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Infection or immunization induces heterogeneous memory T cell subsets, but their origin and protective value against infection are unclear. In this study, we report the functional characterization of two memory Th subsets, defined by expression of integrin CD49b. Stable CD49b expression is induced in up to one-half of all memory Th cells. More importantly, the CD49b- and CD49b+ subsets display distinct helper activities, typified by the production of IL-10 and TNF-α, respectively. Although the inflammatory properties of the CD49b+ subset are protective against intracellular bacterial infection, they are associated with immunopathology in acute viral infection. Modulation of the CD49b-defined memory Th subsets may provide infection type-specific interventions, where either enhancement of the inflammatory response or reduction of immunopathology is essential. The Journal of Immunology, 2006, 177: 968–975.

Owing to MHC restriction of Ag recognition by the TCR, developing T cells lack effector functions until their somatically generated random TCR repertoire is selected against self-reactivity. Newly generated, “naïve” T cells remain in a state of immaturity in the periphery until they encounter their cognate Ag. This induces the final stages of a developmental program, associated with dramatic changes in T cell phenotype, effecter function, cellular architecture, cell cycle control, and migration pattern (1–4). Although many are transient, some of these activation-induced changes persist in the “memory” state (after Ag clearance) and form the basis of immunological memory.

Ag encounter and the context in which it occurs, often induces divergent phenotypic and functional states, resulting in substantial heterogeneity among the responding T cells (5, 6). Some of the heterogeneity in the responding T cells stems from the induction of different developmental programs, committing T cells to distinct lineages. Such an example is the division of the Th response into type 1 (Th1) and type 2 (Th2), exemplified by the production of IFN-γ and IL-4, respectively (7). Alternatively, heterogeneity in the responding T cell population may represent progressive stages in a single, linear differentiation pathway (8). For example, IFN-γ is not produced by all CD4+ T cells that commit to the Th1 pathway (9) and IL-2 is produced only by a proportion of the responding CD4+ T cells (10). Thus, different degrees of antigenic stimulation (as a result of T cell affinity for the Ag, timing of recruitment into the response, local microenvironment, etc.) may drive T cells to different functional states of differentiation (differentiation compartments). Moreover, it is becoming increasingly clear that further heterogeneity exists within conventional subsets, often revealed by the production of cytokines, other than the subset-defining ones. It has been reported that, in addition to several subsets of regulatory T cells, a proportion of classical Th1 cells have the potential for IL-10 production (11). Whether these cells represent a separate subpopulation of Th1 cells is currently unclear. Also, a novel subset of Th2 cells, characterized by the production of IL-17, has recently been described (12, 13). Although IL-17 and IFN-γ production seem to be mutually exclusive, it is suggested that the IL-17-producing Th cells mediate many of the inflammatory and autoimmune disease that have traditionally been ascribed to the IFN-γ-producing Th1 subset (13).

The combination of both commitment to distinct Th lineages and progressive stages of differentiation toward each of these lineages greatly amplifies the heterogeneity of memory Th cells and this complexity has hindered our understanding of the precise lineage relationship of the various memory Th subsets. Furthermore, the protective value of each of the distinct Th lineages and differentiation intermediates against infection with a diverse range of pathogens is still unclear. In this study, we examine the phenotypic and functional heterogeneity of the memory Th pool in mice. Our results identify integrin CD49b as a memory Th differentiation Ag, expressed in as many as one-half of all memory Th cells. Acquisition of CD49b expression by memory Th cells appears to be permanent, indicating a unidirectional differentiation pathway. More importantly, CD49b expression subdivides the memory Th pool into subsets with distinct cytokine production patterns and functional properties. Furthermore, our results indicate that the protective value of CD49b- and CD49b+ memory Th cells is context dependent and relies on the infecting pathogen and the fine balance between immune activation and immunopathology.

Materials and Methods

Mice

A1 TCR-transgenic mice (14) recognize an epitope form the Dby-encoded male Ag (HY) in association with H-2Eβ and were kept on an H2b Rag1−/− genetic background. 6.5 TCR-transgenic mice (15) recognize an epitope form the influenza A/PR/8/34 virus hemagglutinin (HA)β in association with H-2Eβ and were kept on an H2d Rag2−/− genetic background.

