Age-related Defects in CD4 T Cell Cognate Helper Function Lead to Reductions in Humoral Responses

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Abstract

With increasing age, the ability to produce protective antibodies in response to immunization declines, leading to a reduced efficacy of vaccination in the elderly. To examine the effect of age on the cognate function of CD4 T cells, we have used a novel adoptive transfer model that allows us to compare identical numbers of antigen-specific naive T cells from young and aged TCR transgenic (Tg) donors. Upon transfer of aged donor CD4 T cells to young hosts, there was significantly reduced expansion and germinal center (GC) differentiation of the antigen-specific B cell population after immunization. This reduced cognate helper function was seen at all time points and over a wide range of donor cell numbers. In hosts receiving aged CD4 cells, there were also dramatically lower levels of antigen-specific IgG. These age-related defects were not due to defects in migration of the aged CD4 T cells, but may be attributable to reduced CD154 (CD40L) expression. Furthermore, we found that there was no difference in B cell expansion and differentiation or in IgG production when young CD4 T cells were transferred to young or aged hosts. Our results show that, in this model, age-related reductions in the cognate helper function of CD4 T cells contribute significantly to defects in humoral responses observed in aged individuals.

Key words: aging • B lymphocytes • germinal centers • antibody • vaccines

Introduction

The increased incidence and severity of infectious diseases in elderly populations and a reduced ability to produce high affinity antibodies upon immunization correlates with relatively weak and short-lived primary antibody responses in aged individuals (1–5). This can dramatically affect the efficacy of vaccinations, which aim to produce high affinity neutralizing antibodies. Clinically, this issue is important since the elderly are highly encouraged to receive vaccinations for infectious diseases such as influenza and pneumococcal pneumonia (6, 7). It is not surprising that vaccine efficacy is reduced with age since numerous studies have shown that aging leads to decreased germinal center (GC) formation, decreased levels of somatic mutations and the production of antibodies that are less protective (8–12). Furthermore, because GCs are required for memory B cell development (13), the generation of B cell memory is also likely to be reduced with increasing age.

CD4 T cells are absolutely necessary for the generation of GCs (14) and CD4 cognate helper function has been shown

to be reduced with age (12). Additional age-related defects in T cell function have been well documented and include decreased in vitro proliferation as well as reduced graft rejection, delayed hypersensitivity reactions, and rejection of tumors (15). Our previous studies have shown that naive CD4 function decreases dramatically with age (16–19). In both in vitro and in vivo studies, naive CD4 T cells from aged TCR transgenic (Tg) animals produce less IL-2, expand less, and differentiate less upon antigen stimulation compared with those from younger individuals (16, 20). One result of this decreased IL-2 production is the incomplete differentiation of effector populations, resulting in decreased effector function (16). In addition, cognate helper activity of CD4 T cells has been shown to require appropriate expression of cell surface molecules such as CD154, CD28, CD134 (OX40), and CXCR5 (21–23), all of which might be affected by incomplete effector differentiation. Importantly, these age-related decreases in CD4 function could potentially im-

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pact cognate interactions with both CD8 T cells and B cells, resulting in diminished CTL activity and humoral responses, respectively, and ultimately resulting in reduced responses to both infections and vaccines.

In this study, we used an adoptive transfer model, with TCR Tg CD4 T cells, to examine the effect of age on cognate helper activity. Our results show that even when identical numbers of young and aged antigen-specific donor T cells were transferred to young hosts, the cognate helper function of CD4 T cells from aged donors was significantly reduced. This lead to reduced antigen-specific B cell expansion and differentiation as well as decreased IgG production. We also show that this reduction in cognate function was not due to age-related differences in migration of donor CD4 T cells into follicles, but may be attributable to age-related reductions in CD154 expression. Finally, we show that when CD4 T cells from young donors were transferred to young or aged hosts, there was no difference in B cell expansion and differentiation or antigen-specific IgG production. These results indicate that age-related reductions in humoral responses are most likely attributable to defects in CD4 T cell cognate helper function in our model system.

Materials and Methods

Animals. All mice used in this study were bred at the Trudeau Institute Animal Core Facility (Saranac Lake, NY). Intact young (2–4 mo) and aged B10.BR (>20 mo) mice were used in the first set of experiments. Adoptive transfer experiments involved the transfer of antigen-specific CD4 cells into adoptive hosts. Young (2–4 mo) and aged (15–19 mo) as well as TCR Tg mice, on a B10.BR background, were used as the source of donor CD4 T cells. These mice express a Vβ3/Vα11 TCR transgene specific for a peptide fragment of pigeon cytochrome C (PCC; 24). Young (2–4 mo) or aged (20–24 mo) CD4KO mice, backcrossed to B10.Br for 10 generations, were used as the adoptive hosts. All mice were housed in sterilized, Hepa-filtered, individually ventilated caging at the animal facility at the Trudeau Institute until their use. All experimental procedures involving mice were approved by Trudeau Institute Institutional Animal Care and Use Committee.

CD4 T Cell Isolation, Adoptive Transfer, and Immunization. Lymphocytes were harvested from the spleens and peripheral lymph nodes of young or aged TCR Tg mice. For each experiment, lymphocytes from two young and four aged mice were pooled to generate donor T cell populations. The enrichment of naive Tg CD4 T cell populations by negative selection has been described previously (16). The percent TCR Tg+ cells within the resulting enriched CD4 populations were similar for young (97.2 ± 1.9%) and aged (94.5 ± 5.6%) populations. In addition, these experiments have also been performed using FACS sorted Tg+CD4 T cells from young and aged donors with similar results. Donor Tg CD4 cells were transferred i.v. into CD4KO adoptive hosts (10^6/mouse or as indicated). When indicated, donor cells were carboxy fluorescein succinimidyl ester (CFSE) labeled as previously described (16). Control hosts received no CD4 T cells. Mice were immunized i.p. with 200 μg 4-hydroxy-3-nitrophenyl acetyl conjugated PCC (NP-PCC) or PBS in alum. For each experiment, three to five hosts were used in each group and each experiment was performed from two to four times.

