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Better safe than sorry: Naive T-cell dynamics in healthy ageing

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ABSTRACT

It is well-known that the functioning of the immune system gradually deteriorates with age, and we are increasingly confronted with its consequences as the life expectancy of the human population increases. Changes in the T-cell pool are among the most prominent features of the changing immune system during healthy ageing, and changes in the naive T-cell pool in particular are generally held responsible for its gradual deterioration. These changes in the naive T-cell pool are thought to be due to involution of the thymus. It is commonly believed that the gradual loss of thymic output induces compensatory mechanisms to maintain the number of naive T cells at a relatively constant level, and induces a loss of diversity in the T-cell repertoire. Here we review the studies that support or challenge this widely-held view of immune ageing and discuss the implications for vaccination strategies.

1. Introduction

Wisdom comes with age. This commonly used phrase also nicely reflects the essence of our adaptive immune system: it learns with every antigenic encounter. At the same time, the ability to learn new things declines with age, whether it is our ability to learn a new language or the ability of our immune system to respond to new antigens. There is indeed ample evidence that our immune system becomes less functional with age. The process of immune dysfunction at older age is multifaceted, involving both the innate and the adaptive immune system, and is often referred to as immune-senescence or waning immunity. With the increasing life expectancy of the human population, we are increasingly confronted with the effects of this gradual deterioration of the immune system. It leads to increased susceptibility to severe infection and has clear clinical consequences. For example, the chance to become hospitalized or to die after infection with influenza or SARS-CoV-2 [1,2] is much higher for older individuals compared to young adults or children.

The general strategy in Western countries is to protect the elderly against such infections through vaccination programmes specifically targeting older individuals. For example, in the Netherlands, everybody over 60 years of age is called for a yearly vaccination against seasonal influenza. Unfortunately, the effectiveness of such vaccinations also decreases with age [3]. Clinical effectiveness of the influenza vaccine, for example, decreases from 70–90% in younger adults to a mere 30–40% in individuals over the age of 65 [4]. Similarly, vaccine responses to hepatitis B and pneumococcal polysaccharides are severely impaired at older age [5]. The people who need these vaccinations most, unfortunately thus respond the least. It is therefore important to gain a better understanding of the mechanisms underlying the gradual deterioration of the immune system. Depending on these mechanisms, we may have to vaccinate differently, e.g. using different adjuvants, or at an earlier age, when the immune system is still more functional.

Among the clearest effects of ageing on the immune system are the changes observed in the T-cell pool [6]. With age, there is a gradual shift from the naive towards the memory T-cell pool, at least percentagewise [7,8]. Such correlations observed in cross-sectional studies need to be interpreted with care, because they can be confounded by chronic latent infections with herpes viruses, such as cytomegalovirus (CMV). Since CMV infection leads to massive expansion of CMV-specific memory T-cell clones, and the chance to be CMV-seropositive increases with age, part of the correlation between memory T-cell numbers and age may in fact be due to CMV infection. When Wertheimer et al. [9] disentangled the effects of age and CMV infection, an age-related decline in the number of naive $CD4^+$ T cells was found only in people infected with CMV, while a significant decline in naive $CD8^+$ T-cell numbers was observed independent of CMV infection. In line with this, Nicoli et al. [10] more recently reported a much larger decline in naive $CD8^+$ compared to naive $CD4^+$ T-cell numbers during ageing, and found that

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chronic infections with herpes viruses, and in particular with CMV, had a strong impact on the naive $CD4^+$ but not on the naive $CD8^+$ T-cell compartment. Ageing thus seems to go hand in hand with a loss of naive T cells, sometimes truly in absolute numbers, sometimes only in relative terms, and the loss rate may depend on the presence of chronic infections. This is perhaps not surprising, because i) the source of new naive T cells dries up with age due to thymic involution, and ii) at older age more naive T cells will have been recruited into the memory pool. The resulting loss of naive T cells is generally thought to play a key role in the impaired immune response to new antigens at older age. In fact, it remains unclear, whether even a several-fold reduction in naive T-cell numbers, and/or diversity, would have a major impact on the capacity to mount novel immune responses.

The consensus view on immune ageing is built on a collection of observations and assumptions related to the maintenance of naive T cells. Firstly, based on a large body of data, the thymus is generally held at least partially responsible for the maintenance of the naive T-cell pool. There seems to be no clear consensus, however, on the age at which the thymus becomes obsolete. Secondly, the loss of thymic output in older individuals is thought to lead to increased rates of naive T-cell division or increased naive T-cell lifespans, to compensate for the loss of naive T-cell production by the thymus. Thirdly, the idea that the generation of naive T cells gradually shifts from the thymus to peripheral T-cell division, is thought to cause a loss of diversity in the T-cell receptor (TCR) repertoire. The "holes" in the repertoire that are hence thought to be created, are generally held responsible for the failure to respond to new antigens at older age [11–13].

Here, we review the studies that have laid the basis for this consensus, and those that challenge it. We conclude by discussing the impact of these insights for our understanding of the aged immune system and specifically, its implications for vaccination strategies.

2. Is the thymus essential for maintenance of the naive T-cell pool?

Immune responses to new antigens are dependent on the presence of naive T cells with cognate T-cell receptors. In order to gain a better understanding of immune ageing, we therefore need to understand how the body maintains a diverse repertoire of naive T cells. It is commonly accepted that thymic output declines significantly with age, both in mice [14] and in humans [15,16]. But what is the effect of the decline in thymic output during ageing on the naive T-cell pool? To what extent are the naive T-cell pools of mice and humans dependent on thymic output? And what happens to the naive T-cell pool when the thymus is removed?

