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Ex Vivo Enzymatic Treatment of Aged CD4 T Cells Restores Cognate T Cell Helper Function and Enhances Antibody Production in Mice

Eric Perkey,* Richard A. Miller,†‡ and Gonzalo G. Garcia§

Previous in vitro studies showed that CD4 T cells from old mice have defects in TCR signaling, immune synapse formation, activation, and proliferation. We reported that removing a specific set of surface glycoproteins by ex vivo treatment with O-sialoglycoprotein endopeptidase (OSGE) can reverse many aspects of the age-related decline in CD4 T cell function. However, the specific mechanism by which this process occurs remains unclear, and it is unknown whether this enzymatic treatment can also restore important aspects of adaptive immunity in vivo. By using an in vivo model of the immune response based on adoptive transfer of CD4 T cells from pigeon cytochrome C–specific transgenic H-2(k/k) TCR-Vβ1,3,4 mice to syngeneic hosts, we demonstrate that aging diminishes CD28 costimulatory signals in CD4 T cells. These age-associated defects include changes in phosphorylation of AKT and expression of glucose transporter type I, inducible T cell costimulatory molecule, and CD40L, suggesting that the lack of CD28 costimulation contributes to age-dependent loss of CD4 function. All of these deficits can be reversed by ex vivo OSGE treatment. Blocking B7–CD28 interactions on T cells prevents OSGE-mediated restoration of T cell function, suggesting that changes in surface glycosylation, including CD40L, may be responsible for the age-related costimulation decline. Finally, we show that the age-related decline in CD4 cognate helper function for IgG production and long-term humoral immunity can also be restored by OSGE treatment of CD4 T cells prior to adoptive transfer.


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Abbreviations used in this article: CD4KO, CD4 knockout; Glut-1Hi, high Glut-1 expression; OSGE, O-sialoglycoprotein endopeptidase; pAKT(308), phosphorylation of AKT kinase at threonine 308; pAKT(308)T308, high phosphorylation of AKT kinase at threonine 308; PCC, pigeon cytochrome C.

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Address correspondence and reprint requests to Dr. Gonzalo G. Garcia, University of Michigan, Room 3005 BSRB, Box 2200, 109 Zina Pitcher Place, Ann Arbor, MI 48109-2200. E-mail address: gonzola@umich.edu

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*Department of Molecular, Cellular, and Developmental Biology, University of Michigan College of Literature, Science and the Arts, Ann Arbor, MI 48109;
†Department of Pathology, University of Michigan School of Medicine, Ann Arbor, MI 48109; and §University of Michigan Geriatrics Center, Ann Arbor, MI 48109

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n vitro T cell activation involves the physical interaction of the TCR-CD3 chains with costimulatory molecules, such as CD28 (1–4). These costimulatory molecules help to elicit a response leading to the formation of the TCR complex and reorganization of the cytoskeleton (5–7). The interaction between B7 and the CD28 molecule expressed on the surface of T cells enhances the PI3K–AKT signaling pathway, including phosphorylation of AKT kinase at threonine 308 [pAKT(308)] (8–10). Activation of PI3K–AKT also leads to increased expression of several proteins, including Glut-1 (11–15) and ICOS, which is involved in T cell–dendritic cell interactions (16) and follicular Th cell function (17, 18). Progression of T cell activation also leads to expression of CD40L, which interacts with CD40 on B cells to trigger the Ab class switch from IgM to IgG (19). T cells also express negative regulators of activation, such as PD-1 (16). The balance between interactions of these surface molecules and their targets on other immune cells determines the level of T cell response, thereby controlling the amount and diversity of the humoral response to an Ag (20).

