significantly affect the outcome of infection [63] and cancer immunotherapy [64]. In aged mice an increase in Th17 polarized CD4+ T cells [65, 66], as well as higher IL-17 secretion by liver-resident NKT cells have been described [63]. Here we discovered γδ17 T cells as a new critical pathogenic player during ageing.

Taken together, we have identified a novel age-dependent dysregulation of the γδ T cell pool that leads to enhanced tumour progression in old mice. Development of therapeutics specifically targeting γδ17 T cells and correcting the biased γδ T cell pool in the elderly might reduce the susceptibility to age-related diseases including infection and cancer.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from Charles River UK Ltd (Margate, United Kingdom) and housed under specific pathogen-free conditions at the University of Cambridge, CRUK Cambridge Institute in accordance with UK Home Office regulations. Animals were euthanized in accordance with Schedule 1 of the Animals (Scientific Procedures) Act 1986. Every mouse used was macroscopically examined externally and internally and animals with lesions or phenotypic alterations were excluded from analysis.

Tissue processing and flow cytometry

Peripheral lymph nodes (inguinal and axillary, alone or pooled), and spleen were collected from young and old mice, respectively, mashed through a 40 µm (thymus and pLNs) or a 70 µm cell strainer (spleen) (Greiner bio-one) with the plunger of a 2 ml syringe to prepare single cell suspensions. Cells were washed with PBS once and stained with Fixable Viability Dye eFluor™ 780 (Thermo Fisher Scientific). Fc receptors were blocked with TruStain fcX™ (anti-mouse FCGR3/CD16-FCGR2B/CD32, clone 93; Biolegend) in FACS buffer containing 3% FCS (Biosera) and 0.05% Sodium Azide (Sigma-Aldrich) in Dulbecco’s phosphate buffered saline (DPBS; Gibco). Subsequently, cells were stained in FACS buffer with fluorochrome-conjugated antibodies against cell surface antigens (Suppl. Table. 1).

For the characterisation of Vγ6+ T cells, the staining procedure was modified as follows. Before staining of cell surface markers, cells were stained with GL3 antibodies against TCR Vδ followed by 17D1 hybridoma supernatant (kindly provided by Prof. Adrian Hayday, The Francis Crick Institute, London) that recognises both Vγ5 and Vγ6 TCR. PE-conjugated mouse anti-rat IgM monoclonal antibody (RM-7B4, eBioscience) was then used to capture cells stained...
positive with 17D1 hybridoma supernatant. Cells were analysed using a FACS LSR II, FORTESSA or ARIA (BD) instrument and FlowJo software (v10.4, FlowJo, LLC).

**In vitro stimulation**

Single cell suspensions from peripheral LNs were washed twice with complete RPMI medium (RPMI-1640 (Gibco), supplemented with 10% heat-inactivated FCS (Biosera), 1 mM Sodium Pyruvate (Gibco), 10 mM HEPES (Sigma), 100 U/ml penicillin/streptomycin (Gibco) and 50 μM β-mercaptoethanol (Gibco)) and plated in 96-well plate with or without 50 ng/ml PMA (Sigma-Aldrich) and 1 μg/ml ionomycin (Sigma-Aldrich) in the presence of GolgiStop (1:1500 dilution, BD) for 4 hours. After incubation, cells were washed once with PBS and stained with Fixable Viability Dye eFluor™ 780 (Thermo Fisher Scientific) followed by blocking with TruStain fcX™ (anti-mouse FCGR3/CD16 - FCGR2B/CD32, clone 93; Biolegend) and staining with fluorophore-conjugated antibodies against cell surface antigens in FACS buffer. Cells were then fixed and permeabilised using BD Cytofix/Cytoperm™ Plus kit for intracellular staining with fluorochrome-conjugated antibodies against IFN-γ (clone XMG1.2, Biolegend) and IL-17A (clone TC11.18H10.1, Biolegend). Stained cells were run on a BD FACS LSR II cytometer and analysis was performed using FlowJo software (v10.4, FlowJo, LLC).

**Isolation of γδ1 and γδ17 T cells**

Single cell suspensions were prepared from inguinal and axillary LNs collected of young and old mice. To enrich γδ T cells, αβ T cells and B cells were depleted from single cell suspensions by MACS using a biotinylated antibody against TCRβ with anti-biotin microbeads and anti-CD19 microbeads, respectively. Enriched γδ T cells were then stained for FACS sorting as described above. Gating strategy used to identify \( V_{\gamma}1^+\gamma\delta1 \), \( V_{\gamma}4^+\gamma\delta1 \), \( V_{\gamma}4^+\gamma\delta17 \), and \( V_{\gamma}6^+\gamma\delta17 \) T cells is summarised in Suppl. Fig. 5. γδ T cell subsets were sorted with a BD FACS ARIA instrument directly into 3 μl of lysis buffer from SMART-Seq v4 Ultra Low Input RNA kit (1 μl of 10X Reaction Buffer and 2 μl of water) accordingly to the instructions of the manufacturer (Clontech). Cells were centrifuged, immediately frozen in liquid nitrogen, and stored at -80°C.