In adult mice, both thymic output and the number of naive T cells decline significantly with age. In terms of cell numbers, the decline in naive T cells is less drastic than the decline in thymocytes [17,18]. By counting naive $CD4^+$ and $CD8^+$ T-cell numbers in the spleens and lymph nodes of euthymic mice and of mice that had been thymectomized at 7 weeks of age, we quantified the impact of the thymus on the naive T-cell pool. Our data were perfectly described by a model in which naive T cells do not divide and live longer when T-cell numbers decline [17]. The total daily production of naive T cells – as measured by in vivo deuterium labelling analysis – turned out to be as large as the estimated daily output of new naive T cells from the thymus. This suggests that the production of naive T cells in mice is almost entirely dependent on thymic output. In support of this, we found no evidence for dilution of T-cell receptor excision circles (TRECs) in mouse naive T cells with age. Throughout the life of the mice, the average TREC contents of naive $CD4^+$ and $CD8^+$ T cells remained at similar levels as the average TREC contents of their $CD4^+$ and $CD8^+$ single-positive (SP) thymocytes. Two independent studies, one based on Ki67 expression data [19], and one based on a fluorescent division reporter system [20], also found that peripheral T-cell division hardly contributes to the maintenance of naive T cells in mice, because the expected lifespan of naive T cells is much shorter than their interdivision time.

Given the important role of the thymus in mice, one would expect thymectomy to have a large impact on the naive T-cell pools of mice. Indeed, very early papers reported that thymectomy in young adult mice leads to severely reduced numbers of lymphocytes in blood, lymph nodes and spleen [21,22]. In neonatal mice, the effect of thymectomy was even more dramatic, and led to severe immunological defects [23]. Even in adult mice, thymectomy had long-term immunological effects in terms of e.g. antibody production and skin rejection, although these effects were smaller than in mice that had undergone neonatal thymectomy [24]. We also observed a drastic and rather rapid decline in naive T-cell numbers in mice upon thymectomy at the age of 7 weeks [17]. Within fifteen weeks following complete thymectomy, the majority of naive T cells had been lost [25]. This is a direct consequence of the important contribution of the thymus to naive T-cell maintenance and of the relatively short expected lifespan of naive T cells in mice, i.e. about 7 weeks for $CD4^+$ and 11 weeks for $CD8^+$ naive T cells [17]. In mice, the naive T-cell pool is thus maintained rather dynamically, and the replacement of lost cells is largely dependent on thymic output. Interestingly, even in mice thymectomized at 18–28 months of age, thymectomy had a significant (albeit small) impact on naive $CD4^+$ T-cell numbers [26], illustrating the continuous role of the thymus in naive T-cell maintenance throughout the life of a mouse.

The above findings have fuelled the idea that the thymus is essential for the maintenance of the naive T-cell pool, not only in mice, but also in humans. In humans, like in mice, thymic output declines significantly with age [27] and naive T-cell numbers decline less dramatically than thymocyte numbers [8]. Nevertheless, the situation in humans turns out to be completely different. First of all, in humans, naive T cells are maintained in a less dynamic way than they are in mice. In humans, naive T cells are extremely long-lived throughout life, with an expected lifespan of ~6 years for $CD4^+$ and ~9 years for $CD8^+$ naive T cells [25,28], i.e. about 40-fold longer than in mice. Thus, a much smaller fraction of naive T cells need to be replaced on a daily basis. To investigate to what extent lost naive T cells are replaced by thymic output and by naive T-cell division in the periphery, we measured naive T-cell TREC contents. Several studies have shown that the average TREC content of naive T cells in humans declines as much as 10–100 fold during healthy ageing [17,29–32]. Although TREC contents have to be interpreted with care, because they are affected not only by thymic output but also by T-cell division and loss [33], they can be used to quantify the *relative* contribution of the thymus to naive T-cell production [17]. Based on naive T-cell TREC contents, we estimated that in human adults, up to ~90% of daily naive T-cell production comes from T-cell division in the periphery, while only ~10% comes from thymic output [17]. Although the contribution of the thymus is larger in children than in adults, even in childhood the numerical contribution of naive T-cell division was estimated to outnumber that of thymic output [34].

Given the more dominant role of naive T-cell division in humans, one would expect thymectomy to have a smaller impact on the naive T-cell pool in humans than in mice, at least in adulthood. Studying the impact of thymectomy in humans is, however, limited to situations where the thymus is removed as treatment for e.g. myasthenia gravis, in which case the naive T-cell pool may be affected by the disease, or during complicated cardiac surgery, which tends to be performed very early in life. We studied the naive T-cell pools of children who were thymectomized shortly after birth because of cardiac surgery, and observed a significant impact of thymectomy during the first 5 years of life. Both naive $CD4^+$ and $CD8^+$ T-cell numbers, as well as the average TREC content of $CD4^+$ T cells were significantly lower in children who had been thymectomized than in healthy age-matched controls [35]. These results were not unexpected, because the role of the thymus in children is thought to be significant. Remarkably, in older children (i.e. above the age of 5), naive $CD4^+$ and $CD8^+$ T-cell counts and $CD4^+$ T-cell TREC contents were within the normal range [35]. Even thymectomy within the first year of human life thus seems to have a less dramatic long-term