In humans and mice, humoral immunity declines with age, resulting in limited IgG diversity and defects in long-term memory formation (21). It is likely that age-related defects in CD4 T cell function limit the production of a robust humoral response in elderly individuals (22–24). Using an adoptive-transfer model, Haynes and Eaton (23) found that CD4 cells from old donor mice did not proliferate well and had reduced CD40L expression. Furthermore, using CD4 T cells from transgenic AN0 AND mice, whose naive CD4 cells express Vβ3–TCR recognizing aa 88–103 of pigeon cytochrome C (PCC), we showed that age-related changes inhibit the early steps of T cell–APC interactions, including immune synapse formation and TCR signaling (25). We hypothesized that changes in glycosylation of T cell proteins (26) might contribute to these defects in T cell–APC interactions and decline in downstream signaling, including that of CD28 (27–29). To test the functional implications of altered protein glycosylation, we evaluated the effects of removing specific sets of surface glycoproteins by using a bacterial enzyme, O-sialoglycoprotein endopeptidase (OSGE). OSGE digests segments of extracellular proteins that contain O-linked glycans bearing terminal sialic acid residues, including CD43, CD44, and CD45, but it does not cleave the TCR chains and several costimulatory molecules (25). We found that OSGE treatment of CD4 T cells could reverse several age-related defects in T cell activation, including synapse formation, expression of CD25, cytokine production, and cytotoxic function (30–32). More recently, we used an in vivo model of T cell activation based on adoptively transferring naive CD4 T cells from young and old transgenic AND mice (Vβ3–TCRβ) to syngeneic B10.BR hosts primed with PCC. This system documented age-related declines in early activation and proliferation that could be reversed by ex vivo OSGE treatment (33). In this article, we report experiments designed to elucidate the mecha-
nism by which aging affects in vivo CD4 T cell cognate helper function and shed light on its restoration by OSGE treatment. We also show that OSGE exposure of CD4 T cells leads to increased helper function for production of Ag-specific IgGs and enhanced long-term immunity after adoptive transfer into host mice.

Materials and Methods

Animals and reagents

H-2(k/k) TCR-Vα14Vβ6 CD4+ mice (AND mice) and CD4 knockout (CD4KO) mice on the B10.BR background were bred in our facilities from stock generously provided by Susan Swain and Laura Haynes (Trudeau Institute, NY). Specific pathogen–free B10.BR and BALB/c × C57BL/6F1 (CB6F1) mice were purchased from Charles River Laboratories (Kingston, NJ) and from the National Institute of Aging contract colonies at Harlan (Indianapolis, IN), respectively. The mice were housed at the University of Michigan and were given free access to food and water. Sentinel animals were examined quarterly for serological evidence of viral infection; all tests were negative during the course of these studies. Mice found to have splenomegaly or macroscopically visible tumors at the time of sacrifice were not used for the experiments. AND mice were used at 6–8 or 16–18 mo of age, and the B10.BR or CD4KO adoptive host mice were 2–4 mo of age. CB6F1 mice were used in the study at 6–8 or 20–22 mo. Antigenic peptides and PCC were purchased from Sigma (http://www.sigmaaldrich.com), OSGE was obtained from Accurate Chemicals (http://www.accuratechemical.com). All flow cytometry Abs were purchased from BioLegend (http://www.biologend.com) or Becton Dickinson (http://www.bdbiosciences.com). Rabbit anti–Glut-1 was obtained from Epitomics (http://www.epitomics.com), and rabbit anti-pAKT(308) was obtained from Cell Signaling (http://www.cellsignal.com). CTLA4Ig was obtained from Pfizer Pharmaceutical (http://www.pfizer.com). Anti-IgG isotypes coupled to alkaline phosphatase were purchased from Jackson Immunoresearch (http://www.jacksonimmuno.com). DNP-BSA was obtained from Biosearch Technologies (http://www.biosearchtech.com), whereas PCC-DNP was produced in our laboratories by coupling PCC and DNP-e-Aminoacaproyl-Osu (PCC/DNP ratio 1:20). Naïve CD4 cells from the spleen and lymph nodes were obtained based on negative selection. CD4TCR purification kit II and CD26L positive selection, according to the manufacturer’s recommendations (http://www.milenyi.com). Analysis of a typical preparation showed the cells to be 90% positive for both CD3 and CD4.

In vitro T cell stimulation and detergent treatments

Naïve CD4 T cell stimulation and detergent treatments were performed as previously described (34). Briefly, CD4 T cells from young and old CB6F1 mice were purified by negative selection, and 10 × 106 cells were incubated with DNP-BSA in HBSS for 45 min at 4°C. Cells were washed, and TCR complex formation was initiated by cross-linking with 0.5 ml goat anti-rat IgG (5 µg/ml) for 5 min at 37°C. Cells were washed and resuspended in 0.5 ml PBS, 100-µl aliquots were incubated with 1 ml 1% Brij-96 for 15 min at room temperature. The resulting cell suspensions were analyzed by flow cytometry.