**RNA-Seq library preparation and sequencing**

RNA-Seq libraries were prepared using the SMART-Seq v4 Ultra Low Input RNA kit (Clontech). Cells frozen in lysis buffers were directly complemented with cold nuclease-free water plus RNase inhibitor (2 U/μl; Clontech) up to 9.5 μl of total volume. Volume of water was estimated calculating the number of events sorted in the BD FACS ARIA and the average drop size for the 70 μm nozzle used (~1 nl/droplet). ERCC spike-in RNA (Ambion) (1 μl diluted at 1:300,000) and 3' SMART-Seq CDS Primer II A (12 μM) were added to the lysis mix. cDNA was prepared following the SMART-Seq v4 Ultra Low Input RNA kit protocol (Clontech).

After cDNA preparation, RNA-Seq libraries were prepared using Illumina Nextera XT Sample Preparation Kit (Illumina, Inc., USA) and the 96 index kit (Illumina, Inc., USA). As previously described, libraries were prepared by scaling down the reactions one-fourth of the manufacturer instructions [67] and libraries were sequenced using paired-end 125bp sequencing on Illumina HiSeq4000.
**Read alignment of RNA-Seq data**

Prior to read alignment, the *Mus musculus* genome (GRCm38) was concatenated with the sequence of ERCC spike-ins (available at http://tools.lifetechnologies.com/content/sfs/manuals/ERCC92.zip). Sequenced reads were aligned against this reference using gsnap version 2015-12-31 [68] with default settings. Gene-level transcript counts were obtained using HTSeq version 0.6.1p1 [69] with the -s option set to “no” and using the GRCm38.88 genomic annotation file concatenated with the ERCC annotation file.

**Quality control of RNA-Seq libraries**

We excluded libraries with fewer than 40% of reads mapped to annotated exons or fewer than 100,000 total reads. Furthermore, we removed libraries with fewer than 10,000 genes detected with at least 1 count.

**Normalization of RNA-Seq libraries**

We used the Bioconductor R package edgeR [70] for data normalisation. More specifically, we used the calcNormFactors to estimate normalisation factors and computed counts per million using the cpm function implemented in edgeR. Gene-level transcript counts are visualized as Z-score scaled, normalized counts.

**Alignment of reads to T cell receptor genes**

TCR repertoire analysis was performed using the MiXCR software [44, 45]. In the first step, sequencing reads were aligned to the V, D, J and C genes of the T-cell receptor. For this, we used the align function with following settings: -p rna-seq -s mmu -O allowPartialAlignments=true.

**TCR assembly**

The T cell receptor sequences were assembled by calling the assemblePartial function twice to assemble partially aligned sequences. To extend TCR alignments, the extendAlignments function was called. In the last step, the assemble function was used to fully assemble the V, D, J and C genes of the TCR.

**Exporting individual clones after TCR assembly**

Individual clones were collected using the exportClones function while excluding out-of-frame variants (-o option) and stop codon containing variants (-t option). Clones were collected for the different chains (TRD, TRG, TRA, TRB, IGH and IGL) separately. This function returns the count, fraction as well as information on the V, D, J and C chain of the individual clones per library as defined through their CDR3 nucleotide sequence.
Quantitative RT-PCR