In Vitro Stimulation of CD4 T Cells. Culture conditions for CD4 T cells have been described previously (16). Enriched populations of Tg CD4 T cells from young and aged mice were stimulated with PCC peptide and DCEK-ICAM APC cell line (25) at a 2:1 T/APC ratio in 24-well plates. At the indicated time points, T cells were harvested and examined for CD4, Vβ3 and CD154, or CD28 expression by flow cytometry as described below. Data presented representative of at least three separate experiments.
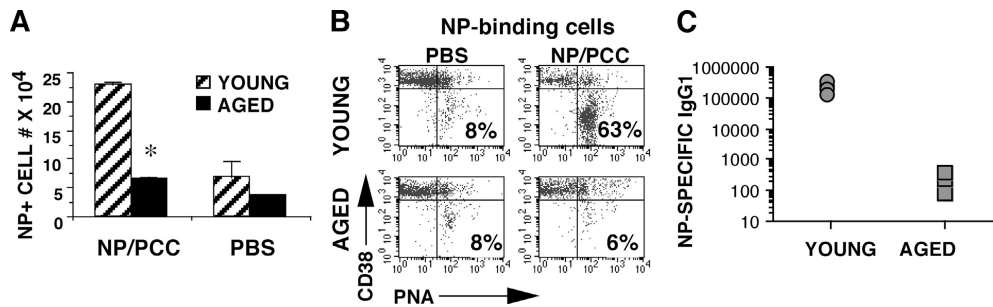
Immunofluorescent Staining. Splenocytes from immunized host animals or from in vitro–stimulated cultures of young and aged TCR Tg CD4 T cells were harvested at the indicated time points and individually analyzed. All staining was done at 4°C in PBS with 1% BSA and 0.1% NaN₃ and all samples were treated with Fc blocking antibody (24G2) before staining. The following antibodies were used (all purchased from BD Biosciences): Cychrome anti-CD4 (RM4-5), PE or biotin anti-Vβ3 (KJ25), PE anti-CXCR5, PE anti-CD154, FITC anti–mouse IgG1, biotin anti-CD134, and PE anti-CD38. Strepavidin conjugated to allophycocyanin was also purchased from BD Biosciences. FITC-peanut agglutinin (PNA) was purchased from Sigma-Aldrich. NP conjugated to allophycocyanin (NP-APC) was prepared as described previously (26). Immediately before analysis, propidium iodide was added to each sample to allow for identification of viable cells. Flow cytometry was performed using a FACS Calibur flow cytometer (Becton Dickinson) and the data were analyzed with Cell Quest software.

Detection of NP-specific Antibodies. Serum was collected from all immunized animals. NP-specific IgM and IgG1 was determined by ELISA with NP-conjugated BSA. Isotype-specific antibodies for detection were purchased from Southern Biotechnology Inc. Titers were determined by the last dilution of serum with detectable antibody above background.

Histology. Spleens were frozen directly in Tissue-Tec OCT (Fisher Scientific) over liquid nitrogen. Frozen tissue blocks were brought to –20°C and 7-μm sections were cut and placed on poly-L-lysine coated slides. Slides were dried at room temperature overnight and then either probed with antibodies or stored at –20°C. Slides were brought to room temperature, fixed in acetone at 4°C for 10 min, and then placed in PBS for 5 min to remove the OCT. All slides were blocked with 5% BSA in PBS for 30 min and then washed before being probed with biotinylated antibodies to various antigens for 30 min in a humidified chamber. Slides were incubated with biotinylated anti-CD4 (L3T4; BD Biosciences) and biotinylated PNA (Vector Laboratories) and were then washed. They were then incubated with streptavidin-Alexa 594, streptavidin-Alexa 488, or streptavidin-Alexa 350 (Molecular Probes) for 30 min, washed, and mounted with Polymount (Polysciences). Sections were also counterstained with Hoechst (Sigma-Aldrich). Slides were viewed with a Zeiss Axioplan 2 microscope and images were recorded with a Zeiss AxioCam digital camera (Carl Zeiss MicroImaging, Inc.) using the Zeiss proprietary software, Axiovision 3.0.6.0. Images, which were originally obtained at 10× magnification, were manipulated in Adobe Photoshop 5.5.

Results

Decreased B Cell Expansion and GC Formation in Aged Mice. To confirm the findings previously published of reduced antigen-specific B cell expansion and differentiation in aged mice (1, 27), nontransgenic young and aged B10.Br mice were immunized i.p. with NP-PCC/alum

Figure 1. Decreased B cell expansion and GC formation in aged mice. Young (2 mo) and aged (23 mo) nontransgenic B10.Br mice were immunized i.p. with NP-PCC/alum (200 μg) or PBS/alum. On day 14, spleens were harvested and stained with NP-APC to detect NP-binding cells by flow cytometry. (A) Bar graph shows the total number of NP-binding cells in young (striped bars) and aged (solid bars) groups (mean ± SE). (B) Representative flow cytometry dot plots gated on NP-binding cells showing CD38 and PNA expression. (C) NP-specific serum IgG1 concentrations in each of the young and aged immunized mice. *P < 0.05.

and on day 14, the NP-binding population was examined by flow cytometry. In NP-PCC immunized groups, there was a threefold reduction in the expansion of NP⁺ cells in aged mice compared with young (Fig. 1 A). There was also reduced differentiation of these NP⁺ cells in aged mice. Fig. 1 B shows dot plots demonstrating that a significant proportion (63%) of the young NP⁺ cells had differentiated to GC phenotype (CD38^{lo}PNA^{hi}), while those in aged mice retained a predominantly naive phenotype (CD38^{hi} PNA^{lo}; 28). In addition, the NP-specific IgG1 titers in immunized young mice were over 100-fold higher compared with the aged mice (Fig. 1 C). Together, these results demonstrate that there was a statistically significant age-related defect in B cell expansion, differentiation and antibody production. However, the frequency and differentiation state of PCC-specific CD4 T cells in these young and aged mice could not be enumerated or controlled. This is an important issue since the number of naive CD4 T cells decreases dramatically with age and the frequency of antigen-specific CD4 cells is also likely to be affected (29, 30). Therefore, we developed an adoptive transfer model to examine the effect of age on CD4 T cell helper function.

