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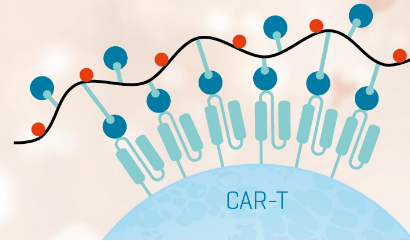

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CD1d-Restricted NKT Cells Contribute to the Age-Associated Decline of T Cell Immunity[1]

Douglas E. Faunce,[2*‡] Jessica L. Palmer,* Kelly K. Paskowicz,* Pamela L. Witte,[†‡] and Elizabeth J. Kovacs*[†‡]

NKT cells are known to regulate effector T cell immunity during tolerance, autoimmunity, and antitumor immunity. Whether age-related changes in NKT cell number or function occur remains unclear. Here, we investigated whether young vs aged (3 vs 22 mo old) mice had different numbers of CD1d-restricted NKT cells and whether activation of NKT cells by CD1d in vivo contributed to age-related suppression of T cell immunity. Flow cytometric analyses of spleen and LN cells revealed a 2- to 3-fold increase in the number of CD1d tetramer-positive NKT cells in aged mice. To determine whether NKT cells from aged mice differentially regulated T cell immunity, we first examined whether depletion of NK/NKT cells affected the proliferative capacity of splenic T cells. Compared with those from young mice, intact T cell preparations from aged mice had impaired proliferative responses whereas NK/NKT-depleted preparations did not. To examine the specific contribution of NKT cells to age-related T cell dysfunction, Ag-specific delayed-type hypersensitivity and T cell proliferation were examined in young vs aged mice given anti-CD1d mAb systemically. Compared with young mice, aged mice given control IgG exhibited impaired Ag-specific delayed-type hypersensitivity and T cell proliferation, which could be significantly prevented by systemic anti-CD1d mAb treatment. The age-related impairments in T cell immunity correlated with an increase in the production of the immunosuppressive cytokine IL-10 by splenocytes that was likewise prevented by anti-CD1d mAb treatment. Together, our results suggest that CD1d activation of NKT cells contributes to suppression of effector T cell immunity in aged mice. ***The Journal of Immunology,*** **2005, 175: 3102–3109.**

The aging immune system experiences a gradual decline in its ability to protect the host from pathogens and tumors. Although the mechanisms that contribute to the age-related decline in immunity are not entirely known, studies conducted over the past two decades have revealed that many aspects of immunity change with age (1–5). Research on aged humans and mice shows phenotypic and functional alterations in both the cellular and humoral arms of the immune response (2–4); however, T cell immunity is the best-studied component of the aging immune system. Earlier reports on T cells from aged subjects led researchers to believe that age-related immune dysfunction resulted solely from a systematic decline in the number and function of naive T cells, due in part, to thymic involution (6) and a decreased capacity of T cells to produce IL-2 (7, 8). However, more recent studies reveal that decreased T cell function in the aged is a multifaceted problem, resulting from not only changes in IL-2 production and responsiveness, but also from shifts in the ratios of naive vs memory T cell populations (9), greater proportions of $CD8^+$ vs $CD4^+$ T cells (10), alterations in cytokine production (11), and changes in TCR signal transduction and activation (12–14).

During recent years, it has become evident that both afferent and efferent aspects of T cell immunity can be regulated by subsets of innate lymphocytes such as $\gamma\delta$ T cells, Treg cells, and in particular, CD1d-restricted NKT cells. Whereas considerable attention is now being focused on the effects of aging on the functional capacity of innate immune cells including dendritic cells, macrophages, and granulocytes, the effects of age on CD1d-restricted NKT cell function remains have not been widely studied. Isolated reports suggest that as age increases, so does the number of cells within the lymphoid compartment that coexpress both NK and T cell surface markers (15–17). However, whether or not these cells are invariant CD1d-restricted NKT cells or whether they contribute to age-associated decline in peripheral T cell immunity is not known. An age-related increase in the number of liver T cells that coexpress NK markers has also been observed in mice (18, 19), but their contribution to immunological changes in aging are not clearly identified. Here, we examined specifically whether the number of CD1d-restricted NKT cells increased with age and whether or not they regulate Ag-specific immunity both in vitro and in vivo. Together, our findings demonstrate that not only does the number of NKT cells increase with age, but that NKT cells stimulated by CD1d in vivo actively suppress the effector phase of T cell immunity in aged mice.

Materials and Methods

Animals

Three-month-old (young) female BALB/c mice used in these studies were obtained from Harlan. Female BALB/c mice (20–24 mo old) were obtained from the National Institutes of Health/National Institute on Aging aged rodent colony at Harlan. All animals were housed on a 12-h light/dark cycle and provided with food and water ad libidum. All mice were treated humanely and in accordance with guidelines set forth by the Loyola University Institutional Animal Care and Use Committee and the National Institutes of Health. After euthanasia, all mice in these studies were

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screened for visible tumors and those found to contain any tumors (i.e., ~5–8%) were omitted from the study.

Abs and immunostaining reagents

Abs used for flow cytometry included CyChrome-conjugated anti-TCR β-chain mAb, FITC-conjugated anti-CD3ϵ mAb, PE-conjugated anti-Ly49C/I mAb (clone 5E6), and purified anti-CD16/CD32 mAb (FcBlock; clone 2.4G2) all obtained from BD Pharmingen, and whole rat IgG (Sigma-Aldrich). Allophycocyanin-CD3ϵ was obtained from eBioscience. Abs used in vivo and in cell culture were obtained from eBioscience and included purified (azide free, low endotoxin) rat anti-mouse CD1d mAb (clone 1B1), hamster anti-mouse CD3ϵ (clone 145-2c11), and rat IgG2b. Anti-CD1d mAb (and control) used in vivo was given i.v. via the tail vein at a dose of 50 μg/mouse in 100 μl of sterile saline. CD1d tetramer conjugated to Alexa-488-labeled protein A was provided by Dr. Jenny Gumperz (Dana-Farber Cancer Center, Harvard Medical School, Boston, MA). α-Galactosylceramide (α-GalCer,[3] KRN7000) was provided by Kirin Brewery Pharmaceuticals Division.