Thymus, spleen and peripheral LNs (both inguinal and axillary) were collected from healthy young and old mice and homogenised with Precellys 1.4 mm Ceramic beads in 2 ml tubes (KT03961-1-003.2, Bertin Instruments) using Precellys 24 lysis and homogenisation unit (Bertin Instruments). Total RNA was extracted from homogenised samples using Ambion Purelink RNA kit (12183025, Invitrogen) according to the manufacturer’s instructions. RNA was quantitated using the NanoDrop Spectrophotometer ND-1000 and diluted in RNase-free water to 100 ng/µl for analysis. Quantitative RT-PCR was carried out using the Superscript III Platinum One-Step qRT-PCR Kit (Life Technologies) and TaqMan™ Gene Expression Assays (Fam) (Life Technologies) to quantify the expression of following genes: Tbp (assay ID: Mm00446971_m1), Hprt (Mm03024075), B2m (Mm00437762_m1), Btn1a1 (Mm00516333_m1), Btnl1 (Mm01281669_m1), Btnl2 (Mm01281666_m1), Btnl4 (Mm03413106_g1), Btnl6 (Mm01617956_mH), Btnl9 (Mm00555612_m1), Skint1 (Mm01720691_m1), Iilb (Mm00434228_m1), Iil2 (Mm00434256_m1), Iil7 (Mm01295803_m1), Iil15 (Mm00434210_m1), Iil17a (Mm00439618_m1 IIL17a), and Iil23a (Mm00518984_m1). qRT-PCR was performed using a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher). For reverse transcription the thermal cycler was set at 50°C for 15 minutes followed by a 2 minutes incubation at 95°C, after which 50 PCR cycles of 15 seconds at 95°C followed by 1 minute at 60°C were run. All samples were run in triplicates and similar results were obtained for all housekeeping genes used (Tbp, Hprt, and B2m).

RNAscope®

Inguinal LNs were isolated from young and old mice, respectively, fixed in 10% NBF (Pioneer Research chemicals Ltd) for 24h, transferred into 70% ethanol for 24h, and embedded into paraffin blocks. Paraffin sections were cut at 3mm onto Superfrost plus slides and baked for 1 hour at 60°C. Probes and Kits (RNAscope® LS Multiplex Reagent Kit, Cat# 322800 and RNAscope® LS 4-Plex Ancillary Kit Multiplex Reagent Kit Cat# 322830) were obtained from Advanced Cell Diagnostics. TSA Plus Fluorescein System for 50-150 Slides (Cat# NEL741001KT), TSA Plus Cyanine 3 System for 50-150 Slides (Cat# NEL744001KT), TSA Plus Cyanine 5 System for 50-150 Slides (Cat# NEL745001KT), and Opal 620 Reagent Pack (Cat# FP1495001KT) were from Perkin Elmer. Probes (automated Assay for Leica Systems) and reference sequences were as follows: RNAscope® LS 2.5 Probe- Mm-Ill7, GenBank: NM_008371.4 (2-1221), RNAscope® 2.5 LS Probe- Mm-Tcrg-V6, GenBank: NG_007033.1: (2-475), and RNAscope® LS 2.5 Probe- Mm-Trdc, GenBank: gil372099096 (9-1098). Fluorescein staining was used as a dump channel for the exclusion of cells with unspecific background staining. Different combinations of fluorochromes were used for each probe to avoid bias in staining. Slides were scanned with Axio Scan (Zeiss) and images were analysed using Halo software (Indica Labs).

In vivo tumour model

3LL-A9 cells were grown in DMEM (Gibco) supplemented with 10% FCS and tested negative for mycoplasma (Mycoprobe® Mycoplasma Detection Kit, R&D systems) and mouse pathogens (M-LEVEL 1 analysis, Surrey Diagnostics). For injection, the right flank of young and old mice was shaved and 3 × 10^6 3LL-A9 Lewis lung cancer cells were injected subcutaneously. Mice were sacrificed on day 14 post inoculation and the tumour and tumour-
draining LN were harvested for characterisation of γδ T cells by flow cytometry. FTY720 (Sigma) was reconstituted in ethanol and diluted in 2% β-cyclodextrin (Sigma) for injections. Mice were injected i.p every other day with FTY720 at 1 mg/kg or with vehicle control 3 times before s.c injection of 3x10⁶ 3LL-A9 cells at right flank. FTY720 treatment was continued on day 1 post tumour cell injection for further 7 times before tissue collection at day 14/15.

Tumour tissue was weighed and minced by surgical curve scissors and then mashed through a 70 µm cell strainer (Greiner Bio-one) with the plunger of 2 ml syringe. Flow-through was passed again through a 40 µm cell strainer (Greiner bio-one) to prepare single-cell suspension. Immune cells were subsequently enriched from cell suspension by gradient centrifugation using Optiprep™ density gradient medium (Sigma-Aldrich). Briefly, cells were resuspended in 10 ml 33.3% Optiprep™ (diluted with PEB containing PBS with 0.5% BSA and 5 mM EDTA) and 5 ml PEB were layered gently on top of cell suspension without disturbing the interface of two layers. Cells were then centrifuged at 500xg for 20 minutes at 4°C without brake in the end of centrifugation. Immune cells at the interface between two layers were collected and washed twice with PBS before flow cytometric analysis.