Adoptive Transfer Model to Study In Vivo Cognate Helper Function. To examine the impact of age on CD4 T cell cognate helper function more precisely, we adoptively transferred AND TCR Tg CD4 cells from young and aged mice into CD4KO recipients. The AND TCR Tg CD4 cells are specific for a peptide of PCC and express a naive phenotype (both cell surface phenotype and functional phenotype) even in very old Tg mice (18). We have extensively characterized the responses of the young and aged AND TCR Tg CD4 T cells and our published results show that the naive Tg CD4 cells from aged mice do not respond to TCR stimulation as well as cells from young mice, resulting in reduced IL-2 production, expansion and differentiation both in vitro and in vivo (16, 18, 20, 31). Therefore, this model is both novel and very advantageous as it allows us to directly compare similar populations of antigen-specific naive CD4 T cells from young and aged mice. It also allows us to eliminate differences in the frequency or antigen experience of CD4 T cells that are likely to occur in intact young and aged mice (29, 30).

AND TCR Tg CD4 cells from young or aged donors were transferred into young or aged CD4KO hosts, which

exhibit no endogenous cognate helper function, as shown in Fig. 2. When young Tg CD4 cells were transferred into young NP-PCC–immunized hosts, there was significant expansion and differentiation to GC phenotype (CD38^{lo} PNA^{hi}) of the NP-binding population (Fig. 2, A and B, respectively, left panels) on day 14. This indicated that the transferred Tg CD4 cells provided effective cognate help that induced good GC differentiation. Upon CD4 T cell
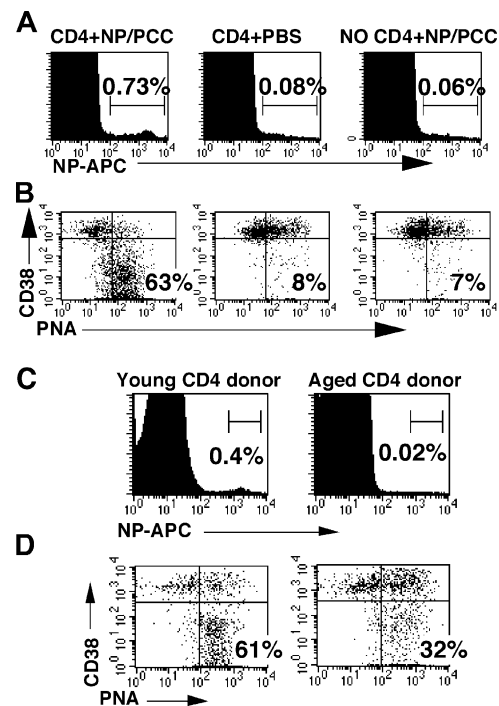

Figure 2. Adoptive transfer model to study in vivo cognate helper function. (A) Young AND Tg CD4 cells (10⁶) were adoptively transferred i.v. to young CD4KO hosts. Control hosts received no CD4 cells. Hosts were then immunized i.p. with 200 μg NP/PCC or PBS in alum. On day 14, NP-specific expansion was examined by staining splenocytes with NP-APC. The percent of NP-binding cells for each condition is shown in the flow cytometry histograms. (B) Dot plots showing PNA versus CD38 staining are gated on NP-binding cells in A. Percentages indicate GC phenotype B cells (CD38^{lo}PNA^{hi}). (C) Flow cytometry histograms showing expansion of NP-specific cells on day 14 after immunization with NP-PCC and transfer of young or aged donor CD4 T cells. (D) Dot plots are gated on NP-binding cells in C; percentages indicate the GC⁺ phenotype population.

transfer and immunization with PBS (Fig. 2, A and B, middle panels) or upon NP-PCC immunization with no transfer of CD4 T cells (Fig. 2, A and B, right panels), there was no NP-specific expansion or differentiation to GC phenotype. Therefore, in these experiments, all of the observed cognate helper function could be attributed to the adoptively transferred Tg CD4 cells.

Decreased Cognate Helper Function of Aged CD4 Cells. To determine whether the age of donor CD4 T cells affected expansion and differentiation of NP+ B cells, we transferred naive TCR Tg CD4 T cells from young and aged donors into young CD4KO hosts and immunized with NP-PCC. Fig. 2, C and D, shows representative flow cytometry results comparing young and aged donor T cell function on day 14. The percentage of NP+ cells generated in hosts that received young donor cells was dramatically increased compared with hosts receiving aged donor cells (Fig. 2 C). In addition, the percentage of NP+ cells that had differentiated to a GC+ phenotype (CD38loPNAhi) was greater in hosts receiving young donor cells (Fig. 2 D).

On days 7, 14, and 21 after immunization, the total numbers and percentages of host NP-binding cells and NP-binding B cells expressing a GC phenotype were determined by flow cytometry. Fig. 3, A and B, shows that at each time point, hosts receiving young CD4 T cells had significantly greater expansion of the NP-binding population compared with those receiving CD4 T cells from aged donors. Fig. 3, C and D, shows that at each time point there is also a significant increase in the percentages and numbers of differentiated GC+NP+ B cells in hosts receiving young donor cells compared with those receiving aged T cells. In PBS-immunized hosts or in hosts receiving no donor TCR Tg CD4 cells very few GC phenotype cells were evident (unpublished data).