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Enrichment was performed with an AutoMACS, using a combination of cells prepared from day 6 bone marrow cultures from A (H2a) mice, which were supplement with GM-CSF, and were pulsed with 1 µM HY peptide (REELAHLQFRS GKR, by preincubation for 4 h). Phycoerythrin (PE)-labeled antibodies (70 µM of the adoptive transfer) were used for the isolation of memory HY-specific T cells 6–14 wk after transfer. HA-specific 6.5 T-cells were generated by adoptive transfer of HA-specific naive T cells to syngeneic wild-type BALB/c mice (5 × 10^5/recipient), which were subsequently infected with influenza, and were analyzed 4–6 wk later. Polyclonal memory Th cells were purified from the spleens of previously infected or immunized mice with a combination of magnetic isolation of CD4^+ cells, using an AutoMACS (Miltenyi Biotec), and subsequent cell sorting. Extralymphoid HY-specific TCR-transgenic and B6 polyclonal memory Th cells were isolated from the peritoneal cavity, lungs, liver, and bone marrow donor mice. Peritoneal cavity cells were isolated by flushing of the femurs and tibias. Single-cell suspensions were obtained from the lungs and liver of PBS-perfused donor mice, using the tissues through a 70 µm nylon mesh. Cells were then stained with mAbs against CD4 and CD49b. Cells were then washed to remove extracellular bacteria. The growth of extracellular bacteria was further blocked by the addition of 10 µg/ml gentamicin. FACS-purified splenic Th cells, from C57BL/6 donors that were immunized 4 wk earlier with 1 × 10^6 heat-killed L. monocytogenes, were added in triplicates cultures (3 × 10^5/well) in a 200-µl final volume, which were incubated for 18 h. Cultures were then washed to remove the antibiotics, macrophages were lysed with PBS containing 0.1% Igepal (Nonidet P-40) and surviving bacteria were counted by plating onto erythromycin-containing brain-heart infusion agar plates.

**Influenza A infection and antiviral Th cell and Ab response**

Nonanesthetized BALB/c mice were infected with 250 HA units (HAU) of the A/PR/8/34 (H1N1) strain of influenza A by instillation onto their nasal cavities. At least 4 wk postinfection, splenic CD4^+ T cells were FACS-purified and adoptively transferred into secondary BALB/c-nude recipients (0.75 × 10^5/recipient). Secondary recipients were infected with 250 HAU of A/PR/8/34 on the day of T cell transfer and their body weight was monitored for 12 days thereafter. The presence of an influenza neutralizing Ab response was tested in sera (Collected on day 6 and 12 postinfection by using a modified Madin-Darby canine kidney (MDCK)-based assay. Serial dilutions of the sera were added to monolayers of MDCK cells in 96-well plates, which were subsequently infected with a 95% tissue culture-infective dose of A/PR/8/34. MDCK cell viability was measured with an Alamar blue-based assay 3 days after infection. Cultures were pulsed with Alamar blue for 1–2 h and fluorescence was measured with a fluorescence plate reader (PerkinElmer LS50B). Antiviral Th cell responses in the spleen of C57BL/6 mice, infected with A/PR/8/34 5–6 wk earlier, were assessed by IL-2 production in response to influenza-infected bone marrow-derived DCs. DCs were infected with 8000 HAU/ml A/PR/8/34 in allantoic fluid for 1 h and then extensively washed. FACS-purified subsets of Th cells (50 × 10^5/well) were incubated with influenza-infected DCs (50 × 10^3/well), for 2 days before supernatants were tested for the presence of IL-2, using an Alamar blue-based CTL-2 assay.