Priming, adoptive transfer, blocking of CD28 signaling with a CTLA4 construct, and analysis of in vivo CD4 activation

Naïve CD4 T cells from young and old AND mice were purified using the Miltenyi CD4 purification kit, based on CD4 and CD62L selection. Then, each cell preparation was divided into two aliquots: one was left untreated, and the other was treated with OSGE, as described elsewhere (25). The cells were resuspended in HBSS and injected into the tail vein of host mice. For the CD26L-blocking experiments, a CTLA4 Ig construct was used as previously described (35, 36), with modifications. In some experiments, as indicated, a single dose of 500 µg CTLA4 Ig was injected i.p. prior to adoptive transfer of CD4 T cells. In these experiments, another dose of 500 µg CTLA4 Ig was injected i.m. 24 h later, followed immediately by adoptive transfer of the CD4 T cells. Then, lymph nodes (pooled cervical, axillary, brachial, and inguinal nodes) and spleens were harvested from each recipient. CD4 T cells were purified by negative selection (37) and stained with CD4 (PECy5), TCR-Vβ3, (PEclone KJ25), ICOS (biotin clone 7E), PD-1 (biotin clone RPM1-30), CD69 (biotin clone H1.2F3), and CD60l (biotin clone MR1), followed by Pacific Blue or Alexa Fluor 700 streptavidin. Intracellular stains were performed as described (38) by permeabilization with 1% Triton in PBS, followed by staining for Glut-1 or pAKT(308) using FITC-coupled goat anti-rabbit secondary Ab. All analyses were performed using a Canto-II Flow Cytometer and FlowJo software, gating for CD4+ and TCR-Vβ3+ cells.

Immunization and Ag-specific IgG quantification by ELISA

Each experiment compared naïve CD4 T cells purified independently from two young and two old AND mice or from CB6F1 donor mice. Cells from each donor mouse were divided into two aliquots, of which one was treated with OSGE. Treated and control CD4 T cells from each donor mouse were then transferred to three individual host mice, as described below. Host mice were primed with a single 100-µl injection of an emulsion containing 200 or 100 µg DNP-PCC inIFA in the left flank of the leg. Fifteen and thirty days later, blood was collected from the tail vein, and reimmunization was performed on day 45 for each host mouse by injecting 100 µg DNP-PCC inIFA. Blood was collected from the tail vein at days 60 and 75 after the first immunization (i.e., 15 or 30 d after the booster injection). For each serum sample, the amount of each IgG isotype (IgG1, IgG3, IgG2a, and IgG2b) against DNP hapten or PCC was measured by ELISA using DNP-BSA and PCC (full protein) as targets.

Statistical analysis

Unless otherwise indicated, results are presented as mean ± SEM. Statistical significance was assessed using a Mann–Whitney U test, with the significance level set at p = 0.05.

Results

Aging reduces CD28 association with the TCR complex on CD4 T cells, but this defect can be reversed by OSGE treatment

We showed, using in vitro assays, that aging causes a significant decline in early TCR signals, cytoskeleton reorganization, and immune synapse formation (39, 40). We also showed that this decline can be restored by treating CD4 T cells ex vivo with OSGE, an enzyme that removes a specific group of surface glycoproteins (25). We hypothesized that age-related defects in TCR signaling could be the result of decreased association between the CD28 costimulatory molecule and the TCR complex and that OSGE treatments may restore CD28 association. During early CD4 activation, the TCR and costimulatory molecules form a complex that associates with the cytoskeleton and becomes resistant to lipid extraction by mild detergents, such as Brij-96 (41–46). We used resistance to Brij-96 solubilization to test whether aging reduced CD28 association to the TCR complex. Purified CD4 T cells were treated with OSGE, stained for CD3, CD4, and CD28 and stimulated via the TCR. CD4 cells were then extracted with Brij-96 and evaluated by flow cytometry to measure the association of CD28 and CD4 with the detergent-resistant TCR complex, as described in Materials and Methods. Fig. 1A shows the levels of CD28 expression in resting CD4 T cells from young mice and the removal of CD28 after detergent treatment; these results implied that CD28 was not associated with the cytoskeleton or TCR before CD4 stimulation. However, after TCR stimulation, the TCR and CD28 formed a complex, rendering CD28 resistant to detergent extraction (Fig. 1B, untreated cells). Treating the cells with OSGE prior to TCR stimulation led to higher association of CD28 with the complex (Fig. 1B, OSGE panel). The effect of OSGE treatment on detergent-resistant CD28 is shown in Fig. 1C. To quantify these effects, we performed a series of four independent experiments (Fig. 1D) using naïve CD4 T cells from young and old donors, normalizing the mean CD28 intensity for each group against that of the control young CD4 T cells (100%). We found no effect of stimulation, age, or OSGE treatment on the intensity of CD28, CD4, or CD3 for controls not exposed to detergent (data not shown), suggesting that neither OSGE nor aging affected Ab binding per se. OSGE treatment increased retention of CD28 in detergent-treated stimulated CD4 T cells from young donors, although this increase did not reach statistical significance (Fig. 1D). In the absence of OSGE exposure, detergent-treated, stimulated CD4 T cells from old donors had a lower mean CD28
fluorescence than did those from young donors (36 and 48%, respectively; $p = 0.009$). These results suggest that age reduces the association of CD28 with the TCR complex following T cell stimulation. OSGE treatment of CD4 T cells from old donors led to significant enhancement of CD28 association with the TCR complex (untreated old = 36%, OSGE-treated old = 76%; $p = 0.0003$). These results suggest that OSGE treatment might enhance CD28 signaling in CD4 T cells from old mice by increasing recruitment of CD28 to the TCR complex.

