Newly generated CD4 T cells in aged animals do not exhibit age-related defects in response to antigen

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Using a T cell receptor transgenic (TCR Tg) mouse model, we have shown that TCR Tg CD4 cells from aged mice retain a naive phenotype, but exhibit reduced proliferation and IL-2 production in response to the antigen compared with cells from young mice. We hypothesize that age-related decreases in T cell function may be partly related to the age of the T cells. Because thymic output is decreased with age, peripheral T cells in older individuals are likely to be older than those in younger individuals. To investigate this possibility, we have manipulated the age of CD4 T cells in the periphery of young and aged mice. The production of new T cells was induced by depleting peripheral CD4 T cells or by creating bone marrow chimeras. In both young and aged individuals where we induced the production of new T cells, these newly generated cells exhibited robust responses to antigen ex vivo and in vivo, exhibiting good expansion, IL-2 production, and cognate helper function. Our results suggest that age-related defects in response to antigenic stimulation, in part, are caused by the age of the CD4 T cells.

It is well established that CD4 T cell function in response to antigen declines with age (1, 2). The consequences of these defects are substantial and lead to increased morbidity and mortality because of infectious disease in the elderly (3–5). We have focused on examining naive CD4 function with age and determining the cause of decreased function. To accomplish this, we have used a T cell receptor transgenic (TCR Tg) model, which allows us to examine antigen-specific naive CD4 T cells from young and aged mice. Naive CD4 T cells from aged Tg mice do not respond to antigen as well as cells from younger mice, leading to reduced IL-2 production and an expansion of these aged cells both in vitro and in vivo (6–8). Effectors generated using CD4 T cells from aged mice produce reduced levels of IL-2 and express lower levels of CD25 compared with young. This age-related defect in CD4 T cells also results in reduced cognate helper function (9) and could affect responses to both pathogens and vaccinations.

Our hypothesis is that age-related defects in naive CD4 T cell function may be related, in part, to the age of the T cells. In aged mice, naive T cells are on the average older, because of decreased thymic output (10, 11), and this increased age may have a negative impact on function. To examine this further, we manipulated the age of CD4 T cells in the periphery using two model systems. First, new CD4 T cells were generated in young and aged Tg mice by depleting the existing populations with anti-CD4 antibody treatment. Second, BM chimeras were generated by transferring BM progenitors from young or aged Tg mice into young or aged hosts. We then examined the ex vivo and in vivo function of these newly generated CD4 T cells. The results of these studies show that newly generated T cells respond well to antigen, regardless of the age of the host in which they develop. Therefore, our results suggest that in our models where we have induced the production of new T cells, the age of the naive CD4 T cells influences antigen-specific T cell responses.

RESULTS AND DISCUSSION

In vitro responses of CD4 T cells from young and aged TCR Tg mice

As shown previously (6, 12), naive T cells from aged Tg mice exhibit diminished responses to antigen. To reiterate this point,
equal numbers of young and aged naive Tg cells were stimulated in vitro with antigen/APC to determine expansion and cytokine production. Fig. 1 A shows that cells from young Tg mice expanded almost threefold more than those from aged mice over the 4-d culture period. In addition, over the first 24 h of culture, cells from young mice produced more than twice as much IL-2 when compared with the cells from aged mice. We hypothesize that T cells in the older animals are older and that this may be one factor that contributes to diminished function. To further our understanding of the basis for this age-related decline in responses, we manipulated the age of the T cell populations in young and aged mice in the experiments that follow.

**In vivo depletion of CD4 T cells in young and aged TCR Tg mice**

To generate new CD4 T cells, young and aged Tg mice were treated with a depleting anti-CD4 or isotype control antibody. The presence of Tg CD4 T cells in peripheral blood was monitored over a period of 2 mo, and the percent Tg CD4 T cells in peripheral blood at day 59 is shown in Fig. 2 A. In the isotype control–treated group, there were significantly fewer Tg+ cells in the PBL of aged mice when compared with the young mice. As shown in a previous study, this is mostly caused by the increased presence of non–Tg-expressing memory CD4 T cells in these aged animals (12). Importantly, there were no apparent differences in the anti–CD4-treated aged groups, with both young and aged animals exhibiting similar levels of Tg expression. The cell surface phenotype of each of these populations is shown in Fig. 2 B. We have shown that the Tg CD4 populations in both young and aged Tg mice express a naive phenotype (CD44lo CD62Lhi CD25lo), even in very old animals (12), and this was also seen in each group in this experiment.