impact on the naive T-cell pool than thymectomy in mice. One possibility is that the naive T cells that are present in children shortly after birth are capable of keeping up a stable naive T-cell pool in the long run thanks to naive T-cell division, without the need for further thymic output. We cannot exclude the possibility, however, that regrowth of thymus tissue may have occurred in these children. Despite the fact that the thymus had been completely removed shortly after birth, in most individuals of 5 years and over, magnetic resonance imaging (MRI) scans indeed suggested the presence of thymic tissue. Although this does not imply that that tissue was also capable of thymopoiesis, in combination with the long-term normalization of TREC contents and T-cell numbers, the most parsimonious explanation for these findings is that renewed thymopoiesis was responsible for the long-term recovery of the T-cell compartment after thymectomy during infancy [35]. Our findings contrasted with two other studies, which found that thymectomy in childhood resulted in reduced naive T-cell counts and TREC contents in adulthood [36,37]. Interestingly, the children in the latter studies were thymectomized at an older age than the children in our study. It would be interesting to investigate whether the thymus in humans has the largest regenerative capacity very early in life. As a consequence, thymectomy in humans at very early age may – rather counterintuitively – have a smaller impact on the naive T-cell pool than thymectomy during later childhood. Whether due to the regenerative capacity of the thymus or due to a larger role of naive T-cell division, thymectomy at very early age in humans seems to have a less dramatic impact on the naive T-cell pool than thymectomy in neonatal mice. It is important to realize that these quantitative differences between mice and men will also have qualitative effects on the naive T-cell pool, because the thymus is capable of producing new T-cell specificities, whereas peripheral T-cell proliferation can only lead to the expansion of existing T-cell clones.

3. Changes in thymic output during healthy ageing

Despite the fact that thymic output undisputably declines with age in both mice and humans, it is still debated whether the thymus remains functional throughout life, or whether instead, above a certain age, the thymus becomes obsolete. It is important to elucidate this, because in the former case, changes in the T-cell pool in disease or during treatment may be due to changes in thymic output, while in the latter case, they cannot. What are the current best estimates of daily thymic output in mice and humans? How quickly does thymic output decline with age? And at what age, if any, does the thymus no longer contribute to maintenance of the naive T-cell pool?

One way to estimate thymic output is to measure the size of the thymic T-cell compartment and assume that thymic output is proportional to the total number of thymocytes. Scollay et al. [38] observed that in mice, the thymus exports ~1% of thymocytes per day, translating to 2×10^6 T cells or 2.5% of the total body naive T-cell count in a 2-months old mouse [39]. A similar export rate of total thymocytes was reported by Berzins et al. [40], who investigated whether thymic export is autonomously regulated. By creating mice with an oversupply (by grafting extra thymic lobes) or undersupply (due to neonatal thymectomy) of thymic output, the effect of thymic output on the peripheral T-cell pool was studied. In all cases, thymic export was estimated to be ~0.7% of the pooled total number of thymocytes in the naturally occurring thymus and the grafted thymic lobes, suggesting that thymic export is indeed proportional to the number of thymocytes and thus strongly decreases with age. In line with this, we used the number of SP thymocytes and naive T-cell numbers in the spleen and lymph nodes of euthymic and thymectomized mice of different ages to determine the fraction of SP thymocytes that are exported to the spleen per day. Using mathematical modelling and a modification of the thymic involution model of Steinmann et al. [16], we estimated that about 3.5% of SP thymocytes were exported to the spleen per day [17]. Assuming that the spleen contains 30–40% of all naive T cells [41] and that SP thymocytes constitute a 0.08–0.12 fraction of all thymocytes, total daily thymic export was estimated to be 0.6–1.4% of all thymocytes, which is nicely in line with the previously estimated export rates of Scollay et al. and Berzins et al. [38,40].

Based on short-term RAG-induced GFP expression in mice, Hale et al. [42] showed that the number of double-positive (DP) thymocytes better reflects thymic output than the number of SP thymocytes, because the SP population may in part consist of mature T cells that have remigrated to the thymus. Over age, the fraction of GFP^+ T cells in the SP population was shown to decrease, while DP thymocytes stayed GFP^+. Since the ratio of GFP^+ DP cells in the thymus over GFP^+ T cells in the spleen remained relatively stable with age, the number of DP cells in the thymus was proposed as a better measure of thymic output. Dulude et al. [43] subsequently showed that intrathymic T-cell precursor proliferation can differ between mouse strains, suggesting that the exact relationship between the number of DP cells and daily thymic output may also differ between strains.

Daily thymic output in humans has also been estimated using different strategies. Assuming an export rate of 1% of thymocytes per day [38], a thymus weight of about 23 gr [16] with $\sim 10^7$ thymocytes per gram of thymic tissue [44] yields and estimated thymic export of 2.3×10^6 cells per day in a human adult of 20 years. Alternatively, several studies have measured the number of DP thymocytes in people of different ages. A recent study by Thome et al. [45] reported that DP thymocytes were virtually absent in the thymus of brain-dead organ donors over 40 years of age, suggesting that the thymus stopped contributing to the maintenance of the naive T-cell pool in these people. Marusic et al. [46], however, found that the frequency of DP cells in thymic tissue removed during open heart surgery varied significantly between individuals. The frequency of DP cells tended to decrease with age, but DP cells were still detectable even in older individuals. The percentage of DP thymocytes in thymus material from the oldest individual in the study (aged 73 years) was even as high as 75%, which was higher than the percentages observed in individuals of 30 or 40 years of age. Although it remains unclear what these differences between studies were due to, it is possible that the different status of the donors (i.e. elective surgery or organ donors) played a role. The observation that even the thymus of people over 70 years of age had normally distributed thymocyte populations [46] suggests, however, that thymic output may persist even in older individuals. Unfortunately, the total amount of thymic tissue was not measured in these studies, which precludes estimating daily thymic output from these data.