Flow cytometric analyses of NKT cells

Flow cytometric analysis of NKT cells was done as previously described (20–22). Briefly, spleens were minced and passed through fine wire mesh, and all debris was removed. Erythrocytes were lysed by ammonium chloride, and the cells were resuspended in RPMI 1640 containing 10% FCS, penicillin-streptomycin, and glutamine. Cell viability was confirmed to be >95% by trypan blue exclusion. Splenocytes were then resuspended in staining buffer (PBS containing 1% BSA and 0.1% sodium azide), and nonspecific staining was blocked with anti-CD16/CD32 (FcBlock) and whole rat IgG. After blocking, cells were then incubated with CyChrome-conjugated anti-TCR β-chain, FITC-conjugated anti-CD3ϵ, and PE-conjugated anti-Ly49C/I (clone 5E6), washed twice in staining buffer, and fixed in 4% paraformaldehyde. For labeling of NKT cells with CD1d tetramers, the tetramers were loaded with the NKT cell-specific ligand, α-GalCer by incubation in a 40 molar excess of α-GalCer in PBS at 37°C overnight. Cells were then immunostained with tetramers as previously described (23) in conjunction with allophycocyanin-CD3ϵ and PE-Ly49C. To control for nonspecific binding of the protein A complexed tetramer to the CD3 and Ly49C Abs, we immunostained cells with anti-CD3ϵ and anti-Ly49C mAbs in conjunction with unloaded CD1d tetramer. Flow cytometric determinations were made using a Becton Dickinson FACSCalibur flow cytometer and CellQuest Pro software.

Depletion of cells by magnetic beads

NK/NKT cells were depleted from splenocyte suspension using magnetic beads. Briefly, erythrocyte-free splenocyte suspensions were prepared from young vs aged BALB/c mice and immunostained with PE-conjugated anti-Ly49C mAb. After thorough washing, the immunostained cells were incubated with anti-PE magnetic microbeads (Miltenyi Biotec) and the LY49C$^+$ cells were removed by passage through MiniMacs MS$^+$ magnetic separation columns (Miltenyi Biotec). The degree of depletion was confirmed by flow cytometry for assessment of remaining PE-positive cells.

Delayed-type hypersensitivity (DTH) and in vivo blockade of CD1d-NKT cell signaling

DTH was induced as previously described (21, 22). Briefly, mice were inoculated subcutaneously at the nape of the neck with 100 μl of an emulsion containing 100 μg of OVA (Sigma-Aldrich) in CFA (Sigma-Aldrich). Seven days later, mice had both ears measured with an engineer's micrometer (Mitutoyo) and were given an intradermal inoculation of 10 μl of PBS containing 200 μg of OVA to the left ear pinna. Some animals received a similar inoculation of human serum albumin (HSA) (Sigma-Aldrich) to the right ear pinna as an Ag specificity control. Twenty-four hours later, ear measurements were made again, and the change in ear swelling was calculated as an index of DTH. All OVA and HSA solutions used for ear challenge were removed of potentially contaminating endotoxin by passage through DetoxiGel polymyxin-B chromatography columns (Pierce-Endogen). For blockade of CD1d activation of NKT cells in vivo, mice were given 50 μg of anti-CD1d mAb i.v. 24 h before ear challenge with OVA.

Lymphocyte proliferation assays

Splenocyte and lymph node cell suspensions were prepared as described above and plated at 2.0×10^5 cells per well in 96-well plates in either RPMI 1640 alone or RPMI 1640 containing OVA (200 μg/ml) (Sigma-Aldrich) or purified plate-bound anti-CD3ϵ mAb (2.5 μg/ml) and cultured at 37°C, 5% CO_2, for 48 h. After 48 h of culture, 1 μCi of [^{3}H]thymidine (Amersham Biosciences) was added to each well, and the cells were cultured for an additional 16–18 h. [^{3}H]thymidine incorporation was assessed by scintillation counting and used as an index of OVA-specific splenocyte proliferation.

Results

The effects of age on the number of CD1d-restricted NKT cells and the expression of cell surface CD1d on professional APCs

Studies of both human and mouse lymphocyte populations have suggested that the frequency of TCR$^+$NK$^+$ lymphocytes increases with age (15, 16). Whether these observations represent an age-related expansion of invariant CD1d-restricted NKT cells remains to be defined. Studies by one laboratory indicated that the age-related increase in TCR$^+$NK$^+$ cells reflects an increase in the NKT cell population (16), whereas additional studies suggest it reflects an age-associated accumulation of senescent conventional T cells that have acquired a memory phenotype (15). Here, we sought to clarify whether quantitative changes in the CD1d-restricted NKT cell population occur with age using immunostaining with both conventional cell surface markers and fluorescent CD1d tetramers. Briefly, single-cell suspensions of splenocytes and lymph node cells from young vs aged (3 vs 22 mo old) female BALB/c mice were prepared as described previously (21) and immunostained with Abs against CD3ϵ, TCRβ, and the NK cell surface marker Ly49C, and analyzed by flow cytometry. Initially, NKT cells were identified as cells within the CD3ϵ^+ population that coexpressed TCRβ and Ly49C (Fig. 1*A*, upper right quadrants of dot plots). In our studies, we observed an age-associated 3- to 4-fold increase in the frequency of splenic CD3ϵ^+ cells that coexpressed the NK cell surface marker, Ly49C (Fig. 1*B*). Similarly, we observed a 2-fold increase in the frequency CD3ϵ^+TCRβ^+ Ly49C$^+$ cells in lymph node cell suspensions from aged mice, compared with young (Fig. 1, *A* and *B*). The increased frequency of apparent NKT cells in aged mice occurred in the absence of significant quantitative changes in splenic CD3, CD4, and B220-positive cell subpopulations and only a minor decrease in lymph node CD4$^+$ T cells and modest increase in lymph node B220$^+$ cells (not shown). Moreover, we observed that the increased frequency of NKT cells also represented an increase in the absolute numbers of NKT cells in aged mice (Fig. 1*C*) and an increase in the ratio of NKT cells to conventional (CD3ϵ^+Ly49C$^-$) T cells (Fig. 1*D*).