**Results**

**CD49b expression in memory Th cells**

To study the heterogeneity of memory Th cells, we used an adoptive transfer system of in vivo memory T cell generation, which involves cotransfer of TCR-transgenic CD4^+ T cells with Ag-pulsed DCs into allogeneic, allogeneic hosts (18, 21). Under these experimental conditions, the resulting memory Th population is heavily skewed toward a Th1 phenotype as 40–60% of them produce IFN-γ upon stimulation (18), while no IL-4 production is detected. Memory Th cells were generated from male Ag (HY)-specific, naive A1 TCR-transgenic T cells and assessed for heterogeneous expression of a number of surface molecules. Surprisingly, a large proportion (~50%) of memory HY-specific T cells, were positive for expression of integrin CD49b, detected with the DX5 mAb (Fig. 1A), a marker usually associated with NK cells (22). This distinguished them from naive HY-specific T cells, which were negative, and also from NK cells, which were uniformly expressing CD49b at higher levels (Fig. 1A). The proportion of memory HY-specific T cells expressing CD49b was monitored over time following their generation and was found to be stable from day 14 to the last time point analyzed (day 220) (data not shown), indicating that memory Th cells can retain CD49b expression for at least this time period. Similarly to memory HY-specific T cells, a large proportion of memory, but not naive, polyclonal CD4^+ T cells from C57BL/6 (B6) mice were expressing CD49b, again at lower levels than NK cells (Fig. 1B). This was additionally confirmed with the HM2a mAb (Fig. 1B), which is also specific for CD49b. In agreement with previous reports (22, 23), the staining pattern obtained with the HM2a Ab was generally

H. Shen (Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, PA). Macrophages were collected by peritoneal lavage of wild-type C57BL/6 mice and plated in flat-bottom 96-well plates at a density of 3 × 10^5/well. Nonadherent cells were removed 2 h later and macrophages were infected with a multiplicity of infection 10 of recombinant L. monocytogenes in a volume of 25 µl and in the presence of 5 µg/ml erythromycin. Cultures were incubated for 1 h to allow internalization of bacteria and were then washed to remove extracellular bacteria. The growth of extracellular bacteria was further blocked by the addition of 10 µg/ml gentamicin. FACS-purified splenic Th cells, from C57BL/6 donors that were immunized 4 wk earlier with 1 × 10^6 heat-killed L. monocytogenes, were added in triplicates cultures (~3 × 10^5/well) in a 200-µl final volume, which were incubated for 18 h. Cultures were then washed to remove the antibiotics, macrophages were lysed with PBS containing 0.1% Igepal (Nonidet P-40) and surviving bacteria were counted by plating onto erythromycin-containing brain-heart infusion agar plates.

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4-fold higher than that obtained with the DX5 Ab, presumably due to the isotype difference (IgG and IgM for HMα2 and DX5, respectively) (22). Thus, the HMα2 Ab allowed a clearer separation of the CD49b+ and CD49b- Th subsets and, as a consequence, a higher proportion of CD49b+ Th cells were detected with this Ab. For this reason, the HMα2 Ab was used in most subsequent experiments. CD49b expression was also induced in homeostatically expanded TCR-transgenic CD4+ T cells, a state which is associated with phenotypic and functional conversion into a memory phenotype (24), and in all cases CD49b expression was independent of expression of other NK cell-related markers, such as NK1.1, NKG2A, C, and E, or Ly49A, C, D, and I (data not shown), thus excluding the possibility that CD49b expression is part of a general NK cell-related gene expression program.

Although absent from HY-specific TCR-transgenic memory Th cells, a sizeable fraction of polyclonal CD4+ T cells express CD25 and are generally accepted to represent a regulatory lineage. These CD25+ regulatory T cells were not responsible for CD49b expression in polyclonal CD4+ T cells as there was minimal overlap between the CD25+ and CD49b+ subsets (Fig. 1C), with only 5–10% of CD25+ CD4+ T cells coexpressing CD49b (in contrast to 30–50% in CD25-CD49b+CD4+ T cells). Furthermore, CD49b expression was largely restricted to memory CD4+ T cells, as there was no expression in naive CD4+ or CD8+ T cells or in the vast majority (>90%) of memory CD8+ T cells (Fig. 1D).