**Aging reduces CD28-dependent phosphorylation of AKT kinase and Glut-1 expression in vivo, and OSGE treatment restores both defects**

To test this hypothesis, we measured two CD28-dependent signaling events: pAKT$_{308}$ and expression of Glut-1 (8–10), using young CD4KO host mice that had received $2 \times 10^6$ naive CD4 T cells purified by positive selection, as described in the Materials and Methods section (24). CD62L positive selection does not appear to affect naive CD4 T cell activation, proliferation, or homing relative to CD4 T cells purified by negative selection (data not shown). We used CD4KO mice as hosts for the adoptive-transfer experiments, because these mice lack endogenous CD4 cells, which, in turn, facilitates the analysis of adoptively transferred CD4 T cells. In the absence of Ag (No PCC in Fig. 2A), gated CD4$^+$ TCR-Vβ$^+$ cells from young and old donors showed a bimodal distribution, with <15% of cells having high Glut-1 expression (Glut-1$^+$) and high phosphorylation of AKT at threonine 308 (pAKT$_{308}$). The vertical line in the scatter plots of Fig. 2 shows the threshold used to define cells as Glut-1$^+$ and pAKT$_{308}$. As expected, priming the adoptive hosts with PCC increased the expression of Glut-1 and pAKT$_{308}$ in CD4 T cells from young donors, with or without prior OSGE exposure (Fig. 2B, top panels). CD4 T cells from aged mice did not show an increase in pAKT$_{308}$ and Glut-1 expression unless they had been treated with OSGE prior to the transfer (Fig. 2B for comparison between OSGE treated and untreated). Fig. 2C shows the mean values for the percentage of cellular expression of Glut-1$^+$ and pAKT$_{308}$ in CD4 T cells isolated from lymph nodes and spleen, illustrating the ability of OSGE to restore CD28-dependent signaling to aged CD4 T cells. Similar effects were also observed in the ICOS-expression assays (Fig. 2C). We found significant age-related declines in the number of CD4 T cells scored as Glut-1$^+$ (2-fold in the lymph nodes, $p = 0.03$, and 4-fold in the spleen, $p = 0.016$). We found similar results in the case of pAKT$_{308}$ expression in CD4 T cells from young donors; however, OSGE treatment significantly increased both signals in CD4 T cells from old mice [lymph nodes: Glut-1, $p = 0.04$ and pAKT$_{308}$, $p = 0.02$; spleen: Glut-1, $p = 0.02$ and pAKT$_{308}$, $p = 0.03$]. These results suggest that in vivo CD28 signaling declines with age and that ex vivo OSGE treatment can restore this costimulatory pathway. It was suggested that de novo ICOS synthesis in CD4 T cells requires CD28 costimulation and that ICOS can play a critical role in humoral immunity (2, 20). We found that aging resulted in a 1–2-fold decline in the numbers of CD4 T cells expressing ICOS after adoptive transfer; however, these differences only reached statistical significance in the spleen ($p = 0.02$). ICOS expression was also restored by OSGE treatment in CD4 T cells from old mice (lymph nodes, $p = 0.05$; spleen, $p = 0.02$).