Equal numbers of enriched Tg CD4 T cells from each group were labeled with the fluorescent dyes carboxyfluorescein succinimidyl ester (CFSE) and stimulated with antigen/APC to examine expansion. Fig. 2 C shows that Tg CD4 T cells from the aged isotype-treated group expanded significantly less than those from young isotype-treated group. In contrast, the young and aged anti–CD4–treated groups responded well to antigen, with both groups showing >10-fold expansion. This was also apparent in the day 4 CFSE flow cytometry profiles of each of these populations (Fig. 2 D). The young and aged anti–CD4–treated groups, as well as the isotype-treated young group underwent many rounds of cell division over this 4-d period. The aged anti–CD4–treated group lagged behind dramatically, having undergone at most four rounds of cell division. Furthermore, Fig. 2 E shows that the aged isotype–treated group also exhibited significantly reduced IL-2 production compared with other groups. Also, it is important to note that no significant differences in the numbers of CD25+ CD4 regulatory T cells were found in these young and aged Tg mice that could account for age-related decreases in function (unpublished data).

**Figure 1. Reduced responses of naive CD4 T cells from aged AND mice.** Lymphocytes from young (gray) and aged (black) AND mice were harvested and enriched for naive CD4 T cells. Each population was stimulated in vitro with Ag/ APC at 2 × 10⁵/ml. (A) Fold expansion of T cells from day 0 to day 4 was determined by counting viable cells. (B) Supernatants were collected from young and aged T cell cultures after 1 d of culture and assayed for IL-2. Data shown are mean ± SE, with n = 3 mice/group; *P < 0.05.

We also performed similar experiments in young and aged B10.BR mice. Repopulation of peripheral CD4 T cells was monitored by tail bleeding over a period of 8 wk after antibody treatment. Fig. 2 F shows that both young and aged anti–CD4–treated animals repopulated peripheral CD4 T cells with similar kinetics. We assayed for the in vivo cognate helper function of CD4 T cells by immunizing with 4-hydroxy-3-nitrophenyl acetyl conjugated pigeon cytochrome c (NP-PCC) in alum. As we have shown recently, the NP-specific B cell response is totally dependent upon the presence of helper CD4 T cells (9). Fig. 2 G shows that expansion of the NP+ B cell population was similar in the anti–CD4–treated young and aged groups, while it was significantly reduced in the aged isotype–treated group. These results indicate that newly generated CD4 T cells in aged mice exhibit robust responses, both ex vivo and in vivo, in both TCR Tg and non–Tg models.

**Generation of BM chimeras in young hosts**

To determine if aged BM stem cells could generate functional T cells in a young environment, chimeras were generated by transferring BM from young or aged Tg mice into lethally irradiated young B10.BR hosts. All of the newly generated Tg CD4 T cells found in the periphery of these chimeras were generated from the host thymus because thymectomy ablated production of new T cells (unpublished data). Repopulation and function of newly produced T cells from 4 to 12 wk after reconstitution was examined. Although aged BM stem cells were slightly slower at reconstitution of the periphery, the in vitro function of the new T cells was similar at all time points (unpublished data). Fig. 3 A shows that there was no difference in the percentages of Tg CD4 T cells in the hosts reconstituted with BM from either young or aged donors by 12 wk after reconstitution. Furthermore, in hosts reconstituted with either young or aged BM, newly generated Tg CD4 T cells (in spleen and peripheral LN) express a naive phenotype (Fig. 3 B).