We next established that CD49b expression can be found in pathogen-specific memory Th cells following the normal course of infection with a pathogen. As shown in Fig. 2A, influenza-specific CD4+ T cells could be detected in both the CD49b+ and CD49b- subset of the memory (CD44high) Th pool of wild-type B6 mice that were previously infected with influenza A. To directly visualize and enumerate the influenza-specific memory Th cells, we adoptively transferred naive 6.5 TCR-transgenic T cells, specific to the HA of influenza, into wild-type BALB/c mice, which were subsequently infected and left to recover. CD49b expression was detected in ~40% of 6.5+ HA-specific memory Th cells (Fig. 2B), which is very similar to the proportion of CD49b+ cells in the endogenous memory Th pool. Together, these results strongly suggest that one-third to one-half of all memory Th cells are positive for CD49b expression.

**Unidirectional lineage relationship of CD49b-defined memory Th subsets**

To further understand the mechanism by which two subsets of memory Th cells, distinguished by expression of CD49b, can arise from CD49b- naive precursors, we examined the possibility that these two subsets represent distinct stages of progressive Th differentiation. To this end, CD49b+ and CD49b- memory HY-specific T cells were transferred separately into secondary alymphoid recipients, with or without further antigenic stimulation, in the form of Ag-pulsed DCs. Following both homeostatic and Ag-driven expansion, about half of the initially CD49b- memory HY-specific T cells expressed CD49b, while almost all the initially CD49b+ memory HY-specific T cells maintained CD49b expression (Fig. 3, top row). This result suggests a linear differentiation pathway of memory Th cells, which progresses from a CD49b- to a CD49b+ stage upon further stimulation. Alternatively, the CD49b+ population that arises from the CD49b- input may represent an outgrowth of CD49b- contaminants in the purified CD49b- population. However, if a growth advantage of the CD49b+ memory T cells over the CD49b- ones were the case, we would expect the total memory T cell recovery from the two recipient groups to be different. In contrast, the total memory HY-specific T cells that were recovered from the respective secondary recipients was similar (Fig. 3, bottom row), suggesting that the expansion potential of both CD49b+ and CD49b- memory HY-specific T cells, in response to both homeostatic and antigenic signals, is comparable.

**Minimal overlap of central memory (TCM) and effector memory (TEM) T cells with CD49b-defined subsets**

Increasing CD49b expression during memory Th differentiation is compatible with the progressive memory T cell differentiation model (8). TCM and TEM T cells, defined by differential expression of the chemokine receptor CCR7, represent the prototypic stages of memory T cell differentiation (25). Therefore, we examined whether the CD49b-defined memory Th subsets correspond to the previously described TCM and TEM subsets. Expression of CD49b in both HY-specific TCR-transgenic and B6 polyclonal memory Th cells showed little correlation with expression of either CCR7.
FIGURE 2. CD49b expression in influenza-specific memory Th cells. A, FACS-purified naive (CD4<sup>+</sup>CD49<sup>b</sup>−/CD25<sup>+</sup>), regulatory (CD4<sup>+</sup>CD25<sup>+</sup>), and CD49b<sup>+</sup> and CD49b<sup>b</sup> memory (CD4<sup>+</sup>CD44<sup>hi</sup>CD25<sup>−</sup>) Th cells from mice that were infected with influenza 5–6 wk earlier were tested in vitro for the presence of an antiviral response elicited by influenza-infected DCs and assessed by IL-2 production. Uninfected DCs and T cells from influenza-naive mice were used as specificity controls. One representative of three experiments is shown. B, CD49b expression in endogenous and influenza HA-specific TCR-transgenic memory (CD4<sup>+</sup>CD49b<sup>+</sup>CD25<sup>−</sup>) Th cells from mice that were infected with influenza 5–6 wk earlier were tested in vitro. The production of NO is part of the macrophage response to infection with intracellular bacteria, which is induced by the synergistic action of TNF-α and IFN-γ and down-regulated by IL-10 and other anti-inflammatory cytokines (26, 27). Importantly, peritoneal macrophages were more strongly induced to produce NO when they presented cognate Ag to CD49b<sup>+</sup> than CD49b<sup>−</sup> memory T cells (Fig. 6A), in line with the respective cytokine production pattern of the two memory Th subsets. To test whether increased NO production by macrophages presenting Ag to CD49b<sup>+</sup> memory Th cells correlates with increased microbicidal activity, we measured the killing of intracellular <i>L. monocytogenes</i> by macrophages receiving help from either CD49b<sup>−</sup> or CD49b<sup>+</sup> memory Th cells, isolated from <i>L. monocytogenes</i>-immunized B6 mice. Intracellular bacteria killing was enhanced by both subsets of memory Th cells compared with naive Th cells (Fig. 6B). However, CD49b<sup>+</sup> memory Th cells further reduced the bacterial load in macrophages compared with CD49b<sup>+</sup> memory Th cells (Fig. 6B). Together, these findings suggest that the CD49b<sup>−</sup> subset of memory Th cells is best equipped to provide inflammatory signals to interacting APCs, a property which is essential for the clearance of intracellular bacteria.