More feasible and routinely-used approaches to measure thymic output involve analysis of specific features of peripheral naive T cells sampled from the blood. These approaches utilize the expression of proteins such as Pecam-1 (CD31), PTK-7, or CD103 to identify $CD4^+$ and $CD8^+$ T cells that "recently" emigrated from the thymus [47–49]. These markers are typically chosen because their expression is lost during the post-peripheral maturation of naive T cells, and their presence thus correlates with recent emigration from the thymus. For CD31 it was later shown that its expression is not necessarily lost when naive T cells divide in the periphery [17,50]. Although CD31 expression can still be used to identify T cells that are most proximal to the thymus, it therefore tends to overestimate daily thymic output [17].

Thymic involution happens in at least two phases [16]. Early in life, in childhood until the age of 35 years, the involution rate has been estimated to be approximately 3% per year on average. Thereafter, the rate declines to ~1% per year [16]. Using Ki67 expression levels and TREC contents of naive $CD4^+$ T cells, Bains et al. [51] estimated the decline in daily thymic output during childhood. They found evidence for a biphasic decline, from 12% per year in the first 8 years of life, to 4% per year between 8 and 20 years of age. Although they originally assumed that Ki67 is expressed for 12 h post-cell division, yielding an estimated thymic output of 3.5×10^8 $CD4^+$ T cells per day at the age of 20 years [51], they later showed that Ki67 is in fact expressed for as long as 3–4 days after mitosis [19]. The estimated daily thymic output would hence be 6–8 times lower, i.e. around 5×10^7 $CD4^+$ T cells per day at the

age of 20 years. The latter estimate is quite well in line with our own estimate in human adults based on a combination of TREC contents, total body naive T-cell numbers and naive T-cell turnover rates [17,52]. We estimated that in young adults, the thymus exports 1.7×10^7 $CD4^+$ T cells per day, while in older individuals between 66 and 72 years of age, the thymus exports ~1 million $CD4^+$ T cells per day [17,52].

Finally, increases in the number of recent thymic emigrants (RTEs) or TRECs in patients during immune reconstitution provide information about thymic output. Naive T-cell TREC contents tend to increase after hematopoietic stem-cell transplantation (HSCT), even in individuals of ~70 years of age [53]. Although this was originally interpreted as a sign of thymic rebound [53], it is most likely a natural consequence of the relatively empty T-cell pool of patients undergoing HSCT [54]. The increasing TREC contents do provide evidence, however, for functional thymic output at older age, since TREC contents can only increase in the presence of functional thymic tissue. In order to provide a more direct measure of thymic output, Lorenzi et al. [55] established a protocol to determine the absolute TREC content of a fixed volume of blood and, as expected, found this to decrease with age. Based on this approach, the increase in the absolute number of TRECs per ml of blood in children after HSCT was found to be between 2500 and 10,000 TRECs in 10 months [56]. Assuming that RTE contain on average 0.25 TRECs per cell, this would amount to a thymic output of at least $2500 \times 4 = 10^4$ T cells per ml of blood in 10 months, and hence to 10^4 x 1000 (ml per liter) x 5 (liter blood) x 50 (assuming that 2% of lymphocytes reside in the blood) / 300 (days) = 8.3×10^6 T cells per day.

Summarizing, thymic output can be present even in older individuals. However, data are relatively scarce and conclusions based on rather incidental cases of not even very old individuals. The question also remains to what extent thymic output contributes to naive TCR diversity in older people, since in the best case, thymic output would add about 10^6 novel TCRs to the naive $CD4^+$ T-cell repertoire per day. Even once the repertoire would be down to ~10^9 clonotypes, this amount of thymic output would add only 0.1% of TCR diversity per day.

4. Changes in T-cell turnover during healthy ageing

The observations that during healthy ageing naive T-cell numbers decline less dramatically than thymocyte numbers [8,17,18] and that the average TREC content of naive T cells in humans declines with age [17,29,31,32,57] have been interpreted as evidence for homeostatic mechanisms kicking in to compensate for the loss of thymic output at older age [17,26,31,58,59]. A possible mechanism is that naive T cells live longer or divide more frequently when thymic output declines, because of reduced competition between cells for survival factors, such as IL7 [59], and/or proliferation factors [60]. Such responses have indeed been observed in lymphopenic mice, in which adoptively transferred naive $CD8^+$ T cells underwent antigen-independent proliferation (and obtained some, but not all, features of memory T cells) [61]. It has been suggested that the sizes of the naive and memory T-cell pools are regulated independently, to ensure that new naive T cells do not go at the expense of old memories, and likewise, that memory T-cell division does not restrict the diversity of the naive T-cell pool [62]. But is there any direct evidence that naive T cells live longer or divide more frequently at older age? What is the evidence that these rates are regulated through cell-density dependent mechanisms? And to what extent are responses to reduced thymic output comparable between mice and humans?

Tsukamoto et al. [26] studied how the expected lifespan of naive $CD4^+$ T cells in mice changes with age, using $CD4^+$ TCR-transgenic (Tg) mice to make a fair comparison between naive T cells carrying the same TCR in young and old mice. These TCR-Tg mice were thymectomized at young (2 months) or old (>18 months) age to follow the decay of Tg cells in the absence of thymic output. The half-life of $CD4^+$ Tg T cells in old mice was about 3-fold longer than that in young mice. Because this difference persisted when young and old $CD4^+$ Tg (or young and old naive polyclonal T cells) were transferred into young mice, the authors concluded that the differences in life expectancies were cell intrinsic (and not restricted to TCR-Tg cells). In line with this, they observed an age-dependent decrease in expression of the pro-apoptotic molecule Bim [26]. Interestingly, donor cells harvested from thymectomized mice which were then transferred into young mice not only survived the longest, but were also the least functional. It was therefore proposed that the intrinsic increase in longevity of naive $CD4^+$ T cells in mice facilitates naive T-cell maintenance, but goes hand in hand with functional defects, which may contribute to immune dysfunction at old age [26].