To test whether OSGE restoration of CD4 T function from old mice was CD28 dependent, B10.BR host mice were treated with a CTLA4Ig construct known to block the CD28/B7 interaction in vivo (35, 36). We treated B10.BR host mice with CTLA4Ig or vehicle prior to adoptive transfers, as described in Materials and Methods. Then, purified naive CD4 T cells from young and old AND mice were labeled with CFSE and treated with OSGE or vehicle, as previously described (25); and $2 \times 10^6$ CD4 cells were adoptively transferred to each B10.BR mouse that was treated or not with CTLA. Eighteen hours later, the lymph nodes and spleen were harvested and stained, as described in Materials and Methods and as shown in Fig. 2 (with modifications), with gating for CFSE$^+$, Vβ$^+$, and CD4$^+$ cells to discriminate between the adoptively transferred and endogenous CD4 T cells. Then, gated CD4 T cells were analyzed for Glut-1, pAKT$_{308}$, and ICOS expression, as well as for CD69 expression as described elsewhere (33). The scatter plots obtained for each stain were similar to those shown in Fig. 2A (data not shown). Fig. 3 shows the mean ± SEM from three experiments. As expected, and similar to the CD4KO model, we found significant age-related declines in the expression of Glut-1, ICOS, CD69, and pAKT$_{308}$, whereas OSGE treatment restored their expression. However, host mice treated with CTLA4Ig (Fig. 3, CTLA4Ig bars), without prior OSGE treatment, showed significantly inhibited signaling and activation of CD4 T cells from young donors, although little effect was noted with nontreated CD4 T cells from old donors. When CD4 T cells were treated with OSGE, we found that CTLA4Ig treatment significantly inhibited OSGE-mediated restoration of CD4 T cell activation in cells from old donors [indicated by significantly decreased Glut-1, pAKT$_{308}$, ICOS, and CD69 staining in OSGE-treated cells]. These findings suggest that the effects of OSGE on CD4 T cell function in old mice are driven by restoration of CD28 signaling.
Of note, CTLA4Ig treatment did not appear to deplete the populations of APCs, such as macrophages (data not shown, CD11b marker in spleen = 1.44% and lymph nodes = 0.54% versus CTLA4-treated spleen = 1.23% and lymph nodes = 0.41%), dendritic cells (CD11c in spleen = 2.3% and lymph nodes = 0.8% versus CTLA4-treated spleen = 2.03% and lymph nodes = 0.63%), or B cells (B220 in the spleen = 58% and lymph nodes = 36% versus CTLA4-treated spleen = 58% and lymph nodes = 32%).

Aging reduces CD40L expression, but not PD-1 expression, of CD4 T cells in vivo, and OSGE treatment can reverse this defect

To test whether OSGE treatments affected the expression of CD40L and PD-1, which are important factors in the regulation of humoral immunity and T cell expansion, respectively (47–49), we performed an adoptive-transfer experiment similar to those shown in Fig. 2 and evaluated CD4 T cells 5 d after cell transfer. As shown in Fig. 4A, CD4 T cells derived from old mice expressed very low levels of CD40L following adoptive transfer, suggesting defects in cognate helper function relative to CD4 T cells from young mice. However, as shown in Fig. 4B, PD-1 was expressed in CD4 T cells from both young and old donors. Quantification based on three independent experiments (a total of eight young and four old AND donors) shows a 2-fold age-related decrease in CD40L expression (Fig. 4A, upper panels; lymph nodes, \( p = 0.009 \); spleen, \( p = 0.009 \)) but no effect of aging on PD-1 expression. OSGE treatments enhanced CD40L expression in CD4 T cells from young mice, although this difference was only statistically significant in the lymph nodes (\( p = 0.02 \)). In contrast, OSGE treatment significantly enhanced CD40L expression in CD4 T cells from old mice in the lymph nodes (\( p = 0.01 \)) and spleen (\( p = 0.03 \)) but did not affect PD-1 expression.

OSGE can reverse the age-related decline in IgG isotype production and recall responses

To test whether adoptive transfer of OSGE-treated CD4 T cells could affect humoral immunity, we performed a pilot experiment that evaluated whether different numbers of OSGE-treated or untreated CD4 T cells from young and old AND mice affected Ab production in response to PCC protein in CD4KO host mice. The results from these experiments are shown in Supplemental Fig. 2. From these experiments, we concluded that transfer of \( \sim 2 \times 10^4 \) OSGE-treated CD4 T cells appeared to be optimal for enhancing the production of IgG1 and IgG2b in response to PCC protein. However, because the diversity of Ags present in the PCC protein can mask the effects of OSGE or aging, we tested whether OSGE treatment could improve T cell support for humoral immunity in response to a specific hapten by immunizing with DNP-PCC and evaluating the production of various isotypes of DNP-specific IgG.