Distinct in vitro helper activity by the CD49b-defined subsets

To further test whether the difference in cytokine production between CD49b<sup>−</sup> and CD49b<sup>+</sup> memory Th cells also translates into differences in functional helper activity, we studied the helper effect of HY-specific TCR-transgenic memory Th cells on APCs in vitro. The production of NO is part of the macrophage response to infection with intracellular bacteria, which is induced by the synergistic action of TNF-α and IFN-γ and down-regulated by IL-10 and other anti-inflammatory cytokines (26, 27). Importantly, peritoneal macrophages were more strongly induced to produce NO when they presented cognate Ag to CD49b<sup>+</sup> than CD49b<sup>−</sup> memory Th cells (Fig. 6A), in line with the respective cytokine production pattern of the two memory Th subsets. To test whether increased NO production by macrophages presenting Ag to CD49b<sup>+</sup> memory Th cells correlates with increased microbicidal activity, we measured the killing of intracellular <i>L. monocytogenes</i> by macrophages receiving help from either CD49b<sup>−</sup> or CD49b<sup>+</sup> memory Th cells, isolated from <i>L. monocytogenes</i>-immunized B6 mice. Intracellular bacteria killing was enhanced by both subsets of memory Th cells compared with naive Th cells (Fig. 6B). However, CD49b<sup>+</sup> memory Th cells further reduced the bacterial load in macrophages compared with CD49b<sup>−</sup> memory Th cells (Fig. 6B). Together, these findings suggest that the CD49b<sup>−</sup> subset of memory Th cells is best equipped to provide inflammatory signals to interacting APCs, a property which is essential for the clearance of intracellular bacteria.

Different behavior of CD49b<sup>−</sup> and CD49b<sup>+</sup> memory Th cells in influenza infection

The cytokine production profile and in vitro helper activity of CD49b<sup>+</sup> memory Th cells suggests that this subset of memory Th cells would induce a higher degree of APC activation and local association with the classical Th1, rather than a regulatory, lineage. Collectively, these results reveal a functional distinction between the two subsets, with respect to production of IL-2 and TNF-α (highly enriched in the CD49b<sup>+</sup> subset). IL-10 (found exclusively in the CD49b<sup>−</sup> subset), but not IFN-γ, a profile which was remarkably similar between TCR-transgenic and polyclonal memory Th cells.

or CD62L, except for a small enrichment of CCR7<sup>+</sup> cells within the CD49b<sup>+</sup> subset (Fig. 4A). Furthermore, the proportion of both HY-specific TCR-transgenic and B6 polyclonal memory Th cells expressing CD49b was comparable between lymphoid and extra-lymphoid tissues, such as the peritoneal cavity, lungs, and liver (Fig. 4B), suggesting that the two subsets display similar overall migration patterns. A possible exception was the bone marrow, where a significantly higher proportion of CD49b<sup>−</sup> memory Th cells were present, compared with other tissues (Fig. 4B). Together, these results argue against an overlap of T<sub>CM</sub> and T<sub>EM</sub> subsets with the CD49b<sup>+</sup> and CD49b<sup>+</sup> ones.