Because Ly49 molecules are reported to be expressed by a subset of memory CD8$^+$ T cells (24), we sought to define whether or not the age-related increase in CD3$^+$TCRβ^+Ly49C$^+$ lymphocytes represented an increase in the CD1d-restricted, Vα14 NKT cell population. To unequivocally identify CD1d-restricted NKT cells, splenocytes and lymph node cells were immunostained with Abs against CD3ϵ and Ly49C in conjunction with murine CD1d tetramers that were loaded with the CD1d/Vα14 TCR-specific ligand, α-GalCer as previously described (23). In agreement with our findings that used conventional cell surface staining, we observed a 3-fold increase in the frequency of CD3ϵ^+Ly49C$^+$ cells that concomitantly bound α-GalCer-loaded CD1d tetramers in the spleens of aged mice, compared with young (Fig. 2). Binding of the tetramer to the NKT cells was ligand-specific, because non-loaded tetramers did not bind CD3ϵ^+Ly49C$^+$ stained cells (not shown). Together, our findings demonstrate that the CD1d-restricted, Vα14 TCR$^+$ NKT cell population increases with age.

[3] Abbreviations used in this paper: α-GalCer, α-galactosylceramide; DTH, delayed-type hypersensitivity; HSA, human serum albumin.

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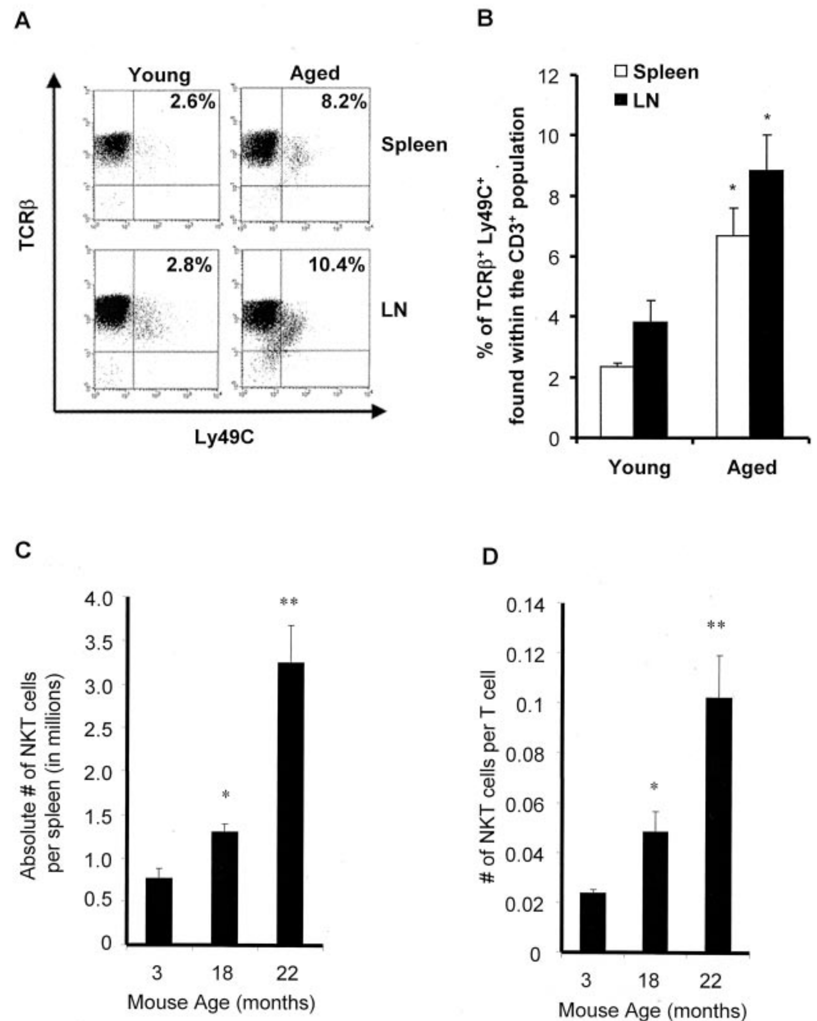

FIGURE 1. Comparison of NKT cell frequency in young vs aged BALB/c mice. *A*, Splenocytes and lymph node (LN) cells were obtained from naive mice and immunostained with anti-CD3e, anti-TCR β-chain, and anti-Ly49C/I (clone 5E6). $CD3^{pos}$ cells that coexpressed TCRβ and Ly49C/I at intermediate densities were identified by flow cytometry and considered to be NKT cells. *B*, A summary of flow cytometric evaluation of NKT cell frequency within the $CD3^{pos}$ population from spleens vs lymph nodes of young vs aged mice. $n = 4$ mice per group for young and $n = 5$ for aged. Data are expressed as mean percentage of NKT cells within the $CD3^{pos}$ population ± SEM. $*$, $p < 0.05$ vs young as determined by a *t* test. *C*, Absolute number of NKT cells per spleen in 3-, 18-, and 22-mo-old mice is shown as number of NKT cells per spleen in millions ± SEM as determined by flow cytometry. *D*, Data shown represent the number of NKT cells per conventional ($CD3^{pos}Ly49C^{neg}$) T cell ± SEM as determined by flow cytometry. Data shown are representative of three experiments in which similar results were obtained. $*$, $p < 0.05$ vs 3 mo, $**$, $p < 0.05$ vs 18 mo.