We transferred \( 2 \times 10^4 \) untreated or OSGE-treated CD4 T cells from young and old AND mice into CD4KO host mice and analyzed the anti-DNP humoral response, as described in Materials and Methods. As expected (23), controls that received CD4 T cells but no immunization did not show detectable levels of anti-DNP IgG or anti-DNP IgM (data not shown). There were no effects of aging or...
OSGE on anti-DNP IgM production at 15 d (for anti-PCC response see Supplemental Fig. 2), consistent with the relative T cell independence of IgM. However, by 30 d, we found IgG1, IgG2b, and IgG3 anti-DNP Abs in all groups. Anti-DNP IgG2a and IgG2c were not detected. Fig. 5 shows a summary of the results, based on three independent experiments with a total of nine young and three old donor AND mice. In the absence of OSGE exposure, CD4 T cells from old donors stimulated 50% less total anti-DNP IgG than did those from the young donor group (*p = 0.03). Each of the individual anti-DNP IgG isotypes also showed a slight effect of donor age, although the differences did not reach statistical significance. Treating young CD4 T cells with OSGE led to a significant 3-fold increase in the production of total anti-DNP IgG. A similar pattern was observed for each isotype, with significant OSGE effects on IgG1 and IgG2b production. OSGE also improved the function of old CD4 T cells, producing a significant 4–5-fold increase in total anti-DNP IgG and in IgG1 and IgG2b production. OSGE-treated old CD4 T cells were at least as effective in supporting Ab production as were untreated CD4 T cells from young mice, demonstrating that OSGE treatment of CD4 T cells could restore the age-related decline of T cell support for the primary humoral response.

Because CD4 T cell memory formation and Ab recall also decline with age (50, 51), we reimmunized the mice with 200 μg of DNP-PCC and again measured total anti-DNP IgG and specific isotypes 60 d after the initial immunization (i.e., at 15 d after boosting). As expected, reimmunization enhanced the production of each IgG isotype relative to the primary response (note changes in y-axis between 30 and 60 d in Fig. 5). The effect of donor age on the performance of untreated T cells was significant at this 60-d time point for IgG2b, IgG3, and total IgG. OSGE treatment resulted in a statistically significant, ∼5-fold enhancement in total anti-DNP IgG production, with a similar pattern noted for each IgG isotype. There was no effect of donor age on T cell help after OSGE treatment. Thus, ex vivo OSGE treatments enhanced the T cell helper function for secondary humoral Ab responses and reversed the age-related decline in CD4 memory function.

CD4KO mice are lymphopenic, and their use as adoptive hosts may distort some features of the normal physiological immune response. Therefore, we performed a similar set of experiments using normal young B10.BR mice as hosts (Fig. 6A).
However, OSGE-treated CD4 T cells from young and old donors with respect to control host mice that had not received CD4 cells. Donor age, did not significantly alter anti-DNP IgG production expected, adoptive transfer of untreated CD4 T cells, regardless of specific transgenic mice. The results are shown in Fig. 6B. As would be higher than in the previous models based on TCR- T cells would initially be quite low, and TCR affinity and diversity CB6F1 recipients. In this model, the frequency of responding CD4 old CB6F1 (nontransgenic) donor mice were transferred to young experiment in which 2

control B10.BR host mice that had not received any transferred cells (‘C’ lanes of Fig. 6A) produced low levels of anti-DNP IgG in the primary response at 30 d and somewhat higher levels during the secondary response at 75 d. Transferring untreated young or old CD4 T cells did not lead to a statistically significant increase in the levels of primary or secondary anti-DNP IgG production with respect to the controls, suggesting that endogenous host CD4 T cells provide ample cognate cell function and that untreated CD4 cells have little additional effect after transfer. In contrast, OSGE-treated CD4 T cells significantly enhanced the production of total anti-DNP IgG 2–6-fold at 30 d, with a similar result for each isotype (with the exception of IgG3 in the primary response). These effects were independent of the age of CD4 T cells transferred. These results suggest that transferred OSGE-treated CD4 T cells provide enhanced cognate cell function, even in the presence of a simultaneous endogenous immune response.