In addition, we examined whether CD25+ CD4 regulatory T cells played a role in this age-related reduction in cognate helper function by removing CD25+ CD4 T cells by FACSorting before adoptive transfer. When the number of total NP+ and NP+GC+ cells was determined on days 7, 14, and 21, the results were similar to those in Fig. 3. Significant reductions in NP+ cell expansion and GC differentiation were observed in hosts receiving aged donor T cells (unpublished data). These results indicate that regulatory CD4 T cells do not play a role in the reduced function of aged CD4 T cells in this model.

In the experiments shown in Fig. 3, A–D, 10^6 Tg CD4 cells from young or aged donors were transferred into the adoptive hosts. To determine if transferring fewer or more aged CD4 T cells would enhance helper function, the number of transferred cells was titrated from 10^3 to 10^7. Fig. 3 E shows that when 10^3 to 10^6 T cells were transferred, CD4 T cells from young donors always induced significantly more NP-specific GC phenotype cells compared with those from aged donors. Interestingly, when 10^7 CD4 cells were transferred, both young and aged populations provided greatly reduced levels of cognate helper activity. For the young helper population at the 10^7 cell concentration, the heightened engagement of CD40 on responding
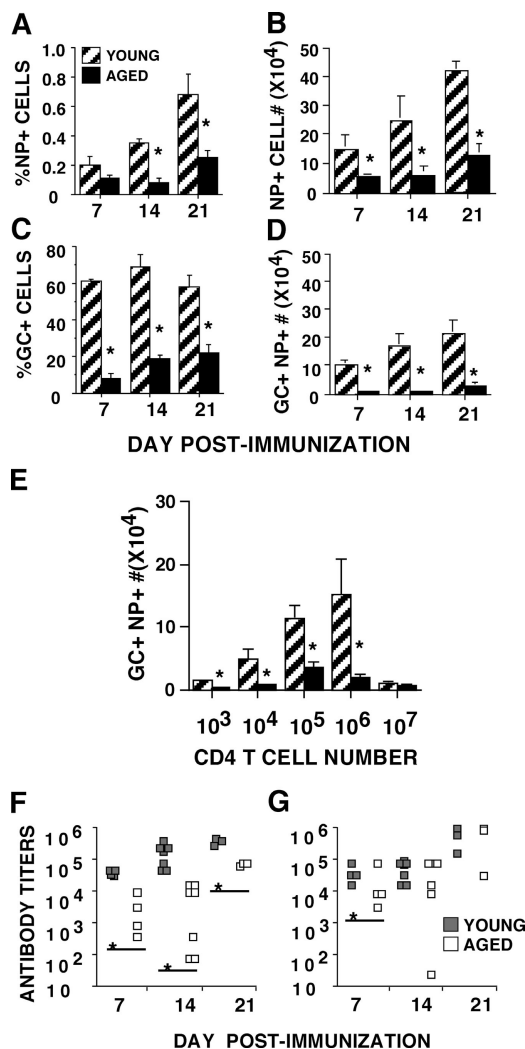

Figure 3. Reduced cognate helper function of Tg CD4 T cells from aged mice. Young (striped bars) and aged (solid bars) AND Tg CD4 cells (10^6) were adoptively transferred i.v. to young CD4KO hosts. Hosts were then immunized i.p. with 200 μg NP/PCC in alum. On days 7, 14, and 21 the (A) percentages and (B) numbers of NP-binding cells were determined by flow cytometry following staining with NP-APC (mean ± SE). On days 7, 14, and 21 the (C) percentages and (D) numbers of GC phenotype (CD38loPNAhi) cells within the NP-binding population was determined by flow cytometry (mean ± SE). (E) Numbers of GC phenotype (CD38loPNAhi) NP-binding cells per spleen on day 14 upon titration of donor T cell numbers. The numbers of young and aged donor cells were titrated from 10^3 to 10^7 per host. Data shown is mean ± SE. (F) NP-specific serum IgG1 titers in NP-PCC immunized hosts receiving young (shaded squares) or aged (open squares) Tg CD4 T cells on days 7, 14, and 21 after immunization. (G) NP-specific serum IgM titers in NP-PCC immunized hosts receiving young (shaded squares) or aged (open squares) Tg CD4 T cells on days 7, 14, and 21 after immunization. For all experiments: *P < 0.05.

B cells, as a result of increased antigen–specific T cell help, has been shown to ablate GC formation and prematurely terminates an ongoing GC response through an unknown mechanism (32). These results indicate that providing greater numbers of antigen-specific aged CD4 T cells does not lead to better cognate helper function.

Production of NP-specific serum antibodies was also examined in each adoptive host. Fig. 3 F shows that on days
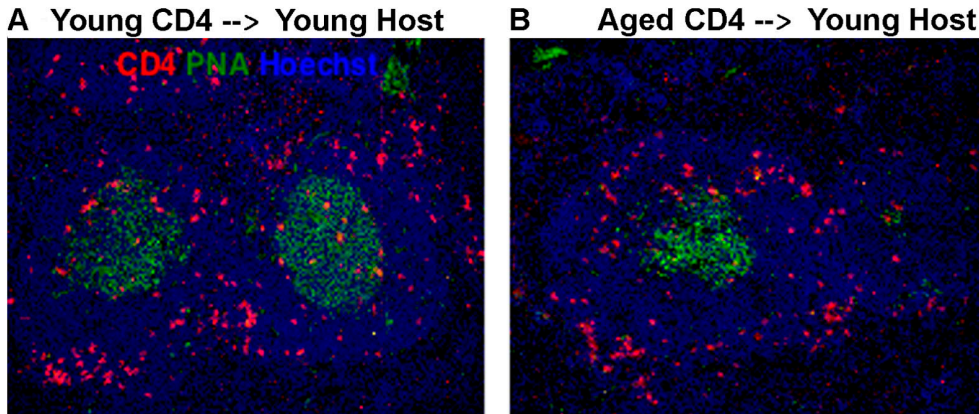
A Young CD4 --> Young Host

B   Aged CD4 --> Young Host

CD4 PNA Hoechst

Figure 4. Young and aged Tg CD4 T cells migrate similarly in young hosts after immunization. On day 14 after immunization with NP-PCC/alum and transfer of young and aged donor T cells into young CD4KO hosts, the migration of donor cells into follicles was examined by fluorescence microscopy. Frozen sections were cut from spleens of young hosts receiving (A) young or (B) aged donor Tg CD4 T cells and stained with anti-CD4 (red), PNA (green), and Hoechst (blue).