Statistical analysis

Statistical analysis was performed using Prism 7 software (GraphPad Inc.). Each data set was firstly analysed by D’Agostino & Pearson normality test for Gaussian distribution. Unpaired t test was used for the comparisons between two data sets (young vs old) both with normal distribution. Comparisons between two groups (young vs old) failed to pass normality test were performed using Mann-Whitney test. Two-way ANOVA with Sidak Multiplicity Correction test was used to compare multiple variables, such as γδ T cell lineages and subsets, between two different groups (young vs old). Descriptive statistics are expressed as mean ± SD (standard deviation) in all figures. All statistical analyses were performed as two-tailed tests, and the level of statistical significance in differences was indicated by p values in all figures (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

Statistical analysis for RNA-Seq data

Principal component analysis of normalized, log₁₀-transformed counts was performed using the prcomp function in R. Differential expression analysis was performed using the Bioconductor package edgeR [70]. A quasi-likelihood negative binomial generalized log-linear model was fitted to the count data after removing lowly expressed genes (averaged expression <10 counts). The glmQLFTest function was used to perform genewise statistical testing incorporating the age of the animals as contrasts. Gene-level differential expression tests with a false discovery rate smaller than 10% were considered as statistically significant. To profile clonal diversity within each library, we calculated the inverse Simpsons index of the clone count as implemented in the R package tcR [71]. To allow the comparisons between libraries, we subsampled the clones to similar numbers within each T cell subset.
**FIGURE LEGENDS**

**Figure 1. γδ T cells from peripheral lymph nodes (pLN)s of old mice are predominantly γδ17-committed.** Inguinal and axillary LNs were isolated from young (3-month old, white circles) and old (>21-month old, red circles) mice. (A) Percentage of γδ T cells in total CD3+ T lymphocytes. Results are from 13 young and 12 old mice (n=5 experiments). (B) Absolute number of γδ T cells in pLN of young and old mice. Results are from 11 young and 10 old mice (n=4 experiments). (C) CD45RB and CD44 expression of mature (CD24hi) γδ T cells in pLN of young and old mice. Left: representative FACS plots (n=11 experiments). Right: Percentage of mature γδ17-committed (CD45RBve CD44hi), γδ1-committed (CD45RB+ CD44+), γδ1-intermediate (CD45RB+ CD44ve) and progenitor (CD45RBve CD44ve) γδ T cells in pLN of young and old mice. Results shown are from 17 young and 16 old mice (n=9 experiments). (D) Cell suspensions from pLN were stimulated with PMA/Ionomycin for 4 hours and examined for their production of IL-17 and IFN-γ. Representative FACS plots are gated on CD24ve γδ T cells. (E) Percentage of γδ T cells in total IL-17-producing CD3+ T lymphocytes upon PMA/Ionomycin stimulation. Results shown (D, E) are from 16 young and 15 old mice (n=6 experiments). Statistical significances for changes in cell proportions were assessed by Mann-Whitney test (A-B), two-way ANOVA (D and F), or unpaired t test (G). ****p<0.0001

**Figure 2. γδ17-committed Vγ4+ and Vγ6+ T cells are the main subsets in pLN of old mice.** (A) Published lineage plasticity of different γδ T cell subsets according to their TCRγ chain usage. (B) Separation of different γδ T cell subsets according to their TCRγ chain usage by flow cytometric analysis. Expression of CD45RB, CD44 and CD27 by each γδ T cell subset was analysed as in Fig. 1 and Suppl. Fig. 1. (C) Proportion of each γδ T cell subset in total γδ T cells from pLN of young and old mice. Results shown are from 23 young and 22 old mice (n=11 experiments). (D) γδ1 and γδ17 lineage commitment of each γδ T cell subset in LNs of young and old mice. Results shown are from 10 pairs of young and old mice (n=6 experiments). Statistical significances for changes in cell proportions were assessed by two-way ANOVA (C and D). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001

**Figure 3. Transcriptomic analysis identifies minimal differences between γδ T cell subsets isolated from young and old mice.** (A) Differentially expressed genes in γδ1 and γδ17 lineages were identified by RNA-Seq analysis using edgeR. Heat map of relevant genes for γδ1 and γδ17 lineage differentiation and function as well as newly identified genes. Genes shown are grouped by their functions and ranked (top to bottom) by log fold change between γδ1 and γδ17 lineages. Expression of genes marked in red were validated at the protein level by flow cytometry (IFN-γ and IL-17A was examined with or without PMA/Ionomycin stimulation in vitro) (Fig. 1 and Suppl. Fig. 1). (B) Segregation of γδ1 and γδ17 T cell subsets by principle component analysis (PCA). Each dot represents one RNA-Seq library. Each library is coloured based on the cell subset. (C) Differential expression (DE) analysis using edgeR identifies genes up-regulated in old (red) and young animals (blue) for each subset. logFC: log fold change in expression.