To examine whether OSGE treatment could enhance humoral responses using a nontransgenic model, we performed a similar experiment in which 2 x 10^6 naive CD4 T cells from young and old CB6F1 (nontransgenic) donor mice were transferred to young CB6F1 recipients. In this model, the frequency of responding CD4 T cells would initially be quite low, and TCR affinity and diversity would be higher than in the previous models based on TCR-specific transgenic mice. The results are shown in Fig. 6B. As expected, adoptive transfer of untreated CD4 T cells, regardless of donor age, did not significantly alter anti-DNP IgG production with respect to control host mice that had not received CD4 cells. However, OSGE-treated CD4 T cells from young and old donors significantly increased all anti–DNP-IgG isotypes tested in both primary and secondary responses, typically by a factor of two to three relative to those of control mice.

**Discussion**

Loss of CD28 expression may be involved in the age-related decline in human T cell function (52, 53). In mice, aging does not reduce overall CD28 expression (data not shown); however, our data now suggest that age may block association of CD28 with the TCR complex, with a consequent decline in CD28 costimulation during the activation process. In addition, our new results suggest that the ability of OSGE treatment to enhance T cell responses may be due to correction of the age-related decrease in CD28 participation in T cell activation. The results shown in Figs. 1 and 3 support a model in which surface proteins (possibly CD45, CD44, and CD43) block CD28 translocation to the TCR complex and (by extension) to the immune synapse. OSGE can cleave a diverse array of surface glycoproteins bearing O-linked, sialylated glycan chains, and it is not yet clear which of these limit T cell activation; aging may block other costimulatory signals in addition to CD28 (i.e., CD4, CD45, LFA-1), contributing to defects in multiple signaling pathways (54–56).

Supporting the idea that restoration of CD28 function may contribute to the effects of OSGE on T cell activation, we found that age reduces, and OSGE restores, CD28-dependent signals, including pAKT(308) and Glut-1 expression. These data correspond
well to our previous in vitro results (25, 29), demonstrating that aging decreases the occurrence of early events dependent on CD28 signals, such as translocation and phosphorylation of Grb-2, Vav, and PLCγ, and that OSGE is capable of restoring these processes. In addition, the effects of OSGE on CD4 T cell function, when CD28/B7 interactions are blocked by CTLA4Ig treatment (Fig. 3), suggest that OSGE modulates CD28 signaling. However, these results do not exclude the possibility that the effects of age and OSGE on Glut-1 and pAKT(308) may reflect the outcome of multiple OSGE-sensitive costimulatory signals, perhaps including activities of CD4, LFA-1, and CD45 (54–56). Nevertheless, to our knowledge, our results are the first to suggest that lack of CD28 signaling is involved in the age-related decline in CD4 T cell function in vitro (Fig. 1) and in vivo (Figs. 2, 3). Interestingly, these results correlate with some of the early reports in humans in which defects in CD4 function were linked to a loss of CD28 expression (53).

The induced expression of surface receptors and ligands on the surface of CD4 T cells plays an important role in the activation of other immune cells, such as dendritic cells and B cells. Because aging can affect CD4 activation, it is not surprising that CD4 T cells from old donors fail to express ICOS (Fig. 2). The lack of ICOS is a novel and interesting result because of its involvement in dendritic cell activation. Mice lacking ICOS expression on T cells show defects in macrophage and dendritic cell opsonization of IgM-coated Ags and cytokine secretion (57, 58), both essential for induction of B cell function in humoral immunity (59). Our data suggest that the age-related decline in T cell ICOS expression could explain some of these defects in dendritic cell function and opsonization (59); therefore, it would be interesting to test whether OSGE-treated CD4 T cells can improve dendritic cell function in old mice.

Age-related defects in CD4 T cell cognate helper function contribute to poor B cell function and decreased humoral responses in the elderly. These defects have been attributed to decreased CD40L expression (51). Our data confirm an age-associated reduction in CD40L expression and show that ex vivo OSGE treatments can restore this aspect of CD4 T cell function. Previous reports (33, 50) and our data (Figs. 1–4) suggest that CD4 T cells from old mice do not proliferate as the result of defective activation and expression of proliferation-related surface proteins. We found that naive CD4 T cells from old mice do not proliferate (Supplemental Fig. 1). However, PD-1 expression data (Fig. 4) show that these cells do respond by increased expression of PD-1, suggesting that the pathways required for PD-1 expression may not be age sensitive. It is possible that PD-1 expression on CD4 T cells from old mice leads to impairments in proliferation and in differentiation to a memory phenotype. In this context, naive CD4 T cells from old mice are known to differentiate into defective memory cells (50); thus, it would be interesting to determine whether PD-1+ CD4 T cells from old mice develop into a defective memory population.