Dissimilar cytokine production by the CD49b-defined subsets

To investigate whether CD49b<sup>−</sup> and CD49b<sup>+</sup> memory Th subsets are also functionally different, we initially assessed the cytokine production profile of both HY-specific TCR-transgenic and B6 polyclonal memory Th cells, following brief in vitro stimulation. As shown in Fig. 5A, the proportion of IFN-γ-producing T cells was comparable in both CD49b<sup>−</sup> and CD49b<sup>+</sup> subsets. In contrast, all the IL-10-producing cells were contained within the CD49b<sup>−</sup> subset (Fig. 5A). IL-2 and TNF-α-producing T cells were detected in both CD49b<sup>−</sup> and CD49b<sup>+</sup> subsets (Fig. 5A). However, for both IL-2 and TNF-α, cytokine-producing cells were highly enriched in the CD49b<sup>−</sup> subset (for example, 30 vs 79% for IL-2 and 36 vs 75% for TNF-α in the CD49b<sup>−</sup> and CD49b<sup>+</sup> subset of B6 polyclonal memory Th cells, respectively). Cytokine coexpression analysis revealed that in the CD49b<sup>−</sup> subset, the majority of IFN-γ-producing T cells also produced IL-2 or TNF-α (Fig. 5B). Because a much larger proportion of CD49b<sup>+</sup> memory Th cells produced IL-2 or TNF-α than IFN-γ, the majority of these cells did not coproduce IFN-γ (Fig. 5B). Lastly, the majority of IL-10-producing cells were also producing IFN-γ (Fig. 5B), indicating an...
inflammation during the course of reinfection, compared with the CD49b⁻ subset. Although this feature would be beneficial in certain types of infection, it may also lead to local tissue damage and immunopathology. The protective value of CD49b⁻ and CD49b⁺ memory Th cells was directly tested in a model of influenza infection. T cell-deficient BALB/c-nude mice were used as hosts of CD49b⁻ and CD49b⁺ memory Th cells from influenza-immune BALB/c donors. Protective immunity against influenza reinfection relies heavily on the presence of high-affinity neutralizing Abs. Thus, the provision of T cell help for Ab class switching and production is a major component of the protective value of Th cells in this type of infection. Therefore, we assessed the neutralizing Ab response of influenza-infected T cell-deficient hosts that had received different Th subsets. Adoptive transfer of naive Th cells into BALB/c-nude mice fully restored the neutralizing Ab response to levels seen in T cell-replete BALB/c mice (Fig. 7A).

Transfer of either CD49b⁻ or CD49b⁺ memory Th cells also restored the Ab response, although it did not significantly alter the kinetics of the Ab response, as titers of neutralizing Abs comparable to T cell-replete mice were not achieved before day 12. However, both subsets of memory Th cells induced a higher titer of neutralizing Abs than naive Th cells (Fig. 7A). Furthermore, the Ab response induced by the CD49b⁻ subset was equivalent to the one induced by the CD49b⁺ subset (Fig. 7A).

In this experimental setting, T cell-deficient mice recover from influenza infection, presumably due to their ability to mount an unmutated IgM Ab response (28) (Fig. 7B). The immunopathological symptoms of the infection in these mice (expressed as weight loss) followed the typical course, with a peak at around day 10 postinfection (Fig. 7B). In line with a T cell-mediated immunopathological mechanism, adoptive transfer of naive Th cells into T cell-deficient hosts exacerbated the clinical symptoms of the infection to some extent, but did not accelerate their onset (Fig. 7B). In contrast, transfer of either subset of memory Th cells accelerated the onset of clinical symptoms by ~3 days (Fig. 7B). More importantly, transfer of the CD49b⁻ subset led to a rapid recovery, starting from day 8 and resulting in an ameliorated overall disease compared with transfer of naive Th cell precursors (Fig. 7B). In stark contrast, transfer of the CD49b⁺ subset led to a deterioration of the clinical course, compared with the CD49b⁻ subset, but also with T cell-deficient hosts (Fig. 7B).