Because CD1d expression on professional APCs such as macrophages and dendritic cells is a requisite for the full activation of NKT cells to perform their effector functions, we examined whether advanced age was associated with changes in CD1d expression. Total splenocytes were obtained from young vs aged mice and immunostained for CD1d cell surface expression in addition to either CD11c or F4/80 to identify dendritic cells and monocytes/macrophages, respectively. We observed that neither the number of CD1d-positive APCs or the relative magnitude of CD1d cell surface expression changed with age (Fig. 3 and Table I).

Depletion of NK/NKT cells from unfractionated splenocytes prevents the age-related defect in T cell proliferative response to polyclonal stimulation

Studies by a number of laboratories support the concept that T cell responsiveness is suppressed in aged mice and humans, compared with young (6–13). Because NKT cells are known to modulate T cell function, we next investigated whether their removal from whole splenocyte populations from aged mice altered T cell responsiveness in vitro. Unfractionated splenocyte suspensions were prepared from young vs aged mice and immunostained with PE-conjugated anti-Ly49C mAb. Using anti-PE magnetic microbeads, the Ly49C-positive (NK and NKT) cells were depleted from the splenocyte suspensions (Fig. 4*A*) and the proliferative response of splenic T cells to anti-CD3ϵ polyclonal stimulus was compared between Ly49C-depleted vs intact splenocyte suspensions. In response to immobilized anti-CD3ϵ, splenic T cells from aged mice demonstrated only one-third the proliferative capacity that T cells from young mice did (Fig. 4*B*). However, removal of the NK/NKT cell population resulted in a T cell proliferative response in cells from aged mice that was statistically insignificant from that seen in young (Fig. 4*B*). However, it should be noted that our depletion technique removed both NK and NKT cells due to their shared expression of Ly49C. Thus, it cannot be ruled out that the effect of $Ly49C^+$ cell depletion resulted solely from NKT cell removal, but instead from the removal of both NK and NKT cells.

Treatment of splenocyte cultures with anti-CD1d mAb prevents the age-associated impairment of in vitro Ag-specific T cell proliferation

Because the approach used in the experiments described above removed both NK and NKT cells from the splenocyte preparations, we could not conclude that the age-associated impairment in T cell proliferation was mediated by the NKT cell population exclusively. Because the majority of NKT cells are restricted in their activation by CD1d found on the surface of professional APCs, we next examined the proliferative capacity of Ag-specific T cells from young vs aged mice in cultures in which the ability of the NKT cells' invariant TCR to engage CD1d molecules was blocked by an anti-CD1d mAb. The ability of anti-CD1d to block NKT cell function in vitro was previously shown (25). Briefly, splenocytes were obtained from young vs aged mice that had been immunized

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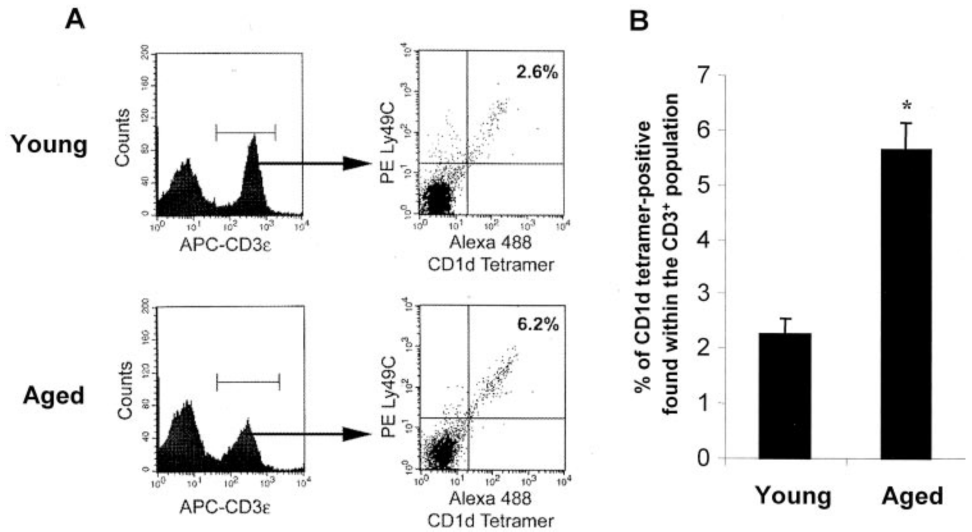

FIGURE 2. Comparison of CD1d-restricted NKT cell frequency in young vs aged BALB/c mice. *A*, Splenocytes were obtained from naive young vs aged mice and immunostained with allophycocyanin-conjugated anti-CD3ε, PE-conjugated anti-Ly49C (clone 5E6) and CD1d tetramer-conjugated to Alexa 488-conjugated protein A. CD1d-restricted NKT cells were identified as CD3ε$^+$ cells that coexpressed CD1d tetramers and Ly49C. *B*, A summary of CD1d tetramer staining in spleens from young vs aged mice (n = 4 mice per group). Data shown are representative of two experiments in which similar results were obtained. Data are expressed as mean percentage of CD1d-restricted NKT cells within the CD3ε$^+$ population ± SEM. *, $p < 0.05$ vs young as determined by a t test.

with OVA in CFA 1 week earlier. Splenocytes were placed in culture for 48 h with or without soluble OVA. [^{3}H]Thymidine was added for an additional 18 h and the proliferative capacity of T cells was determined by scintillation counting. Compared with T cells from young mice, we observed a 50% reduction in the proliferative capacity of T cells from aged mice in response to in vitro rechallenge with OVA (Fig. 5). However, OVA-specific T cell proliferation was not impaired in splenocyte cultures from aged mice that included anti-CD1d mAb (Fig. 5). Because reactivity to CD1d is limited to the NKT cell population, these results suggested that in splenocyte preparations from aged mice, NKT cells suppressed T cell proliferative capacity, a finding that appears consistent with the NK/NKT cell depletion studies shown in Fig. 4.