7, 14, and 21 after immunization, there were significantly higher levels of NP-specific IgG1 in the hosts receiving CD4 cells from young mice compared with those receiving cells from aged donors. Interestingly, there was a significant age-related difference in IgM production only on day 7, but not on days 14 or 21 (Fig. 3 G), which was not surprising as IgM production is less dependent on CD4 cognate help compared with IgG1 (33).

In Vivo Migration and Expansion of Donor Tg CD4 T Cells. Because CD4 T cells from aged donor mice exhibited reduced cognate helper function, the next set of experiments examined whether the in vivo migration and expansion of the aged donor T cells was defective. Fig. 4 shows photomicrographs of splenic sections from young hosts receiving young (Fig. 4 A) or aged (Fig. 4 B) donor T cells. Although the GCs observed in the sections from donors receiving young CD4 T cells were larger and more frequent compared with those receiving aged donor cells, we found no difference in migration of the donor T cell populations. Both young and aged CD4 T cells (Fig. 4, red) migrated similarly and could be found in the B cell follicle (blue) and in the GC (green), with no age–related differences. We also examined the expression of the chemokine receptor CXCR5 by these young and aged donor CD4 T cells by flow cytometry. As shown in Fig. 5 A, we found no difference in CXCR5 expression on the young and aged donor cells. This correlates well with the histology results shown in Fig. 4, since CXCR5 expression is required for appropriate T cell migration into GCs (23).

Because the CD4 T cells from aged mice seem to migrate appropriately, they must lack the ability to provide cognate help once they reach the B cell follicle. Given that CD28 and CD134 (OX40) have been implicated in the cognate functions of CD4 T cells (22, 34), we examined the expression of these molecules on young and aged Tg CD4 T cells after 4 d of culture with Ag/APC. We found no difference in the expression of either CD134 or CD28 on naive (unpublished data) or in vitro activated young and aged Tg CD4 T cells (Fig. 5, B and C). Another molecule that has been shown to play an important role in T–B cognate interactions is CD154 (CD40 ligand), which is expressed by activated helper T cells (33). We examined the
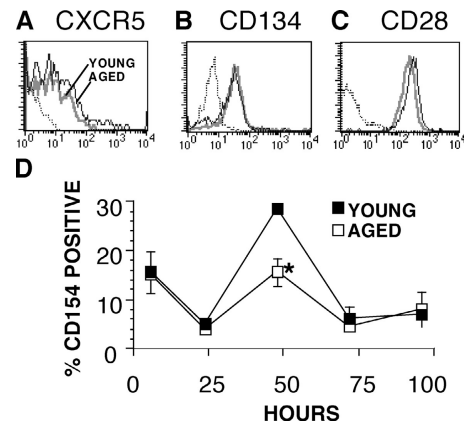

Figure 5. Phenotype of young and aged CD4 T cells. (A) Young (bold gray line) and aged (thin black line) donor T cells from the experiment in Fig. 4 (day 14 after immunization) were prepared for flow cytometry and stained for expression of CD4, Vβ3, and CXCR5. The flow cytometry dot plots are gated on CD4+Vβ3+ T cells and show the percent positive for CXCR5 expression. (B) Naive young and aged Tg CD4 T cells were stimulated with Ag/APC for 4 d. Effector populations were harvested and stained for CD4, Vβ3, and CD134 expression; histogram is gated on Vβ3+CD4+ cells. (C) 4 d young and aged Tg effectors were also stained for CD4, Vβ3, and CD28 expression; histogram is gated on Vβ3+CD4+ cells. (D) Young (filled squares) and aged (open squares) Tg CD4 T cells were stimulated in vitro with Ag/APC. At 6, 24, 48, 72, and 96 h, cultures were harvested, stained for CD4, Vβ3, and CD154 expression and analyzed by flow cytometry. T cell populations were gated on CD4+ Vβ3+ T cells and the percent CD154 positive in young and aged populations were determined. Graph shows the mean ± SE; *P < 0.05. Representative of three experiments.

expression of CD154 on young and aged Tg CD4 T cells after in vitro stimulation with Ag/APC. CD154 expression occurs in two phases, one very early after TCR stimulation (6 h) and one much later (40–60 h) (35). Fig. 5 D shows that both young and aged Tg CD4 T cells up-regulate CD154 similarly at the early time point, but the second phase of expression is significantly lower on aged cells. As we find that there is a significant reduction in CD154 expression on the aged cells, this could have a dramatic impact on cognate helper function and could account for the results shown in Fig. 3.