To determine whether the disease-enhancing effect of CD49b⁺ memory Th cells were due to increased migration into the infected organs, compared with the CD49b⁻ subset, we measured the numbers of adoptively transferred memory Th cells present in the lungs. Comparable, if not higher, numbers of donor-origin CD4⁺ T cells were found in the lungs of hosts that received CD49b⁻ memory Th cells (4.94 ± 2.25 × 10⁶, n = 2), compared with hosts that received CD49b⁺ memory Th cells (2.11 ± 1.75 × 10⁶, n = 2) at the peak of clinical symptoms (day 7 postinfection). In contrast, the lungs of hosts that received naive Th cell precursors, in which clinical symptoms had not yet reached their peak at this time

FIGURE 4. CD49b expression in T CM and T EM. A, CD49b expression relative to CCR7 and CD62L expression in total CD4⁺ HY-specific (A1) TCR-transgenic or B6 polyclonal memory Th cells. Numbers within the plots indicate the percentage of cells that stained positively with each respective marker and are representative of six to eight mice. B, Mean ± SEM percentage of CD49b⁺ cells in CD4⁺ HY-specific (A1) TCR-transgenic or B6 polyclonal memory Th cells isolated from the lymph nodes (LN), spleen (SP), peritoneal cavity (PC), lung (LU), liver (LI), or bone marrow (BM). The mean value of three to five mice is shown. The dashed line represents the mean percentage of CD49b⁺ memory Th cells in the spleen (*, p < 0.05 between SP and BM, two-tailed Student’s t test; nonsignificant between SP and all other tissues).

FIGURE 5. Cytokine production profile of CD49b⁻ and CD49b⁺ memory Th cells. A, Intracellular cytokine detection in relation to extracellular CD49b expression in MACS-purified HY-specific (A1) TCR-transgenic or B6 polyclonal memory Th cells, following 4 h in vitro stimulation. B, Intracellular cytokine coexpression in electronically gated CD49b⁻ and CD49b⁺ HY-specific (A1) TCR-transgenic or B6 polyclonal memory Th cells, following 4 h in vitro stimulation. Numbers within the plots indicate the percentage of cytokine-producing cells in either the CD49b⁻ or the CD49b⁺ memory Th subset and are representative of six to eight mice.
point, contained significantly fewer CD4<sup>+</sup> T cells (0.75 ± 0.10 × 10<sup>6</sup>, n = 2). In all cases, the lung-infiltrating Th cells were uniformly CD44<sup>high</sup> and, similarly to what we observed with HY-specific T cells (Fig. 3), many (75–77%) of the initially naive or CD49b<sup>−</sup> memory Th cells were now expressing CD49b (geometric mean fluorescence intensity: 250–271), while almost all (93%) of the initially CD49b<sup>+</sup> memory Th cells maintained expression of CD49b, at a higher level (geometric mean fluorescence intensity: 391). These preliminary results suggest that the distinct disease outcome associated with transfer of either CD49b<sup>−</sup> or CD49b<sup>+</sup> memory Th cells is not fully explained by differential migration into the infected lungs and further highlight the functionally different helper profiles of the two subsets.

Overall, these findings suggest that the CD49b<sup>−</sup> subset of memory Th cells is highly efficient at providing help for the neutralizing Ab response to influenza infection and, at the same time, mediates minimal immunopathology, compared with naive or the CD49b<sup>+</sup> subset of memory Th cells.

**Discussion**

In this study, we report the division of memory Th cells into functionally distinct subsets, based on the expression of integrin CD49b, which are associated with qualitatively different types of helper activity. The enhanced proinflammatory properties of CD49b<sup>+</sup> memory Th cells correlate with their increased TNF-α production, as well as the absence of IL-10 production, and although they can be beneficial in the protection against intracellular bacterial infection, they can also cause immunopathology in acute viral infection. Therefore, each of the CD49b-defined memory Th subsets may have a distinctive role in orchestrating a complex and tailor-made type of immune response that is most effective against a given pathogen with the least damage to the host.