Systemic administration of anti-CD1d mAb prevents age-associated defects in Ag-specific T cell immunity

To address the contribution of NKT cells specifically to age-associated defects in T cell immunity in vivo, we next asked whether CD1d activation of NKT cells contributed to age-related dysfunction of Ag-specific T cell immunity in vivo. Because generation of sufficient numbers of 22-mo-old NKT cell-deficient mice was not feasible for our studies, we chose instead to block the CD1d activation of NKT cells in vivo by systemic administration of anti-CD1d mAb. Previous studies by our laboratory and others have shown that systemic administration of CD1d mAb effectively blocks NKT cell regulatory function, cytokine production, and chemokine production both in vivo and in vitro, without depletion of CD1d$^+$ APCs (21, 22, 26). This approach, instead, serves only to prevent engagement of the invariant Vα14 TCR by CD1d on the surface of APCs. Briefly, parallel groups of young and aged mice were immunized subcutaneously at the nape of the neck with OVA in CFA. Seven days later, mice were given 50 μg of either anti-CD1d mAb or rat IgG2b isotype control systemically (i.v., 100 μl total injection per mouse). Twenty-four hours later, mice were given an intradermal ear challenge with either OVA (200 μg in 10 μl HBSS) or HSA as an irrelevant Ag specificity control. Changes in ear thickness were determined with a digital micrometer 24 h later and used as an index of Ag-specific DTH.

Twenty-four hours after ear challenge with OVA, young mice given systemic control IgG had robust OVA-specific DTH responses (Fig. 6). In contrast, aged mice given control IgG had DTH responses that were only one-third to one-half the magnitude seen in young mice. Systemic administration of anti-CD1d mAb in aged mice yielded OVA-specific DTH responses that were comparable to those seen in young mice (Fig. 6).

As an additional measure of the effects of CD1d-restricted NKT cells on T cell responsiveness in aged mice, lymph node and spleen cells from young vs aged OVA-immunized mice were examined for their ability to proliferate in response to re-challenge with OVA in vitro. In agreement with the DTH findings mentioned above, we observed that both lymph node (Fig. 7*A*) and splenic (Fig. 7*B*) T

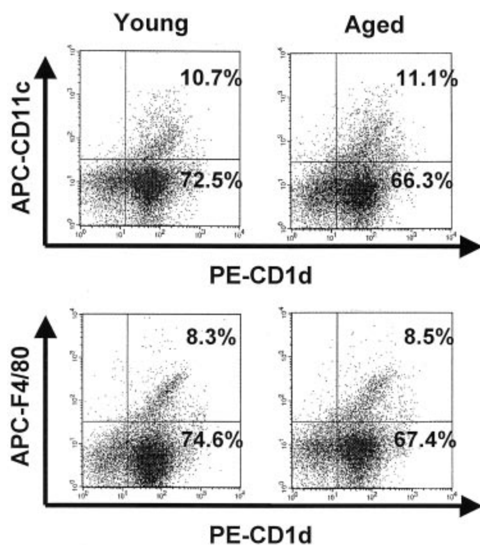

FIGURE 3. Comparison of CD1d expression on APCs from young vs aged BALB/c mice. Splenocytes were collected from young vs aged mice and immunostained with PE-conjugated anti-CD1d and either allophycocyanin-conjugated CD11c or F4/80 mAbs. Expression of CD1d on CD11c^{pos} and F4/80^{pos} cells was determined by flow cytometry. The frequency of CD1d^{pos}CD11c^{pos} and CD1d^{pos}F4/80^{pos} cells was compared between groups. A summary of the data is provided in Table I.

Table I. *Comparison of the frequencies of CD1d$^+$ APCs and the relative magnitude of CD1d cell surface expression in young vs aged mice*

	Frequency (Percentage of Total Spleen Cells)	Mean Fluorescence Intensity of CD1d
Young CD11c$^+$CD1d$^+$	8.7 ± 0.2	143.8 ± 3.8
Aged CD11c$^+$CD1d$^+$	10.1 ± 1.3	136.4 ± 4.3
Young F4/80$^+$CD1d$^+$	9.7 ± 0.6	165.7 ± 6.7
Aged F4/80$^+$CD1d$^+$	8.3 ± 1.0	146.1 ± 15.7