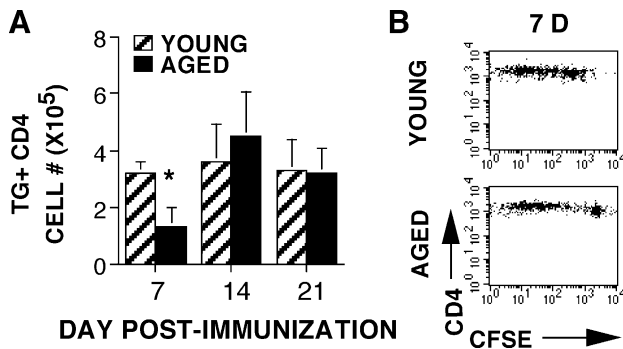Additionally, the in vivo expansion of these donor CD4 populations in young hosts was examined. We have shown

Figure 6. Recovery of young and aged donor T cells in young hosts. Young and aged Tg CD4 T cells were CFSE labeled and transferred to young CD4KO hosts that were then immunized with NP-PCC. (A) The number of young (striped bars) and aged (solid bars) donor T cells was determined by flow cytometry analysis on days 7, 14, and 21. Data shows mean ± SE. (B) Flow cytometry dot plots, gated on $V\beta3^+CD4^+$ donor cells, showing CFSE profiles of young and aged cells on days 7, 14, and 21 after immunization.
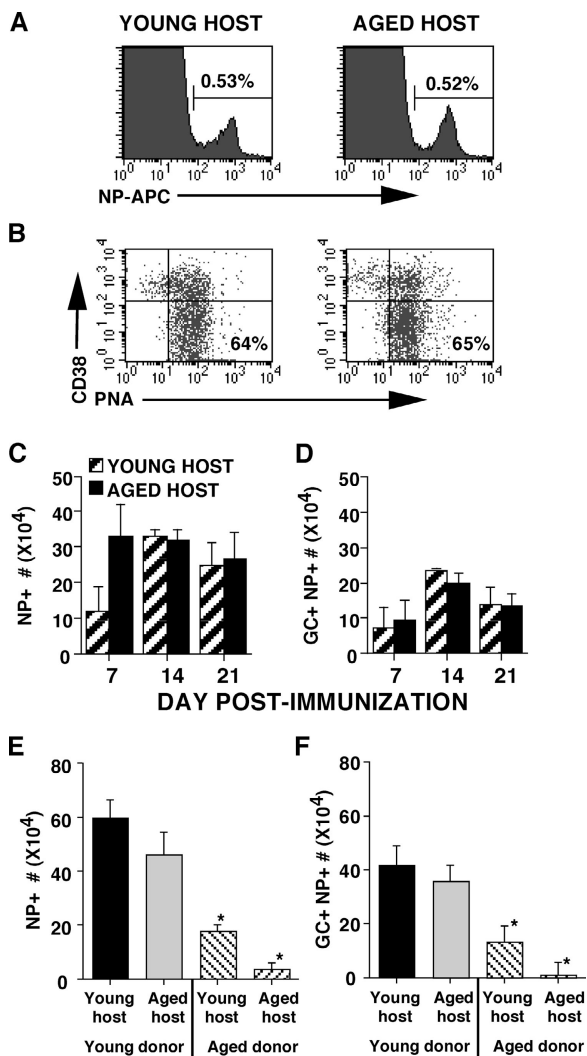

Figure 7. Cognate helper function of young donor T cells is similar in young and aged hosts. Young Tg CD4 T cells (10^6) were adoptively transferred i.v. to young or aged CD4KO hosts. Hosts were then immunized i.p. with 200 μg NP/PCC or PBS in alum. (A) On day 14, the percentage of NP$^+$ cells in young and aged hosts was determined by staining splenocytes with NP-APC. Representative flow cytometry histograms are shown. (B) Flow cytometry dot plots are gated on NP$^+$ cells from A. Percentages indicate GC phenotype cells (CD38^{low}PNA^{hi}) in young and aged hosts. (C) On days 7, 14, and 21 the numbers of NP-binding cells in young (striped bars) and aged (solid bars) hosts were determined by flow cytometry after staining with NP-APC. (D) On days 7, 14, and 21 the numbers of GC phenotype (CD38^{lo}PNA^{hi}) cells within the NP-binding population in young (striped) and aged (solid) hosts was determined by flow cytometry. (E) Young or aged donor Tg CD4 T cells (10^6) were transferred into young or aged CD4KO hosts, which were immunized with NP-PCC. On day 14, the number of NP-binding cells was determined by flow cytometry. (F) The number of GC phenotype NP-binding cells was also determined on day 14 by flow cytometry. For all, data shown is mean ± SE; *P < 0.05 when aged donors compared with young donors.

previously that the early (through day 6) in vivo expansion of aged CD4 T cells is reduced compared with young cells (17, 20). Fig. 6 A shows that on day 7 after immunization there was significantly greater expansion of donor cells from young mice compared with those from aged mice, but by days 14 and 21, expansion had reached a plateau and similar numbers of young and aged donor cells were recovered. These results could be due to the fact that the young donor effectors had either stopped proliferating or had begun to undergo apoptosis. In either case, this is likely due to host-related factors controlling the number of peripheral T cells. Because these CD4 T cells from young and aged donors were labeled with the fluorescent dye CFSE before transfer, we could also examine the extent of cell division after immunization. Fig. 6 B shows that on days 7, 14, and 21, cells from young donors (top panel) had undergone more rounds of cell division compared with those from aged donors (bottom panel). These results indicate that even though the total cell recoveries were the same on days 14 and 21, there may be other age-related differences in the in vivo–generated CD4 effector populations derived from young and aged donors.