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Equal numbers of Tg CD4 T cells from chimeras generated with young or aged BM were stimulated with antigen/APC. Fig. 3 C shows that expansion of these populations was similar, with both showing over 10-fold expansion. In addition, Fig. 3 D shows that there was similar IL-2 production by both of these T cell populations. These results show that BM from aged Tg donors can produce new mature T cells that exhibit robust in vitro responses to antigen.
equal to those produced from young BM and also equal to that seen in Tg CD4 T cells from young mice (Fig. 1).

**Generation of BM chimeras in young and aged hosts**

In the previous experiment, we showed that BM from aged donors can generate highly functional T cells in a young host environment, but the effects of an aged environment on newly generated T cell function were not addressed. Thus, we generated BM chimeras using GFP BM from young donors transferred into young or aged B10.BR hosts. This allowed us to identify newly generated T cells by GFP expression. The use of GFP-marked donor BM was necessary because the repopulation of the aged hosts was significantly diminished compared with young hosts, as shown in Fig. 4 A. There were about three times as many Tg CD4 T cells in the PBL of the young hosts compared with the aged hosts, most likely because of the increased presence of memory phenotype lymphocytes, which are more radiation resistant, in the aged animals (13). The phenotype of newly generated Tg CD4 T cells (from spleens and peripheral LNs) is shown in Fig. 4 B. Importantly, regardless of whether T cells were generated in a young or aged host, they expressed a naive T cell phenotype.

At 12 wk post-BM transfer, newly generated Tg GFP CD4 T cells from young and aged hosts were sort purified, and equal numbers were stimulated with antigen/APC.

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**Figure 3.** Responses after reconstitution of young hosts with BM from young or aged AND Tg mice. BM from young (gray) and aged (black) AND Tg mice was used to reconstitute lethally irradiated young B10.BR hosts. (A) Repopulation of the periphery with donor AND Tg BM-derived lymphocytes was monitored by examining the presence of Tg (Vβ3) CD4 T cells in total PBL at 12 wk after reconstitution is shown. (B) Cell surface phenotype of lymphocytes pooled from spleens and LNs 12 wk after reconstitution. Flow cytometry histograms are gated on Tg (Vβ3) CD4 cells. Dotted histograms represent isotype control staining. (C) At 12 wk after reconstitution, lymphocytes were harvested, enriched for naive CD4 T cells, and stimulated with antigen/APC at 2 x 10^5/ml. Graph shows the fold expansion over the 4-d culture period. (D) Supernatants were collected from the cultures after 1 d and assayed for IL-2. Data shown are mean ± SE, with n = 3 mice/group.

**Figure 4.** Responses after reconstitution of young or aged hosts with BM from young AND Tg mice. BM from young AND × GFP mice was used to repopulate lethally irradiated young (gray) and aged (black) B10.BR hosts. (A) Repopulation of the periphery with donor AND × GFP Tg BM-derived lymphocytes was monitored by flow cytometry. The percent Tg (Vβ3) GFP CD4 in total PBL at 12 wk after reconstitution is shown. (B) Cell surface phenotype of lymphocytes pooled from spleens and LNs 16 wk after reconstitution. Flow cytometry histograms are gated on Tg (Vβ3) CD4 lymphocytes. Dotted histograms represent isotype control staining. (C) At 12 wk after reconstitution, lymphocytes were harvested, FACSort purified for Tg GFP CD4 T cells, and stimulated with antigen/APC at 2 x 10^5/ml. Graph shows the fold expansion over the 4-d culture period. (D) Supernatants were collected from the cultures after 24 h and assayed for IL-2. Data shown are mean ± SE, with n = 6 mice/group; *, P <0.05.
Interestingly, there was no difference in the in vitro expansion (Fig. 4 C) or the IL-2 production (Fig. 4 D) of the newly generated Tg CD4 T cells in either young or aged chimeras. These results indicate that, even in an aged environment, newly generated T cells can respond well to antigen.