Motivated by our finding that naive T-cell numbers in euthymic and thymectomized were perfectly described by a model in which naive T cells live longer when T-cell numbers decline [17], we also searched for more direct evidence that naive T cells are longer-lived at old age. To this end, we performed an in vivo deuterium labelling experiment in 85-weeks-old mice. We found that the expected life span of naive $CD8^+$ T cells was nearly 50% longer than that in young adult mice. Naive $CD4^+$ T cells in these older mice, in contrast, had similar expected life spans as naive $CD4^+$ T cells in 12-week-old mice. Importantly, even in the older mice, naive $CD4^+$ and $CD8^+$ T-cell proliferation hardly contributed to naive T-cell maintenance [17]. The fact that the average TREC contents of naive $CD4^+$ and $CD8^+$ T cells stayed constant with age provided further support that neither $CD4^+$ nor $CD8^+$ naive T cells start to divide when mice age. We found that only in thymectomized mice, the average TREC content of naive T cells was moderately lower than in young euthymic mice, suggesting that in the absence of the thymus, naive T cells in mice may ultimately start to proliferate [17].

Although the above findings are well described by a model with cell-density dependent naive T-cell loss rates, they do not prove that cellular loss rates are regulated in a cell density-dependent manner. Hogan et al. [19] studied the dynamics of T cells in mice using the transplant-conditioning drug busulfan, which ablates hematopoietic stem cells while leaving the peripheral T-cell pool intact. After transplantation of congenic donor bone-marrow cells, they followed the displacement of host T cells in the periphery by newly generated donor-derived T cells. This revealed that the $CD4^+$ and $CD8^+$ naive T-cell pools in mice are kinetically heterogeneous. While most host naive T cells were gradually displaced by new donor-derived T cells, a significant fraction of host naive T cells, referred to as incumbent cells, were resistant to displacement by donor-derived cells [19]. The authors proposed that these incumbent cells are established early in life, have a competitive advantage, and thereby form a progressively larger part of the naive T-cell pool as mice age. This would provide an alternative explanation for the increased lifespan of naive T cells in older mice [19].

Rane et al. [63] subsequently tested different mathematical models for naive T-cell homeostasis on all datasets described above: i) the data obtained from busulfan chimeras [19], ii) naive T-cell numbers in euthymic and thymectomized mice [17], and iii) the co-transfer data of naive T cells of different ages into the same host [26]. The model that described all three datasets best was the model in which the fitness of naive T cells (defined as the difference between their rate of division and loss) increased with their time spent in the periphery [63]. By including data from very young mice, Rane et al. [64]subsequently developed a unified model of naive T-cell dynamics across the lifespan of the mouse. Again using mathematical models and a diverse set of experimental data, they managed to tear apart the effects of host age, cell age and cell numbers, each of which separately may influence the dynamics of naive T cells. They found that the model in which naive T cells progressively become longer-lived provided a remarkably good description of all data sets. They also pointed out that the high level of Ki67 expression of naive T cells in neonatal mice, which has previously been interpreted as evidence for naturally occurring lymphopenia-induced T-cell proliferation [65], most likely reflects the high level of proliferation of these cells when they were still in the thymus. Taken together, these studies suggest that peripheral T-cell proliferation hardly contributes to the maintenance of the naive T-cell pool throughout the life of the mouse, and the

fact that naive T cells become longer-lived with age seems to be a direct consequence of their time spent in the periphery, and not of density-dependent homeostatic mechanisms [64].

Given that naive T-cell division plays a more significant role in humans, how do these insights from mice translate to the human situation? Several studies have reported increasing levels of naive T-cell proliferation, as measured by Ki67 expression, during healthy ageing in humans [12,66] and rhesus macaques [67]. This may reflect a compensatory response to lower thymic output at older age, but these data by themselves do not provide direct evidence for such a homeostatic response. One of these studies found that the level of Ki67 expression of naive T cells remained remarkably stable until the age of 70, after which it increased [12]. Since thymic output is thought to decline throughout adulthood, this already casts some doubts on the cause of the increased levels of Ki67 expression in older individuals. What is the evidence that the increased rates of naive T-cell proliferation in older individuals reflect a compensatory response to declining thymic output? Could they instead reflect other age-dependent effects, such as increased levels of inflammation? And is there any evidence that naive T cells become progressively longer-lived during healthy ageing in humans?

We studied the dynamics of naive $CD4^+$ and $CD8^+$ T cells in humans over 65 years of age using in vivo deuterium labelling and compared them to the dynamics in young adults under 25 years of age [68]. To rule out the possibility that T-cell dynamics were affected by inflammatory processes, which occur more frequently in older individuals, all individuals were strictly selected on having a very good health status. Although this population of older individuals may not be representative of the general older population, this allowed us to study the effect of chronological age, and the accompanying reduced level of thymic output, on the turnover of the naive T-cell population. We found that naive $CD8^+$ T cells in the older cohort had a higher turnover rate (and hence a shorter expected lifespan) than in younger individuals, but this effect could be fully ascribed to the increased size of the $CD95^+$ sub-population (which was unfortunately included in our naive $CD8^+$ T-cell gate) in the older cohort. For naive $CD4^+$ T cells, we observed no significant differences in turnover rates between young and older individuals despite a significant $>$ 10-fold decline in thymic output [68]. Our interpretation of these findings is that the contribution of thymic output in terms of cell numbers in human adults is so small that changes in thymic output require no homeostatic compensation through increased naive T-cell lifespans or proliferation rates [68]. The observation that in less strictly selected cohorts of older people, the level of Ki67 expression of naive T cells tends to be increased [12,66] may in fact be a sign of (low grade) inflammation rather than a compensatory response to decreased thymic output.