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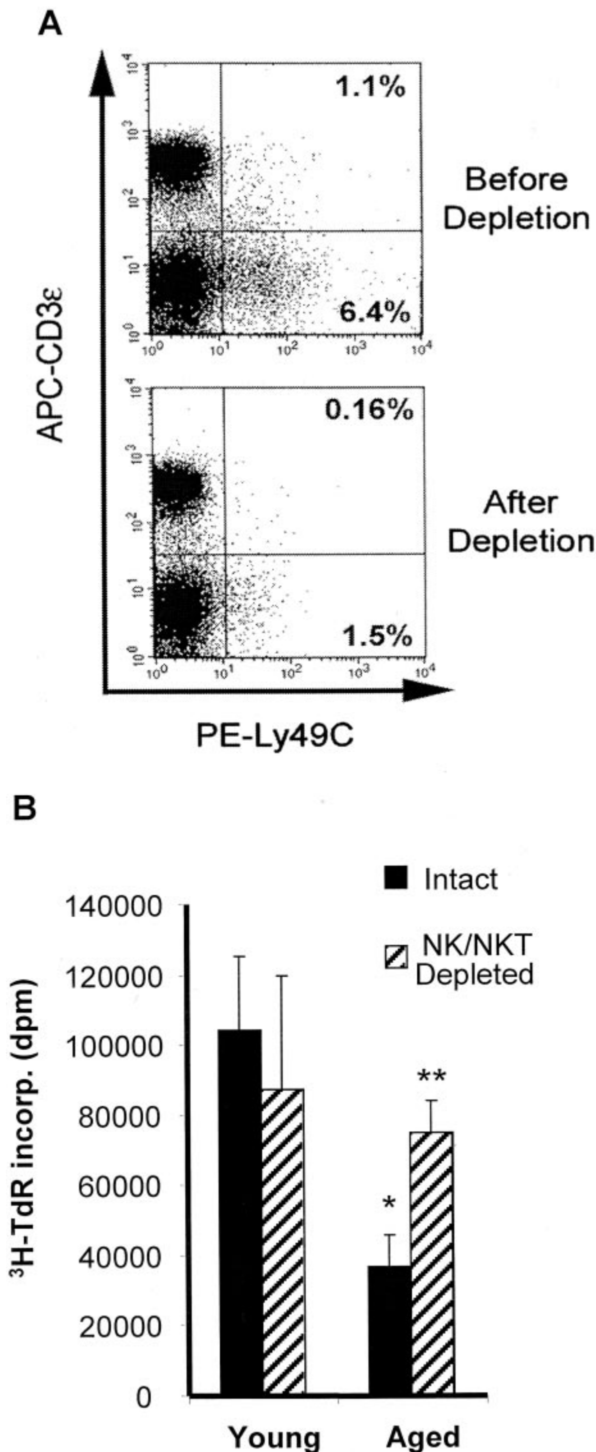

FIGURE 4. Effect of NK/NKT cell depletion on the proliferative capacity of splenic T cells from young vs aged mice. *A*, Splenocyte suspensions were prepared from young vs aged BALB/c mice and immunostained with PE-conjugated anti-Ly49C mAb and anti-PE magnetic beads. The Ly49C^{pos} (NK and NKT) population was removed by single passage over magnetic columns. Depletion of NK and NKT cells was confirmed by flow cytometry for remaining PE-positive cells. *B*, Splenocyte populations that were either left intact, or removed of their NK/NKT populations were cultured for 48 h in the presence of plate-bound anti-CD3ε mAb and then pulsed with [^{3}H]thymidine for an additional 16 h. Thymidine incorporation was determined by scintillation counting. $n = 3$ mice per group for young and $n = 6$ for aged. Data shown are representative of two experiments in which similar results were obtained. $*$, $p < 0.05$ vs intact young, $**$, $p < 0.05$ vs intact aged as determined by two-way ANOVA, followed by Newman-Keuls post hoc analysis.

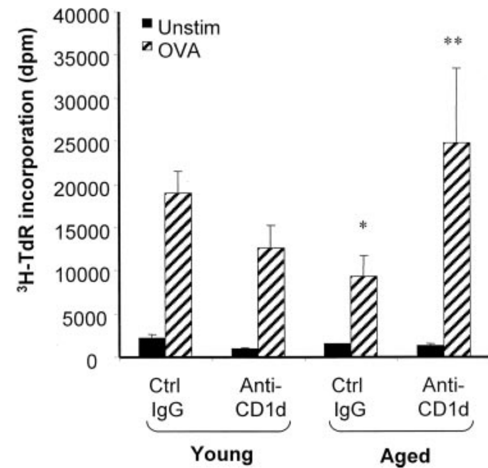

FIGURE 5. Effects of anti-CD1d mAb on Ag-specific T cell proliferation in vitro. Seven days after s.c. immunization with OVA, splenocyte suspensions were prepared from young vs aged mice. Splenocytes were placed in culture for 72 h in the presence or absence of OVA (200 μg/ml) with or without anti-CD1d mAb (10 ng/ml). T cell proliferation was measured by [^{3}H]thymidine incorporation. $n = 4$ per group. $*$, $p < 0.05$ vs young + control (Ctrl) IgG, $**$, $p < 0.05$ vs aged + Ctrl IgG as determined by ANOVA.

cells from aged mice had decreased ability to proliferate in response to OVA re-challenge in vitro, compared with cells from young mice. However, T cells obtained from aged mice given anti-CD1d mAb systemically, demonstrated markedly improved in vitro proliferative responses to OVA compared with T cells from aged mice given control IgG (Fig. 7).

Systemic administration of anti-CD1d mAb prevents the age-associated increase in IL-10 production

IL-10 is an immunoregulatory cytokine that is known to be produced by NKT cells (27) and other regulatory T cell subsets and can potently suppress a variety of immune parameters including Ag presentation and T cell activation (28–30). Here, we examined whether IL-10 production differed with age and whether or not its

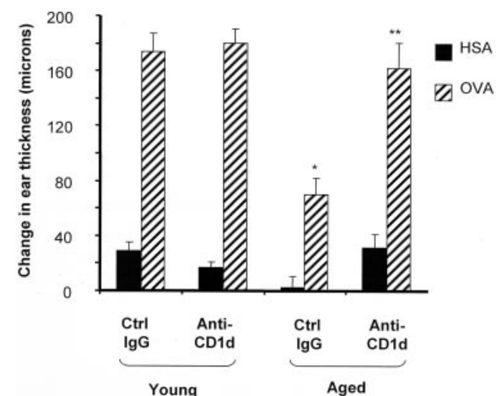

FIGURE 6. Effects of systemic anti-CD1d mAb treatment on in vivo T cell immunity in aged mice. Seven days after s.c. immunization with OVA in CFA, young vs aged mice received either control (Ctrl) rat IgG or anti-CD1d mAb, i.v. Twenty-four hours later, mice were given an ear challenge (i.d.) with OVA in the right ear pinnae and human serum albumin (HSA, Ag specificity control) in the left ear pinnae. Twenty-four hours following ear challenge, the ears were measured again and changes in ear thickness were calculated. $n = 4$ mice per group. $*$, $p < 0.05$ vs young + Ctrl IgG, $**$, $p < 0.05$ vs aged + Ctrl IgG as determined by ANOVA.