**Phenotype of newly generated T cells**

As shown previously, a hallmark of aged Tg CD4 T cells is incomplete phenotypic differentiation of effector populations (6). Fig. 5 A shows that effectors generated using Tg CD4 T cells from aged mice exhibit reduced CD62L down-regulation and dramatically reduced up-regulation of CD25 compared with those generated from young mice. We examined the phenotype of day 4 effector populations generated from BM chimera populations in the preceding experiments. In chimeras generated by transferring young or aged BM into young hosts (Fig. 5 B) and young BM into young or aged hosts (Fig. 5 C), no age-related differences in effector phenotypes were observed. All populations exhibited an activated phenotype with reduced CD62L and increased CD25 expression. Once again, these results indicate that newly generated CD4 T cells, whether derived from young or aged BM, in either young or aged hosts, behave like young cells in response to antigen.

In our models, where we induced production of new T cells by depleting the periphery, we saw that new CD4 T cells in aged animals function well in primary responses to antigen. Upon stimulation with antigen/APC, new T cells, all of which expressed a naive phenotype, expanded and produced levels of IL-2 similar to young T cells. They also differentiated to a highly activated effector phenotype, unlike Tg CD4 T cells from intact aged mice, which do not differentiate well. Importantly, newly generated CD4 T cells in aged mice also exhibit robust cognate helper function, leading to good antigen-specific B cell expansion (Fig. 2 F). These results also indicate that other components of the immune system, such as B cells, are less affected by aging. Importantly, our results suggest that enhancing production of new T cells in older individuals should improve immune responses and may benefit responses to infectious agents and vaccines in the elderly.

While our findings imply that the age of the naive CD4 T cells influences their function and may be responsible for age-related defects observed in T cell responses, it is quite possible that other environmental influences are also involved. Age-related environmental influences are likely to also contribute to declining T cell function, including the cytokine milieu and the presence of other T cell populations, such as regulatory T cells and memory phenotype T cells. Unfortunately, our model does not address these environmental influences and this must be kept in mind when interpreting our results. Furthermore, it must be pointed out that we are only examining primary responses to antigen for each population and it is possible that some age-related defects are present but are not apparent in our assays. In fact, our studies have shown that even though we can generate robust primary responses from aged naive CD4 T cells when exogenous cytokines are added, these aged cells differentiate into poorly functioning memory cell populations (14).

Some differences in repopulation between the two models that we used in this study were apparent. No differences were seen when peripheral CD4 T cells in young and aged animals were depleted by antibody treatment. This was in contrast to the BM chimera studies, where a reduction in the repopulation of aged hosts was seen. A likely explanation is that depletion via antibody treatment was more complete compared with lethal irradiation, possibly because of the fact that aged hosts have increased numbers of memory cells that are radiation resistant (13). This results in less space in the periphery of irradiated mice, leading to reduced levels of reconstitution. Defects in repopulation of aged BM stem cells have been reported (for review see reference 15), but because we are only examining the function of the newly generated CD4 T cells, this is not especially relevant to our studies.

One main defect in the function of CD4 T cells from aged mice may originate at the immune synapse. Studies have shown that naive CD4 T cells from aged animals exhibit decreased immune synapse formation including reduced movement of signaling molecules after stimulation with antigen (16, 17). These researchers hypothesized that
age-related decreases in T cell activation are caused by alterations in the plasma membrane, resulting in decreased immune synapse formation and subsequent T cell activation. If initial T cell activation is reduced, this could lead to reduced levels of T cell responses that are observed with age. This hypothesis is especially interesting in light of our findings. Older CD4 T cells will have been in the periphery for some period of time and will have been exposed to many things including oxidative damage. Oxidative stress decreases IL-2 production by T cells (18) and preferentially reduces the function of naïve T cells (19). Oxidative stress can dramatically affect lipids (20), causing alteration of lipids in the plasma membrane. This can negatively affect formation of immune synapses in response to TCR stimulation, resulting in decreased T cell activation and function. By depleting older, damaged CD4 T cells in the periphery and replacing them with newly generated T cells, we have removed one defect because of age.