A more recent study did find evidence for progressively increasing lifespans of naive T cells in humans [69]. By quantifying nuclear bomb test-derived ^{14}C in the genomic DNA of naive T cells, the average lifespans of naive $CD4^+$ and $CD8^+$ T cells were estimated in healthy individuals between 20 and 65 years of age. The authors found evidence for turnover of the complete naive T-cell pool in humans, a significant loss in thymic output during ageing, and a positive correlation between the age of an individual and the average lifespan of naive $CD4^+$ and $CD8^+$ T cells [69], reminiscent of the correlation observed in mice [64]. The mechanism underlying this correlation remains unclear. If there is heterogeneity in the expected lifespans of naive T cells, one naturally expects that the cells with the longest lifespans are enriched at older age, without any requirement for a compensatory mechanism. It also remains unclear why the latter study found evidence for increasing lifespans of naive T cells during healthy ageing, while Westera et al. [68] did not. The fact that Mold et al. [69] included people under the age of 65 while Westera et al. [68] studied individuals over the age of 65 makes the differences between these studies even more remarkable. Taken together, there is no unequivocal evidence that lower thymic output in humans induces a homeostatic response, neither by increased peripheral T-cell proliferation, nor by an increased life span of naive T cells.

5. Quantification of naive T-cell repertoire erosion using TCR sequencing

To investigate the effect of the gradual decline in thymic output during ageing on the diversity of the naive T-cell repertoire, one can nowadays sequence the TCRs of millions of T cells using next generation sequencing (NGS). To deduce from such data whether healthy ageing goes hand in hand with erosion of the naive T-cell repertoire remains challenging, however. Several authors have reported a decline in T-cell diversity in the total $CD4^+$ and $CD8^+$ T-cell pools over age [70–72]. Given the typical changes in naive and memory T-cell frequencies during ageing, it remains inconclusive whether the diversity of TCRs within the naive T-cell pool is truly lower in older individuals [73]. It is thus important that sequencing is performed on sorted naive or memory T cells. Another complicating factor in T-cell repertoire studies is that TCR diversity can be defined in multiple ways, including (1) the total number of naive T cells with different TCRs (i.e. a measure that is dependent on the total number of naive T cells in the body), (2) the number of different TCRs per million naive T cells (i.e. a measure independent of the naive T-cell pool size), and (3) a variety of diversity indices, such as the Shannon or Simpson's diversity index, which take into account the frequencies of different T-cell clones (i.e., increased repertoire skewing will lead to a lower diversity measure of the sample).

Studying the diversity of the *naive* T-cell pool is particularly challenging, because naive T-cell repertoires are enormously diverse, and because any blood sample, no matter how large it is, will represent only a small fraction of the total body naive T-cell pool. Even sequencing the TCRs of as many as 10^6 sorted naive T cells from the blood of young and older individuals is naturally expected to result in similar diversities, because 10^6 T cells correspond to no more than a 10^{-5} fraction of the total body naive T-cell pool, which is estimated to contain $\sim 10^{11}$ cells. In such a small sample of the total naive T-cell pool, an abundant clonotype (i.e. unique αβ TCR) is only expected to be represented by multiple T cells if its abundance is larger than 10^6 cells. As a thought experiment, consider sampling 10^6 cells from a total naive T-cell pool of 10^{11} cells. If the naive T-cell pool were to be maximally diverse, i.e. consist of 10^{11} different TCRs, all T cells in the sample would have a unique TCR. If the diversity of the total naive T-cell pool were to be 1000-fold lower, i.e. 10^8 different TCRs, the chance to find only unique TCRs in the sample of 10^6 cells would still be $(1–10^{-8})\hat{}10^6 = 0.99$. Thus, single samples from very diverse repertoires provide little information on the total diversity of the repertoire, and even quite large differences in the diversity of naive T-cell repertoires of young and older individuals will be hard to detect. On top of that, enumerating repeated TCRs is prone to experimental error, as even in an experiment in which the cDNA transcribed from TCR-encoding mRNAs are extended with unique molecular identifiers (UMI), a sizeable fraction of sequences will come from cells that contributed more than one mRNA, and thus more than one UMI [74]. Finding more than one UMI per TCR sequence may thus incorrectly be interpreted as evidence for multiple cells in the sample expressing the same TCR. A similar problem holds for finding duplicate α or β chains in a single sample, as these are likely to come from cells with different αβ TCR combinations [74]. Estimating total body diversity and abundance profiles from single samples of naive T-cell repertoires is therefore notoriously unreliable.