**Figure 4. γδ TCR repertoire analysis and Vδ segment usage of γδ T cell subsets in pLN of young and old mice.** (A) The diversity of TCRγ (top) and TCRδ (bottom) repertoires was evaluated in sorted Vγ1+ γδ1, Vγ4+ γδ1 and Vγ4+ γδ17 subsets of young and old mice and is represented as inverse Simpsons index. As a control the analysis of TCRγ and TCRδ diversity...
in the Vγ6+ γδ17 subset yielded a Simpsons index around 1 indicative of the invariant nature of this subset (data not shown). (B) Vδ chain usage in Vγ1+ γδ1, Vγ4+ γδ1 and Vγ4+ γδ17, and Vγ6+ γδ17 T cell subsets from young and old mice (Vδd3 is a pseudogene). The fraction of in-frame rearrangements of Vδ gene segments within the sorted populations is shown. (C) Emerging and declining clones defined by CDR3 nucleotide sequence in old mice. Heat map and indicated frequency show the abundance of specific clones in each young and old individual. (Inset right) Percentage of the canonical CDR3 amino-acid sequence ASGYIGGIRATDKLV in sorted Vγ4+ γδ17 T cells from the pLNs of young and old mice. Data shown are from 4-6 mice/condition (3 independent experiments). **p<0.01; ***p<0.001; ****p<0.0001

Figure 5. IL-7 is highly expressed in pLNs of old mice and creates a niche for Vγ6+ γδ17 T cell expansion. (A) Expression of IL-1b, IL-23, IL-2, IL-15 and IL-7 mRNA in the pLNs of young (n=4) and old (n=4) mice was analysed by qRT-PCR and normalised to Tbp as a housekeeping gene. Similar results were obtained when using Hprt and B2m as housekeeping genes (data not shown). Results are representative of two independent experiments each with 4 young and 4 old mice. (B) Protein expression of IL-7Rα by γδ1 and γδ17 T cells from young and old mice. Results shown are from 7 young and 7 old mice (n=3 experiments). (C) Serial sections of inguinal LNs from young (top) and old (bottom) mice were stained with H&E or with specific probes targeting the constant region of TCRδ, Vγ6 TCR or IL-7 mRNA as indicated. Representative images shown are from 3 pairs of young and old mice. (D) Density of total γδ T cells and Vγ6+ T cells. (E) Proportion of Vγ6+ T cells of total γδ T cells. (F) Expression of IL-7 mRNA in the T cell zone and the follicle of the pLN. (G) Localisation of γδ T cells within the follicles. (H) Average distance between Vγ6+ γδ T cells and the nearest IL-7 producing cell. Statistical significances for changes in expression levels were assessed by Mann-Whitney test (A) or two-way ANOVA (B). *p<0.05; ***p<0.001

Figure 6. Infiltration of selectively activated Vγ6+ γδ17 T cells from the draining LN into the tumour is associated with faster tumour progression in old mice. (A) Infiltration of γδ T cells from draining LN into tumour was blocked by the treatment with FTY720. Mice were injected every other day with FTY720 or vehicle control from day -5 to day 13 and 3x10^6 3LL-A9 Lewis lung carcinoma cells on day 0. (B) 3x10^6 3LL-A9 cells were injected subcutaneously into young and old C57BL/6 mice and tumours were analysed 14 days post injection. (C) Tumour weights shown are from 19 young (n=5 experiments) and 5 old mice (n=2 experiments). (D) γδ1 and γδ17 lineage-commitment of total γδ T cells within the tumour of young and old mice. (E) γδ T cell subsets recovered from the tumour. (F) Linear regression fit between the weight of tumours and the proportion of Vγ6+ T cells in total tumour-infiltrating γδ T cells. (G) Activation and exhaustion of total tumour-infiltrating γδ T cells in young and old mice. FACS files acquired for each individual mouse were concatenated for analysis and the results are shown as representative dot plots. (H) Activation and exhaustion of different γδ T cell subsets in tumours from young and old mice. Results shown (D-H) are from 8 young and 5 old mice (n=2 experiments). Cell populations with the total cell number less than 10 were excluded from analysis. Statistical significances for differences were assessed by Mann-Whitney test (A and C) or two-way ANOVA (D and E). *p<0.05; ****p<0.0001
ACKNOWLEDGEMENTS

Special thanks and gratitude go to Julia Jones and Cara Brodie from the Histopathology/ISH Core at the CRUK Cambridge Institute for their support in conducting RNAscope® experiments and analysis; Angela Mowbray and Matthew Clayton from BRU for expert animal care; and the Flow cytometry core for cell sorting. We thank Prof. Adrian Hayday and Dr. Anett Jandke at King’s College London for supplying 17D1 hybridoma supernatant and a FACS staining protocol. We also thank Dr. Matthias Eberl at Cardiff University for constructive comments on the manuscript.