### MATERIALS AND METHODS

#### Animals.
AND TCR Tg mice on a B10.BR background were used for these studies. They express a 
Vβ3/Vα11 TCR. Tg specific for a peptide of PCC (21). For some studies, AND Tg mice were crossed with GFP Tg mice (22) and used as a source of BM cells. TCR Tg mice were used at 2–4 mo (young) and 16–20 (aged) mo old. B10.BR mice were also bred and maintained at the Trudeau Institute and were used as young (6–8 wk old) or aged (at least 18 mo old). All mice were housed at the animal facility at the Trudeau Institute until their use. Experimental procedures were approved by Trudeau Institute Institutional Animal Care and Use Committee.

#### In vivo depletion of CD4 T cells.
Young and aged AND Tg mice or B10.BR mice were injected i.p. with 200 μg anti-CD4 antibody (GK1.5) or isotype control (IgG2b) as described previously (23). The repopulation of peripheral CD4 T cells was monitored by flow cytometry analysis of peripheral blood over a period of 8 wk.

#### Preparation of BM chimeras.
BM was obtained from the bones of the hind limbs of two to three young or aged AND Tg or AND × GFP Tg mice. Mature T cells were depleted by treatment with anti-CD4, anti-CD8, and anti-Thy1.2 antibodies plus complement. No CD4- or CD8-mice. Mature T cells were depleted by treatment with anti-CD4, anti-CD8, and anti-Thy1.2 antibodies plus complement. No CD4- or CD8-positive cells could be detected by FACS analysis after depletion. Young or aged syngeneic B10.Br hosts were lethally irradiated (950 Rads) and reconstituted with 3–5 × 10^6 BM cells. At specified time points after BM transfer, lymphocytes from spleens and peripheral LNs of young and aged chimera mice were assayed. In the experiments with AND × GFP BM, newly generated GFP+ Tg CD4 T cells were purified by FACSorting on a cell sorter (model FACS Vantage/Diva; Becton Dickinson).

#### Cell culture and effector generation.
Enrichment and culturing of CD4 cells from spleen and peripheral LNs has been described previously (6). Enriched naive CD4 T cell populations exhibited no age-related differences in Tg expression within the CD4 T cell compartment. The percent TCR. Tg+ cells within the resulting enriched CD4 populations were 97.2 ± 1.9% young and 94.5 ± 5.6% aged. DCEK-ICAM cells (24) were used as APC at a 2:1 T cell/APC ratio. CD4 effector cells were generated by culturing 2 × 10^6/ml Tg CD4 cells with 5 μM PCC peptide and mitomycin c-treated DCEK-ICAM cells (2:1 T cell/APC) for 4 d. Where indicated, CD4 T cell populations were labeled with the dye CFSE (Molecular Probes) before culture (6). To determine cytokine production, supernatants were collected after 1 d and IL-2 production was determined. After 4 d of culture, effector populations were washed twice and counted to determine fold expansion. The cell surface phenotype of these effectors was examined by flow cytometry as described in Immunofluorescent staining section. Each experiment was performed at least three times.

#### Cytokine detection.
Culture supernatants were assayed for the presence of IL-2 in a bioassay with the NK-3 cell line. IL-2 concentration is expressed as units per milliliter; 1 U = 1.2 ng IL-2.

#### Immunization.
Young and aged B10.BR mice were immunized i.p. with 200 μg NP-PCC in alum. On day 14 after immunization, splenocytes were harvested. The number of NP-binding splenocytes was determined by flow cytometry after staining with NP conjugated to allophycocyanin (NP-APC) as described previously (9). Each experiment was performed twice with three to five mice per group.

#### Immunofluorescent staining.
All staining was performed at 4°C in PBS with 1% BSA and 0.1% NaN3. Antibodies were purchased from BD Biosciences and included the following: Cy-chrome anti-CD4 (clone RM4-5), PE anti-CD34 (clone HJ12), PE anti-CD62L (clone Mel14), PE anti-CD25 (clone PC61), isotype control antibodies, biotin and PE anti-Vβ3 (clone KJ25), and streptavidin-APC. Flow cytometry was performed using a FACSCalibur flow cytometer (Becton Dickinson), and the data were analyzed with Cell Quest software.

#### Statistical analysis.
Statistical significance was determined by Student’s t test. Values of P < 0.05 were considered significant.

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