To reliably estimate TCR diversity and abundances, it is much better to draw multiple samples, and amplify them separately. If TCRs are then observed in multiple samples, one can be sure that they came from different cells rather than from different RNA molecules of the same cell. The fraction of sequences overlapping between such biological replicates thereby provides a much more reliable measure for total body diversity, and the "incidence" of clonotypes over all replicate samples (i.e., the number of biological replicates in which the TCR was present) provides a more reliable measure for the abundance of clonotypes. Since

the repertoire of TCRα sequences is much less diverse than that of TCRβ sequences, finding the same TCRα in multiple samples still fails to provide solid evidence for an overlapping clonotype, as the overlapping TCRα chains are likely paired with different TCRβ chains [74]. Being much more diverse, overlapping TCRβ sequences in repeated samples from bulk TCR experiments provide a better proxy for the true diversity of the T-cell pool than overlapping TCRα sequences. To translate the incidence of overlapping TCRs between different samples into an estimated total naive TCR diversity, one typically uses the Chao2 estimator [75]. This measure was originally developed in ecology for estimating total species richness, and is based upon the ratio of unique over duplicate species in different samples. It is important to note that ecological samples – for which the Chao2 estimator was developed – are typically much less diverse and represent a much larger fraction of the total pool of species than TCR samples. Although it is currently the best estimator of TCR diversity, even the Chao2 index tends to be noisy because of the extremely high diversity of naive T-cell receptors and the natural limitations on sample sizes from the large number of naive T cells in the body.

A now almost 10-year old paper by Qi et al. [75] still provides the most reliable information on the erosion of naive T-cell repertoires in humans. Using plasma apheresis, Qi et al. [75] took relatively large samples of naive $CD4^+$ and $CD8^+$ T cells from four young (20 – 35 years of age) and five older (70 – 85 years of age) individuals and split each blood sample into five subsamples before mRNA extraction. We have analysed these seminal data in detail [73] and estimated that in each subsample of 10^6 $CD4^+$ naive T cells, about 2×10^5 cells contributed at least one RNA molecule, and that in young individuals almost every naive T cell expressed a unique TCRβ sequence. In the older individuals, about 90% of the $CD4^+$ naive T cells expressed a unique TCRβ sequence [73]. This illustrates that even in relatively large samples of naive TCR repertoires, the vast majority of TCRβ sequences are unique. Qi et al. [75] used information from multiple subsamples and the Chao2 index, to estimate that the total diversity of the naive TCRβ repertoire is about 10^8 TCRβ sequences for both $CD4^+$ and $CD8^+$ naive T cells in young individuals, and about 10^7 to 5×10^7 TCRβ sequences in older individuals [75]. Since each TCRβ chain is expected to pair with about 25 TCRα chains [76], the total body diversity of naive T cells was estimated to be about 2.5×10^9 TCRs in young individuals, and 2.5×10^8 to 10^9 TCRs in older individuals [75].

The study by Qi et al. [75] has thereby provided two important insights: i) the diversity of the naive T-cell repertoire declines about two- to five-fold during healthy ageing, and ii) the naive T-cell repertoire is so diverse that even relatively large samples taken from older individuals tend to yield only unique TCR sequences. As a result, naive T-cell counts in fact provide a relatively good proxy for naive TCR diversity. Indeed, previous studies [70,77] also reported that the reduction in naive T-cell repertoire diversity during aging tends to go hand in hand with the loss of naive T cells. Since the total body richness of naive $CD4^+$ and $CD8^+$ T-cell clonotypes in older donors was estimated to be very high, i.e., more than 10^8 clonotypes [75], one may wonder whether this level of erosion has functional consequences. Let us estimate the expected breadth of an immune response to a single epitope: At an estimated precursor frequency of 10^{-6} of the naive T cells [78], and a conservatively assumed naive T-cell pool size of more than 10^{10} cells, even in older individuals, one would still expect more than 10^4 naive T cells and more than 100 different clonotypes to be specific for a single epitope. As also pointed out by Qi et al. [75], the erosion of the naive T-cell repertoire during healthy ageing may therefore not severely impact the chance to make an immune response.

Having multiple biological replicates is not only important for estimating total T-cell diversity, it also comes with the advantage that the individual abundances of naive T-cell clonotypes can be estimated more reliably by their incidence over the biological replicates. Using the incidence as a measure for abundance, we found in two unrelated data sets that a small fraction of the TCRα and TCRβ sequences in naive T-cell repertoires are present in more than two replicate samples, suggesting that these sequences correspond to TCRα and TCRβ chains that are frequently used [74,79]. We found that the abundant TCRα sequences had a relatively large chance to be produced in the thymus and could hence pair with different TCRβ sequences. The abundant TCRβ sequences, in contrast, were primarily derived from very large T-cell clones, and their high frequencies were only partially explained by the probability by which these TCRβ sequences are generated during VDJ-recombination [74]. This raises the question what these large T-cell clones in the naive T-cell pool represent. We found that the most abundant TCRβ sequences tended to have shorter CDR3 regions, and often lacked the D-segment [79]. Interestingly, such clonal expansions accumulate with age [75,79–81], and this increased clonality in older individuals may explain the (modest) decrease in diversity per million naive T cells.

Thus, the properties of the TCRs present in the naive T-cell repertoire seem to change during healthy ageing. On average, the CDR3 region becomes shorter, the number of non-template added N nucleotides decreases, and the D-segment is more often absent [79–81]. Additionally, the usage of TRVB and TRJB gene segments differs between young and older individuals [80,81]. Moreover, the physicochemical properties of the amino acids in the central part of the CDR3 loop differ somewhat between young and older individuals, because older individuals tend to have a lower abundance of strongly interacting amino acids in this region [80]. This may be readily explained by a preferential recruitment of naive T cells expressing more "sticky" TCRs into the memory pool by the various antigens encountered over a lifetime. The fact that naive T-cell clonotypes with shorter CDR3 lengths survive better into old age is more difficult to explain. Several studies have demonstrated that T cells produced during the foetal period tend to have shorter CDR3 lengths, and a lower number of N additions due to the relatively late expression of TdT [70,82–86]. Gaimann et al. [87] proposed that T cells that are produced early in life have expanded in a relatively empty pool, and that it therefore takes much longer for those clones to be replaced compared to clones that were generated later in life, similarly to what has been observed in mice [88,89]. An elegant NGS study among monozygotic twins indeed suggested that these large germ-like clonotypes that are created during pre-natal life, survive for decades as circulating naive T-cell clonotypes, and only slowly decay with age [86].