This work has been funded by the Cancer Research UK (H-CC, LMOB, DTO, JCM, and MdlR), Wellcome Trust Sir Henry Dale Fellowship (WT107609, MdlR), Janet Thornton Fellowship (WT098051, CPJM), European Research Council (DTO), EMBO Young Investigators Programme (DTO), EMBL (NE and JCM), Wellcome Trust Sanger Institute (CPMJ, JCM, and DTO) and NIHR pump priming grant (H-CC).

AUTHOR CONTRIBUTION

HCC and MdlR designed the experiments; HCC, CPMJ and LMOB performed experiments and experimental analyses; NE performed computational analyses; LMOB provided technical assistance and support; DTO provided the ageing colony. HCC and MdlR wrote the manuscript. All authors commented on and approved the manuscript.

DATA AVAILABILITY

The RNA-Seq data will be made available on ArrayExpress under the accession number E-MTAB-7178. Analysis scripts for the RNA-Seq and TCR analysis can be found at: https://github.com/MarioniLab/GammaDeltaTcells2018

REFERENCES


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Figure 6. Infiltration of selectively activated Vγ6+γδ17 T cells from the draining LN into the tumour is associated with faster tumour progression in old mice. (A) Infiltration of γδ T cells from draining LN into tumour was blocked by the treatment with FTY720. Mice were injected every other day with FTY720 or vehicle control from day -5 to day 13 and 3x10^6 3LL-A9 Lewis lung carcinoma cells on day 0. (B) 3x10^6 3LL-A9 Lewis lung carcinoma cells were injected subcutaneously into young and old C57BL/6 mice and tumours were analysed 14 days post injection. (C) Tumour weights shown are from 19 young (n=5 experiments) and 5 old mice (n=2 experiments). (D) γδ1 and γδ17 lineage-commitment of total γδ T cells within the tumour of young and old mice. (E) γδ T cell subsets recovered from the tumour. (F) Linear regression fit between the weight of tumours and the proportion of Vγ6+ T cells in total tumour-infiltrating γδ T cells. (G) Activation and exhaustion of total tumour-infiltrating γδ T cells in young and old mice. FACS files acquired for each individual mouse were concatenated for analysis and the results are shown as representative dot plots. (H) Activation and exhaustion of different γδ T cell subsets in tumours from young and old mice. Results shown (D-H) are from 8 young and 5 old mice (n=2 experiments). Cell populations with the total cell number less than 10 were excluded from analysis. Statistical significances for differences were assessed by Mann-Whitney test (A and C) or two-way ANOVA (D and E). *p<0.05; ****p<0.0001
Supplementary Table and Figures

Altered composition of the γδ T cell pool in lymph nodes during ageing enhances tumour progression

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*These authors contributed equally to this study.

Supplementary Table

Suppl. Table 1. Antibodies used in this study

Supplementary Figures

Suppl. Fig. 1. CD44 and CD45RB expression by γδ T cells in peripheral lymph nodes (pLNs) identifies IL-17-producing (γδ17) and IFN-γ-producing (γδ1) lineages.

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Suppl. Fig. 5. Differentially expressed genes between γδ1 and γδ17 T cell subsets isolated from young and old mice.

Suppl. Fig. 6. Quality controls of repertoire analysis.

Suppl. Fig. 7. FTY720 treatment blocks egress of αβ T cells from the pLNs to the tumour.

Suppl. Fig. 8. Activation of different γδ T cell subsets in the tumour of young and old mice.

Suppl. Fig. 9. Vγ6+ γδ17 T cells are the only γδ T cell subset that becomes activated in the draining LN of tumour-bearing young and old mice.