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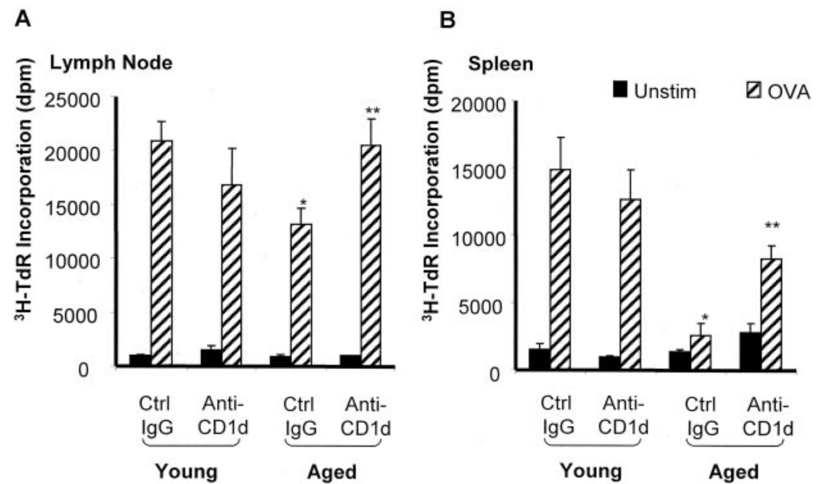

FIGURE 7. OVA-specific spleen and lymph node T cell proliferation after systemic anti-CD1d mAb treatment. Seven days after s.c. immunization with OVA in CFA, young vs aged mice received either control (Ctrl) rat IgG or anti-CD1d mAb, i.v. Twenty-four hours later, lymph node (*A*) and spleen (*B*) cells were collected and cultured for 48 h with or without OVA. OVA-specific proliferation was assessed by [^{3}H]thymidine incorporation and scintillation counting. Data are expressed as mean [^{3}H]thymidine incorporation in dpm ± SEM. n = 4 mice per group. Data are representative of two experiments in which similar results were obtained. *, $p < 0.05$ vs young + Ctrl IgG, **, $p < 0.05$ vs aged + Ctrl IgG as determined by ANOVA.

production was regulated by administration of anti-CD1d mAb. Briefly, splenocytes were obtained from young vs aged mice that had been given systemic anti-CD1d mAb 24 h earlier, and placed in culture with plate-bound anti-CD3ϵ mAb to stimulate all TCR-bearing cells. Twenty-four hours later, culture supernatants were collected and examined for IL-10 via ELISA. We observed that CD3-stimulated splenocytes from aged mice produced levels of IL-10 that were ~10 times greater than splenocytes from young mice (Fig. 8). However, the age-related increase in inducible IL-10 from aged splenocytes was significantly abrogated in splenocytes obtained from aged mice given systemic anti-CD1d (Fig. 8). We also observed an age-related increase in the production of inducible IL-4, but systemic anti-CD1d treatment did not significantly prevent IL-4 production (data not shown). When removed from the mice and placed in culture with anti-CD3 mAb, splenocytes from aged mice produced levels of IFN-γ that were comparable to

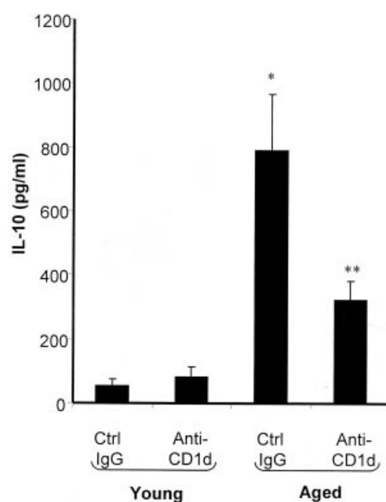

FIGURE 8. Effects of systemic anti-CD1d mAb on CD3-stimulated production of IL-10 by splenic lymphocytes from young and aged mice. Splenocytes were isolated from young and aged BALB/c mice given either control (Ctrl) IgG or anti-CD1d mAb systemically (i.v.) and placed in culture with plate-bound anti-CD3ϵ mAb (2.5 μg/ml) for 24 h. At the end of culture, supernatants were collected and examined for IL-10 content via ELISA. Data are expressed as mean levels of IL-10 in pg/ml ± SEM and are representative of two experiments in which similar results were obtained. *, $p < 0.01$ vs young + Ctrl IgG, **, $p < 0.01$ vs aged + Ctrl IgG as determined by ANOVA.

splenocytes from young mice (1319.8 ± 203.8 pg/ml in young vs 1357.7 ± 38.8 pg/ml in aged).

Discussion

Age-related immune dysfunction presents serious health concerns for today's society, as the population of individuals over the age of 65 years old continues to expand. The consequences of immunosenescence are obvious, as aged individuals are less able to ward off bacterial, viral, and fungal infections, have higher incidences of cancer, and have overall decreased responses to protective vaccines compared with younger individuals (4, 14). Age-related changes in adaptive immunity are well documented, whereas less is known about the effects of age on the innate immune system, with particular regard to innate lymphocytes such as CD1d-restricted NKT cells. Here, we show that as age advances, the number of CD1d-restricted NKT cells increases and that these cells in the aged immune microenvironment actively suppress, rather than support efferent T cell immunity. Additionally, our findings support the concept that NKT cells may suppress efferent T cell immunity via mechanisms that involve excess production of IL-10.