Summarizing, on a per naive T cell basis, the erosion of repertoire diversity seems to remain modest, but total body naive T-cell richness does decline when naive T-cell counts decline. Moreover, the properties of the TCRs expressed by naive T cells change during healthy ageing, most likely due to preferential survival and recruitment of certain T-cell clones into the memory pool. If aging would indeed induce an enrichment of naive T cells expressing a more specific TCR, this would contribute to the accrual of holes in the naive T-cell repertoire [13]. For future characterization of the effect of aging on naive T-cell repertoire diversity, it would be helpful to improve our estimates for the total body number of naive T cells, as this provides a reasonable proxy for total body naive T-cell repertoire diversity, and to perform single-cell analysis on large numbers of naive T cells, to better estimate the loss of true clonotypes.

6. Conclusions and future directions

In summary, a large number of quantitative studies have provided important – albeit not always unequivocal – insights into the mechanisms responsible for the long-term maintenance of naive T cells. While thymic output declines significantly in both mice and humans, the impact of this decline differs fundamentally between mice and men, because naive T cells in mice are much more dependent on thymic output than naive T cells in humans [17,19]. Although correlations between age and naive T-cell lifespans and proliferation rates have generally been ascribed to homeostatic mechanisms compensating for the loss of thymic output, various recent studies have questioned this

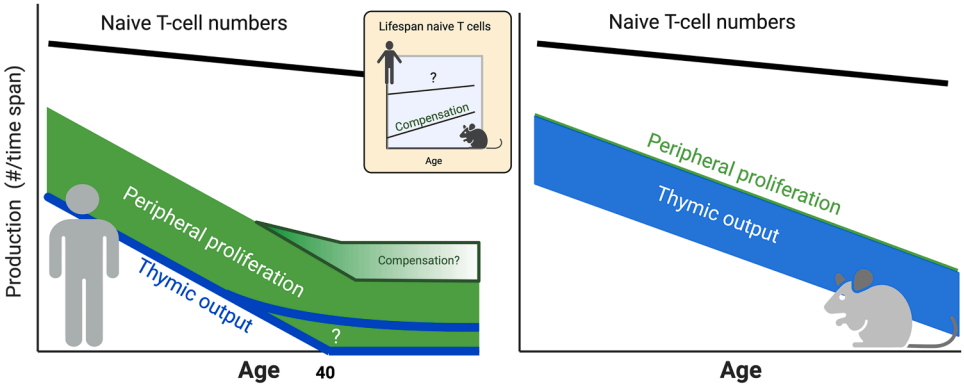

Fig. 1. Graphical summary of the observed/estimated changes in thymic output, and naive T-cell numbers, lifespan, and peripheral proliferation rates during healthy ageing in humans and mice. In both mice and humans, thymic output declines significantly, and more drastically, with age than naive T-cell numbers. In mice, this discrepancy is thought to be (at least partially) due to increased naive T-cell lifespans at older age (see Inset). In humans, it remains unclear whether naive T cells undergo a density-dependent increase in lifespan (see Inset) or proliferation rate during ageing. In human adults, naive T cells are mostly generated through peripheral T-cell proliferation (green), while in mice, the vast majority of new naive T cells are generated by the thymus (blue). It remains unclear whether the thymus in humans becomes obsolete at older age, or whether it is still functional (albeit much less than in the young) well into old age. Figure created with BioRender.com

causal link. They have instead proposed that the altered dynamics of naive T cells at older age may reflect the gradual accumulation of cells with increased lifespans [64] or result from low-grade inflammation in older people [68]. While even changes in naive T-cell dynamics and their causes are difficult to interpret, it is even harder to study the TCR repertoire of naive T cells. Even though the accumulation of holes in the naive T-cell repertoire seems a very intuitive and attractive way to explain why immune responses to new antigens and responses to vaccination tend to be impaired in older people, this remains very hard to prove. Because of its immense diversity, it remains challenging to investigate changes in the diversity of the naive T-cell repertoire during ageing. As a result, it remains unclear whether a decline in naive TCR diversity is responsible for the impaired immune responses in older people. Alternatively, the different characteristics of naive T cells in older people, including their different TCR composition [79–81], reduced telomeric length [31], or changes related to their longer residence time in the periphery [64] may be responsible for the impaired immune responses to new antigens at older age.

Future studies into the long-term effects of thymectomy on the naive TCR repertoire in humans would be helpful to understand to what extent the thymus is required for the maintenance of a diverse naive TCR repertoire in humans. Likewise, studies into the breadth of T-cell responses to newly encountered antigens in young versus older individuals would be helpful to investigate the impact of the changes in the naive T-cell pool at older age on the induction of new immune responses. Better insights into these processes are important for the optimization of vaccination strategies for older people. Depending on the underlying mechanisms of immune senescence, vaccinations that aim to protect us at older age may have to be given at a younger age, when the immune system still has the capacity to respond vigorously and to generate functional memory responses. In that case it would be crucial for vaccinations to target the most conserved parts of pathogens, to ensure long-term protection against evolving pathogens. The relatively naive immune system at younger age could hence be trained to provide protection at older age (Fig. 1).

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