Suppl. Fig. 10. Activation and tumour infiltration of Vγ6+ γδ17 T cells in the LL2 tumour model in young and old mice.
### Table 1. Antibodies used in this study

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*hybridoma supernatant
Suppl. Fig. 1. CD44 and CD45RB expression by γδ T cells in peripheral lymph nodes (pLNs) identifies IL-17-producing (γδ17) and IFN-γ-producing (γδ1) lineages. (A) Maturation status of γδ T cells in pLNs of young and old mice according to expression level of CD24. Results shown are from 8 independent experiments with 17 young and 16 old mice. (B) γδ T cells were harvested from pLNs of young and old mice, and stimulated with 50 ng/ml PMA and 1 μg/ml ionomycin in the presence of GolgiSTOP. After 4 hours, cells were stained with antibodies against cell surface markers and intracellular IL-17 and IFN-γ. FACS plots are representative of 6 independent experiments with 16 young mice. γδ T cells isolated from old mice showed similar results (not shown). (C) Expression of characteristic γδ1 and γδ17 lineage makers by populations separated via CD44 and CD45RB expression. FACS plots shown are representative for the number of mice indicated.
Suppl. Fig. 2. Lineage polarisation, phenotype and function of γδ T cells in the pLNs of young and old mice. (A) CD45RB and CD44 expression segregates mature (CD24−) γδ T cells in LNs of young (3 months, n=17), mid-age (12 months, n=4) and old (> 21 months, n=16) mice into γδ17-committed (CD45RB− CD44+), γδ1-committed (CD45RB+ CD44+), γδ1-intermediate (CD45RB+ CD44−) and progenitor (CD45RB− CD44−) γδ T cell subsets. (B) Percentage of γδ17 T cells, characterized by lack of CD27 expression, from total γδ T cells in old and young pLNs. Results shown are from 3 independent experiments with 7 young and 7 old mice. (C) CD44 and CD62L expression profile of γδ17-committed (CD45RB− CD44+), γδ1-committed (CD45RB+ CD44+), γδ1-intermediate (CD45RB+ CD44−) and progenitor (CD45RB− CD44−) γδ T cells from pLNs of young mice. (D) Representative FACS plots and analysis of the memory/activation status of γδ T cells in the pLNs of young and old mice. Results shown are collected from 3 independent experiments using 7 young and 7 old mice. (E) IL-17 and IFN-γ production by γδ T cells from old and young mice. γδ T cells were harvested from pLNs and stimulated with PMA and ionomycin for 4 hours in the presence of GolgiSTOP. Results shown are from 6 independent experiments with 16 young and 15 old mice. Statistical significance for changes were assessed using Mann-Whitney test (A and E) or two-way ANOVA (D). ***p<0.001; ****p<0.0001
Suppl. Fig. 3. Characterisation of the splenic γδ T cell pool in young and old mice. (A) Absolute numbers of γδ T cells in the spleen of young and old mice. (B) Proportion of γδT cells in total CD3+ T lymphocytes in the spleen of young and old mice. (C) Maturation status, as determined by CD24 expression, of γδ T cells in the spleen of young and old mice. (D) γδ1 and γδ17 lineage commitment and (E) proportion of γδ T cell subset of mature γδ T cells in the spleen of young and old mice. Results shown are collected from 7 independent experiments with 17 young and 17 old mice. Statistical significance for difference was assessed by Mann-Whitney test (A-C) or two-way ANOVA (D and E). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001
Suppl. Fig. 4. Isolation of γδ1 and γδ17 T cells from different γδ T cell subsets from pLNs of young and old mice. (A) Four populations of γδ T cells were isolated from the pLNs of young and old mice for RNA-Seq. (B) FACS gating strategy: Lymphocytes were gated by forward scatter (FSC-A) and side scatter (SSC-A). Cell doublets were excluded according to area and width of the forward scatter (FSC-A/FSC-W). Dead cells were removed using viability dye. From mature T lymphocytes (CD3+ CD24-ve), γδ T cells were determined by TCRδ expression. γδ T cells were then further segregated into 4 cell subsets according to their expression of different TCRγ chains. Vγ1+, Vγ4+ and Vγ6+ T cells were separated by the staining profile of cells with antibodies against Vγ1+, Vγ4+ and Vγ5+ TCR and 17D1 hybridoma supernatant against Vγ5/Vγ6 TCR. γδ1 (CD44+ CD45RB+), γδ17 (CD44hi CD45RB-) T cells were characterised within Vγ1+, Vγ4+ and Vγ6+ T cells. γδ1 T cells were isolated from Vγ1+ (red) and Vγ4+ (blue) T cell subsets and γδ17 T cells were isolated from Vγ4+ (green) and Vγ6+ (orange) T cell subsets.
Suppl. Fig. 5. Differentially expressed genes between γδ1 and γδ17 T cell subsets isolated from young and old mice. Genes expressed at different levels in γδ1 and γδ17 T cells were identified by RNA-Seq analysis. Heatmap shows the Z-score scaled, normalised expression of selected marker genes.