Our results also show that OSGE-treated cells provide helper function in three models of adoptive immunity: TCR-transgenic CD4 T cells transferred into either lymphopenic CD4KO mice or into intact B10. BR mice or nontransgenic T cells transferred into syngeneic CB6F1 hosts. Aging impairs function only in the first of these systems, and T cells from both young and old mice show substantial OSGE-dependent improvements in helper function for primary and secondary responses in all three transfer systems. Similar conclusions emerge when sera are evaluated for Abs to the PCC carrier protein (Supplemental Figs. 2, 3), instead of the DNP hapten featured in Figs. 5 and 6. Our results suggest that the age-related decline in CD4 T cell function and humoral responses can be restored by OSGE and that one mechanism for this restoration is the improvement of CD28 recruitment to the TCR and CD28 signaling. Furthermore, the ability of OSGE to improve helper function of CD4 T cells from young mice suggests that OSGE-sensitive targets, perhaps related to CD28 signals, may be limiting for some aspects of T cell function in mice of any age. In addition, our data do not rule out the idea that the improvement in humoral responses after OSGE treatment of CD4 T cells reflects enhanced activation, proliferation, and CD40L expression in CD4 T cells (Fig. 3, Supplemental Fig. 1), as well as enhanced cytokine production. In vitro, we previously showed augmented cytokine production, in vivo, as a consequence of OSGE treatment of CD4 T cells (31).

It is possible that the ability of OSGE to enhance support of CD4 T cells for humoral immune responses might someday prove useful in specific clinical settings, such as those in which T cells are activated or expanded in culture prior to reintroduction into patients (60) or in other situations in which a decline in the humoral immunity results from poor CD4 T cell responses (21, 22).

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Aging impairs CD4 T cell proliferation in adoptively transferred CD4 cells, while OSGE improves cell proliferation. Analysis of CFSE-labeled CD4 T cells from the same experiments shown in Figure 4. Untreated CD4 T cells from young (left graphs) or old (right graphs) AND mice were adoptively transferred to CD4KO hosts. The dot plot shows that PCC priming increased the proliferation of gated CD4VB3+ cells from young, but not from old, donor mice (arrow in dot plot indicates undivided CD4 T cells from old mice). The middle panel shows the overlapping histogram of cell proliferation in untreated (light lines) and OSGE-treated (dark lines) CD4 T cells from young and old mice, suggesting that OSGE can restore cell proliferation in CD4 T cells from old mice. The bottom bar graphics shows the mean percentage (± SEM) of non-dividing CD4 T cells in untreated and OSGE-treated cells, in addition to unprimed host controls (C), from 8 young and 4 old AND donor mice. The asterisk (*) indicates statistical significance with respect to untreated young CD4 T cells (open bars).
**Supplemental Figure 2.** A) The effect of transfer of different numbers of untreated or OSGE-treated CD4 T cells from young AND mice on the anti-PCC IgG isotype response in CD4KO host mice, analyzed at 15 days after transfer. The asterisk (*) indicates statistically significant increases the IgG response when 4x10⁴ untreated CD4 T cells were transferred, while the (^) represents statistically significant increases when CD4 T cells were treated with OSGE. B) Analysis of the anti-PCC-specific IgG isotype response in CD40KO hosts at day 30 after adoptive transfer of CD4 T cells from young and old AND mice as described in Figure 5. C) Analysis of the anti-PCC-specific IgG isotype secondary response in CD40KO hosts at 60 and 75 days after re-immunization with PCC as described in Figure 5. The asterisk (*) indicates statistically significant differences in the CD4 T cells response from young OSGE untreated CD4 T cells.
**Supplemental Figure 3.** Analysis of the anti-PCC-specific IgG isotype response in B10.BR (panel A) and CB6F1 background (panel B) hosts after adoptive transfer of untreated (-) or OSGE-treated (+) naive CD4 T cells from young (Y) or old (O) AND mice. Blood was collected 30 and 75 days later after re-immunization as described in Figure 5. Then the anti-DNP-specific total IgG and IgG1, IgG2, and IgG3 isotypes were measured in the serum. Each bar represents the mean concentration (± SEM) of each anti-DNP IgG from 8 young or 3 old AND donor mice. The asterisk (*) indicates statistical significance with respect to untreated (-) young CD4 cells T (open bars) for each group.