Effect of Host Age on Humoral Responses. To determine if there were age-related defects in host components, including B cells and follicular dendritic cells (FDCs), we modified our adoptive transfer model such that TCR Tg CD4 T cells from young mice were transferred into young and aged hosts. In these studies, the adoptive hosts were 20 to 24 mo old. On day 14 after immunization, we found no difference in expansion (Fig. 7 A) or GC differentiation (Fig. 7 B) of the NP$^+$ populations in young and aged hosts. We also examined other time points and found no significant differences in either the number of NP-specific cells (Fig. 7 C) or the number of GC phenotype cells (Fig. 7 D) in young versus aged hosts. Finally, we compared the cognate function of young versus aged donors transferred into young versus aged hosts in the same experiment. We found
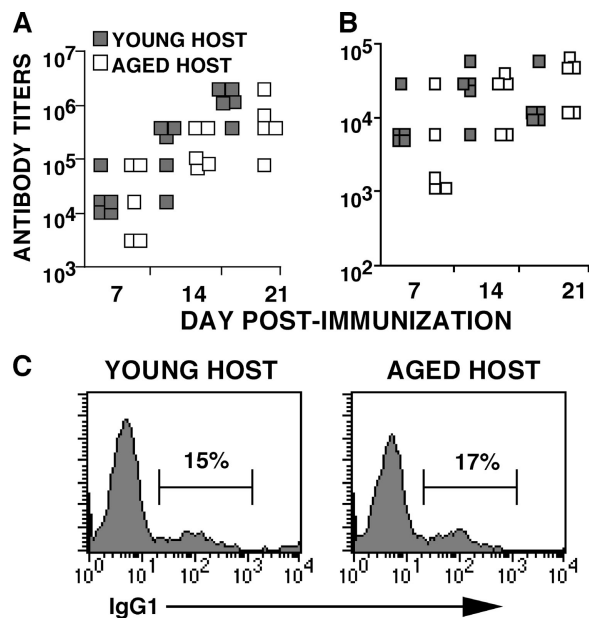
Figure 8. Antibody production in young and aged hosts. Young and aged CD4KO hosts were given young donor CD4 T cells and immunized as described in the previous figure. Serum was collected on days 7, 14, and 21 after immunization. (A) NP-specific IgG1 titers were determined by ELISA from serum of young (filled squares) and aged (open squares) hosts. (B) NP-specific IgM titers were determined by ELISA from serum of young (filled squares) and aged (open squares) hosts. (C) Splenocytes from young and aged hosts were stained for NP binding and surface IgG1 expression on day 14 after immunization. Representative flow cytometry histograms are gated on NP^+ splenocytes; percentages indicate the positive staining for IgG1.

no difference in the expansion of NP^+ B cells when young donor T cells were used, but there was significantly reduced expansion when aged donor cells were used, regardless of host age (Fig. 7 E). The same pattern was also seen for GC differentiation of the NP^+ B cells (Fig. 7 F). Although it does appear that there was a reduction in the number of NP^+ and GC^+ B cells when aged donor T cells were transferred into aged hosts, this difference was not significant. Importantly, this experiment shows that transferring a population of antigen-specific TCR Tg–aged donor cells into aged hosts does not overcome the defect that we observed in intact aged mice as shown in Fig. 1.

As it has been shown that aging negatively impacts B cell Ig class switching (36), we examined serum IgG pro-

duction and surface IgG expression on NP-specific cells from these young and aged hosts. Fig. 8 A shows that there was no difference in serum titers of NP-specific IgG1 in the young and aged hosts over a period of 3 wk after immunization. Additionally, we also found no difference in serum IgM titers (Fig. 8 B). To examine whether similar proportions of NP-specific cells in the young and aged hosts were undergoing class switching, we examined surface IgG1 expression by flow cytometry. Fig. 8 C shows representative histograms indicating that similar percentages of NP-specific cells from young and aged hosts stained positive for IgG1. Therefore, in this model, we found no age-related defects in the ability of NP-specific cells to undergo Ig class switching.

We also examined the expansion of young donor CD4 T cells in the young and aged hosts. Fig. 9 A shows that there was no difference in the number of the donor cells recovered in either the young or aged hosts at days 7, 14, or 21 after transfer and immunization. In addition, Fig. 9 B shows the CFSE profiles of the donor cells at each time point. There were no differences in these profiles, indicating that the young donor T cells responded similarly in the young and aged hosts, with no age-related differences in donor T cell priming. Taken together the results shown in Figs. 7, 8, and 9 indicate that the age of the host components have no effect on the response to immunization in our NP-PCC model and that age-related differences in CD4 T cell cognate function could account for the reduction in antibody production that we have observed in our model.

Discussion

Aging has severe effects on antibody production, which can impact vaccine efficacy in the elderly. In older individuals, the primary antibody response is very weak and short-lived with reduced affinity maturation and reduced memory B cell generation (1–5). These effects are thought to be due to reduced GC formation in the aged, because GCs are required for all of these events to occur (13). The goal of our study was to determine which lymphocyte populations are responsible for the observed age-related declines in antibody production. To accomplish this, we used an adoptive transfer model using CD4 T cells from TCR Tg mice. Our previous studies have shown that T cells from aged AND Tg mice express a naive phenotype, with respect to both cell surface
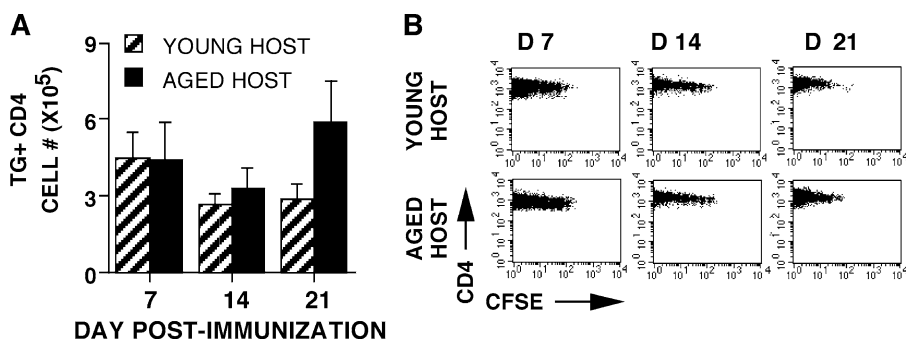

Figure 9. Recovery of young donor T cells in young and aged hosts. Young Tg CD4 T cells were CFSE labeled and transferred to young and aged CD4KO hosts that were then immunized with NP-PCC. (A) The number of young donor cells in young (striped bars) and aged (solid bars) hosts was determined by flow cytometry analysis on days 7, 14, and 21. (B) Flow cytometry dot plots, gated on $V\beta3^+CD4^+$ donor cells, showing CFSE profiles from young and aged hosts on days 7, 14, and 21 after immunization.