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Heatmap shows the Z-score scaled, normalised expression of selected marker genes.
Suppl. Fig. 6. Quality control of repertoire analysis. TCRγ and TCRδ chains were assembled from bulk RNA-Seq data of highly pure, FACS-sorted (A) Vγ1+ and Vγ4+ γδ1 cells and (B) Vγ6+ and Vγ4+ γδ17 cells. MiXCR was used in RNA-Seq mode to reconstruct TCRγ and TCRδ chains. As a quality control, the fraction of assembled TCRγ chains was plotted for each sample. Only T cell subset-specific sequences were used for further analysis. The detected non-specific sequences are likely due to alignment errors resulting from high level of homology between Vγ1 and Vγ3 (A).
Suppl. Fig. 7. FTY720 treatment blocks egress of αβ T cells from the pLNs to the tumour. Young mice were injected every other day by i.p with FTY720 (1 mg/kg) or with vehicle control from day -5 to day 13. 3x10^6 3LL-A9 cells were injected s.c on day 0 and tumours were harvested on day 14 or day 15 for flow cytometry analysis. Number of CD4 (A) and CD8 (B) T cells in the tumour of control and FTY720-treated mice. Results shown are from 2 independent experiments with 11 control and 10 FTY720-treated mice. Statistical significances for difference in cell densities and cell proportions were assessed by Mann-Whitney test (A and B) or two-way ANOVA (C and D). ****p<0.0001
Suppl. Fig. 8. Activation of different γδ T cell subsets in the tumour of young and old mice. (A) Percentage of γδ T cells in total tumour-infiltrating CD3+ T lymphocytes from young and old mice. (B) Density of γδ T cells in tumours from young and old mice. (C) γδ1 and γδ17 lineage commitment of tumour-infiltrating Vγ1+, Vγ2/3/7, Vγ4+ and Vγ6+ T cells in young and old mice. (D) Activation of γδ1 and γδ17 T cell subsets in the tumour is determined by PD-1 and Tim-3 expression. Representative FACS plots (left) show the analysis with concatenated FACS data acquired for each individual young mouse. Results shown (right) are obtained from 2 independent experiments with 8 young and 5 old mice. Cell populations with a total cell number less than 10 were excluded from analysis. Statistical significances for differences were assessed by Mann-Whitney test (A and B) or two-way ANOVA (C and D).
Suppl. Fig. 9. Vγ6+γδ17 T cells are the only γδ T cell subset that becomes activated in the draining LN of tumour-bearing young and old mice. (A) Proportion of γδ T cell subsets in the tumour-draining LN of young and old mice. (B) γδ1 and γδ17 lineage commitment of γδ T cells in the tumour-draining LN of young and old mice. (C) Activation status of each γδ T cell subsets in the tumour-draining LN were characterised by PD-1 and Tim-3 expression. (D) γδ1 and γδ17 lineage commitment of each γδ T cell subsets in the tumour-draining LN were characterised by PD-1 and Tim-3 expression. FACS files acquired for each individual mouse were concatenated for the analysis and the results are shown as representative dot plots. Results are from 2 independent experiments with 8 young and 5 old mice. Cell populations with the total cell number less than 10 were excluded from analyses. Statistical significances for differences were assessed by two-way ANOVA (A and B). **p<0.01; ***p<0.001; ****p<0.0001
Suppl. Fig. 10. Activation and tumour infiltration of $V_{\gamma}\delta$ T cells in the LL2 tumour model in young and old mice. (A-E) Young and old mice were injected subcutaneously with 3x10^6 LL2 Lewis lung carcinoma cells. Tumour and tumour-draining LN were harvested at indicated times for flow cytometry analysis. (A) $\gamma\delta$ lineage commitment of $\gamma\delta$ T cells in the tumour of young and old mice at day 14. (B) Proportion of different $\gamma\delta$ T cell subsets in the tumour of young and old mice at day 14. (C) Activation status of each $\gamma\delta$ T cell subsets in the tumour of young mice were characterised by PD-1 and Tim-3 expression at day 11. Activation status of $V_{\gamma}\delta^+$ T cells in the tumour (D) and tumour-draining LN (E) of young and old mice at day 14. FACS files acquired for each individual mouse were concatenated for the analysis and the results were shown as representative dot plots. Results shown in (A, B, D and E) are obtained from an experiment with 4 young and 3 old mice. Results shown in (C) are obtained from experiments with 5 young mice. Statistical significance for differences was assessed by two-way ANOVA (A and B). *p<0.05.