The appearance of increased numbers of T cells bearing NK cell surface markers in aged humans and mice was shown previously by other investigators (15, 16, 31). Before this report, it was unclear whether this observation reflected an increase in the number of CD1d-restricted NKT cells, non-CD1d-restricted NKT cells, or an expansion of CD8 T cells bearing a memory phenotype, because the latter was reported previously by other investigators (15). Using CD1d tetramers loaded with α-GalCer, a ligand specific for the invariant Vα14 CD1d-restricted TCR, we identified the increase in T cells bearing NK cell surface markers as canonical CD1d-restricted NKT cells. Although our data indicated that there were indeed greater numbers of NKT cells overall in the immune compartments of aged mice, additional investigation will be required to determine whether the increased numbers of NKT cells in immune organs reflects an expanding NKT cell population either centrally or peripherally, alterations in recruitment or retention of circulating NKT cells to lymphoid organs, or alterations in NKT cell lifespan as age advances. Age-related alterations in recruitment and retention, lifespan, and proliferative homeostasis have all been reported for conventional T and B cells (2, 32, 33). Additionally, although the aged mice in our studies had greater body weights and their spleens were modestly larger than their young counterparts, our experiments were not controlled for obesity or body size, because that was beyond the scope of this study. Nonetheless, our data showed that in aged mice, regardless of spleen

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size or body weight, the frequency of NKT cells per conventional T cell was significantly greater, indicating an age-related selective increase in the CD1d-restricted NKT cell population.

Studies by our laboratory and many others have demonstrated the regulatory capacity of CD1d-restricted NKT cells over both T cell priming and effector T cell function in young mice under various pathological and non-pathological conditions (21, 26, 34–36). Together, our studies shown here provide the first evidence that under nonpathological conditions in aged mice, NKT cells suppress the activation of Ag-specific T cells both in vitro and in vivo. In fact, we observed that by preventing CD1d engagement of NKT cells in vivo in aged mice, we achieved OVA-specific DTH and proliferative responses that rivaled those seen in young mice. Because the OVA-specific DTH responses being measured here rely on adequate numbers of OVA-specific $CD4^+$ effector T cells having been generated during the priming phase, our findings strongly suggest that in aged mice, effector T cell generation can occur reasonably well. These results further imply that the age-related decline in protective immunity may involve an element of active suppression of effector T cell function that is CD1d-NKT cell dependent, in addition to the inadequate production of cytokines and T cell growth factors reported by others.

It is reasonable to propose that the immune microenvironment changes with age in a manner that might either fail to support or actively suppress immune function, but whether the age-related decline in protective immunity arises from alterations in T cell function at the priming vs effector stages of the immune reflex arc, or both, remains controversial. Here, we immunized both aged and young BALB/c mice with a foreign protein (OVA) in CFA subcutaneously. We sought to test effector T cell responses within the context of an aged immune microenvironment containing aged APCs, aged naive T cells, and an aged inflammatory cytokine profile. Despite immunization with a potent inflammatory adjuvant, we consistently observed decreased T cell immunity (DTH) in aged mice, unless NKT cell activation was blocked just before secondary challenge. Given this, our results indicate that the events necessary for T cell priming remain somewhat intact in aged mice, whereas effector T cell activation is impaired. Our findings contrast with recent studies by Haynes et al. (37) in which naive $CD4^+$ T cells from aged AND TCR Tg (PCC_{88-104}) mice adoptively transferred to young syngeneic CD4-deficient hosts failed to expand upon immunization of the young recipients with PCC and alum, suggesting that priming of aged naive cells fails even within the context of a "young" immune microenvironment. Interestingly however, the authors observed that if the recipients of adoptively transferred, aged TCR Tg $CD4^+$ T cells were immunized with either CFA or proinflammatory cytokines, expansion of aged effector T cells occurred equally as well as T cells from young mice. Perhaps differences in adjuvants used, route of Ag delivery, adoptive transfer models, animal strains, etc., may contribute to the differences in T cell priming observed by different investigators. Nonetheless, our observations together with those of other laboratories reveal different mechanisms whereby aged T cells are rendered less responsive at multiple stages in the development of protective immunity.

The precise mechanism by which NKT cells suppress effector immunity in aged mice remains to be identified. Because NKT cells are known to produce significant quantities of immunomodulatory cytokines including IL-4, IL-10, IL-13, and TGF-β upon primary stimulation (27, 38, 39), it is possible that NKT cells respond to the altered circulating cytokine milieu that occurs with aging, and as a result, these cells could acquire a cytokine phenotype of their own that suppresses, rather than supports, effector T cell activation. Here, we presented data to show that in aged mice, there was increased production of CD3-inducible IL-10, a cytokine known to suppress T cell immunity via several parameters. Support that the IL-10 production was NKT cell dependent (either directly or indirectly) was shown by our ability to prevent age-associated production of IL-10 via systemic administration of anti-CD1d mAb. It has been proposed that ligation of CD1d on the surface of cells can trigger cytokine production, namely IL-10 by intestinal epithelial cells (40), therefore it should be considered that systemic anti-CD1d mAb could modulate immunity via non-NKT cell-dependent mechanisms as well. Additional support for NKT-dependent cytokine production comes from studies by Poynter and colleagues (16), who showed that the age-related increase in inducible IL-4 production was partially NKT cell dependent. Specifically, they observed that the majority of IL-4 produced by lymphocytes from aged mice was from memory T cells ($CD44^{high}CD45RB^{low}NK1.1^{neg}$) in a manner that was regulated by NKT cells.

In conclusion, we identified a role for CD1d-restricted NKT cells in suppression of protective immune function in the aged. Because in young individuals, NKT cells have been shown to be critical for numerous aspects of protective immunity including antitumor responses (34, 41), clearance of microbial and viral infections (35, 42), and autoimmunity (43, 44), it is reasonable to postulate that defects in NKT cell function in the aged may contribute to any number of immunosenescent manifestations. Future investigations may reveal beneficial effects of manipulation of NKT cell activation and phenotype, either through the use of anti-CD1d mAb or pharmacological manipulation, as an approach to improve protective immunity in the aged population.

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Disclosures

The authors have no financial conflict of interest.

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