molecules (CD44^{lo}CD45RB^{hi}CD62L^{hi}CD25^{neg}) and in vitro function (16, 18, 37). This model eliminates the differences that are found in polyclonal CD4 T cell populations from young and aged mice and allows us to directly compare homogeneous populations of naive CD4 T cells of identical antigenic specificity, with the only difference being that they were obtained from young or aged Tg mice. Furthermore, this model involves the transfer of young and aged Tg CD4 T cell populations into CD4KO hosts, which lack CD4 T cells and, therefore, have no endogenous cognate helper activity. This allows us to specifically examine the helper function of the young and aged donor CD4 T cells.

The results presented in this study show that when we transfer Tg CD4 T cells from aged donors into young hosts, we observe significant reductions in antigen-specific B cell expansion and differentiation as well as reduced IgG production. These differences were evident at time points over a 3-wk period and over a wide range of donor cell concentrations. As we found that there was no difference in the migration of the young and aged donor cells, we surmised that the aged donor cells exhibited defects in cognate helper activity once they had trafficked to the B cell follicle. Therefore, we examined cell surface molecules that have been shown to be involved in CD4 T cell cognate function (21–23). Even though the aged donor cells expressed normal levels of CXCR5, CD134 and CD28, they did not up-regulate CD154 to the same extent as young donor cells. This could have a significant impact on their cognate helper activity and on GC formation in the young hosts, considering CD40-CD154 interactions are required for GC formation as well as antibody affinity maturation and class switching (33, 38). Interestingly, we have shown that these aged donor CD4 T cells exhibit reduced NFκB activation after TCR stimulation (20), which may negatively impact up-regulation of CD154 as this transcription factor is important for appropriate CD154 expression by T cells (39–41).

In this study, our model also shows that host components, such as B cells and FDCs, do not exhibit the same degree of age-related defects that we observe in aged CD4 T cells. When we transfer young donor CD4 T cells into aged CD4KO mice, we find no reduction in antigen-specific B cell expansion or differentiation or serum IgG production. This is in contrast to other reports that have demonstrated defects in both B cell populations and FDCs with increasing age. Whereas many age-related defects in B cell responses can be attributed to reduced cognate function of aged CD4 T cells, other defects have been hypothesized to be intrinsic to B cell populations. Most notably, an age-related decline in B-lineage precursors leading to reduced production of new mature B cells has been described. Interestingly, this defect does not result in a decline in the generation of new B cells or in the numbers of peripheral mature B cells (42, 43). Other studies have examined the antigen-specific responses of young versus aged B cells using splenic fragment cultures. Although the numbers of B cells responding to haptens such as NP was shown to decline with age, the amount of antibody produced per cell remained constant (44). We do not find differences in the number of NP-specific B cells in aged hosts in our model, but our studies are quite different from the splenic fragment culture experiments. Most notably, in the splenic fragment culture assay, antigen-specific B cells were enumerated by their ability to produce antibodies, whereas our assay involves only the ability to bind NP. Therefore, it is not surprising that we observed somewhat different results.

In addition, other studies, using in vitro systems, have shown defects in the ability of aged B cells to undergo Ig class switching, independent of aging effects on CD4 cognate helper function (36). We do not find this in our antigen-specific in vivo model, which is quite different from in vitro polyclonal stimulation of B cell populations. When we transfer young Tg CD4 T cells into young or aged hosts, we find no age-related differences in either serum IgG1 titers or in the proportion of NP-specific cells expressing surface IgG1 after immunization. It is possible that there are other undetected defects in affinity maturation or somatic mutation of the NP-specific B cells in the aged hosts. We are examining these parameters in ongoing experiments.

Our model is well suited for this sort of analysis because in both the young and aged CD4KO hosts there is no endogenous CD4 helper activity (Fig. 2). This allows us to compare predominantly naive B cell populations in both the young and aged hosts (something that is not possible in immunologically intact models, where memory B cells accumulate with increasing age; 45). One other possibility to account for our results is that B cells in the CD4KO model do not undergo age-associated changes that are observed in normal mice. This is probable because, unlike normal wild-type mice, B cells in unimmunized aged CD4KO mice do not show a decrease in follicular (CD21^{int} CD23^{int}) B cells or a decrease in B220 expression (unpublished data), both of which are associated with aging B cell populations (46).

One other cell type that has been implicated in age-related reductions in GC formation is the FDC. It has been shown, using in vitro models, that FDCs from older animals bind fewer antigen–antibody immune complexes, thus reducing the amount of cross-linked antigen available to B cells. This is thought to lead to age-related reductions in GC formation, affinity maturation, and the development of memory B cells (27, 47). Although these studies definitively show age-related defects in FDCs, it is not clear that they might have an impact on in vivo GC formation. Other researchers have shown that FDCs are not absolutely necessary for GC formation (48–50), and we see no evidence of decreased GC development in aged hosts using our model. It is probable that our immunization protocol, using NP conjugated to PCC at a 3 to 1 ratio along with alum precipitation, provides enough cross-linking to adequately stimulate NP-specific B cells, thus bypassing any age-related functional defects in FDC populations.

In summary, our results show that age-related reductions in humoral responses are likely the result of decreased CD4 T cell helper activity and that other cell types, such as B cells and FDCs, are less affected by age. In addition, we hypothesize that this defect is related to significantly reduced

CD154 expression by the aged CD4 T cells, leading to decreased B cell expansion, GC formation and IgG production. By defining specific age-related defects in specific lymphocyte populations, we can then target strategies for enhancing humoral responses. Ultimately, this will allow us to improve vaccine efficacy in the elderly.

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