The CD49b (α<sub>6</sub>) and CD29 (β<sub>1</sub>) integrin heterodimer constitutes the VLA-2, a receptor for extracellular matrix components, such as collagen and laminin (29, 30). Although expressed on a variety of hemopoietic and nonhemopoietic cell types, VLA-2 was originally identified in activated T cells (30), hence the designation. Despite that, reactivity with the DX5 mAb (which recognizes the CD49b integrin (22)) was originally considered to be an NK cell-specific property. Recently, CD49b expression in conventional CD4<sup>+</sup> and CD8<sup>+</sup> T cells during viral infections has also been reported (31–33), in conjunction with (31) or separately from (32) expression of other NK cell-related molecules, such as NK1.1. Our data show that CD49b expression can be stably induced in a large proportion of conventional monoclonal TCR-transgenic and polyclonal memory Th cells, independently of any other NK cell-related molecule. Therefore, caution in interpreting the results of studies, which rely on CD49b expression to distinguish between NK cells and memory Th cells, is warranted. In contrast to CD4<sup>+</sup> memory T cells, our data suggest that only a small percentage of CD8<sup>+</sup> memory T cells display CD49b expression. This finding is in line with previous reports, in which CD49b expression in virus-specific CD8<sup>+</sup> T cells was found to be transient and following resolution of the acute infection CD49b<sup>+</sup> CD8<sup>+</sup> memory T cells were reduced in proportion or completely disappeared (31–33). More recently, significant CD49b expression in CD8<sup>+</sup> memory T cells was shown to be maintained for more than a year after viral infection, which was however reduced, compared with earlier time points (34). It is therefore possible that, although CD49b induction in CD4<sup>+</sup> memory T cells is stable, the rate with which CD49b expression is lost...
in CD8+ memory T cells is dependent on the type and strength of the stimulus.

The lineage relationship of the CD49b- and CD49b+ subsets of memory Th cells would appear to be linear, with CD49b- memory Th cells originating from CD49b+ precursors. FACS-purified CD49b+ memory Th cells retain CD49b expression following transfer into secondary hosts, indicating that their development is unidirectional. The homeostatic expansion associated with transfer of T cells into lymphocyte-deficient hosts causes CD49b expression in FACS-purified CD49b- memory Th cells and thus could also interfere with physiological CD49b down-regulation in FACS-purified CD49b+ memory Th cells under nonlymphopenic conditions. However, the proportion of CD49b+ HY-specific memory Th cells was maintained at a constant level for over 200 days following their generation in primary adoptive hosts, in which a homeostatic equilibrium is achieved (35), indicating that memory Th cells can retain CD49b expression even under nonlymphopenic conditions. A linear lineage relationship of the CD49b- and CD49b+ subsets would imply that the CD49b+ subset may represent a more advanced stage of a linear memory Th differentiation pathway, while the CD49b- memory Th cells are intermediates. Such a scheme would be analogous to the progressive differentiation model, proposed for the TCM and TEM subsets (8). However, there are several observations that are incompatible with such an interpretation. The functional differences between CD49b- and CD49b+ memory Th cells are also in quality, and not only in quantity, as exemplified by their cytokine production profile. Furthermore, both CD49b- and CD49b+ Th subsets contain similar proportions of TCM and TEM cells (defined by CCR7 or CD62L expression), indicating that they have undergone a comparable degree of differentiation. Lastly, although CD49b+ precursors can give rise to CD49b- memory Th cells upon further activation, there are many CD49b- memory Th cells that fail to express CD49b even after repeated in vivo activation (our unpublished observations). An alternative explanation would be that the CD49b- and CD49b+ subsets represent the products of divergent Th differentiation pathways. As both originate from CD49b- naive Th precursors, it is possible that the CD49b+ subset of memory Th cells has a CD49b- intermediate. Thus, the CD49b- fraction of memory Th cells would be heterogeneous, containing both differentiated CD49b+ memory Th cells as well as separate CD49b- precursors of the CD49b+ subset. Recently, induction of CD49b expression has been shown to occur in the first few days of in vitro stimulation of naive TCR-transgenic Th cells (23). Furthermore, CD49b expression was found only in about half of the responding Th cells during the first week of stimulation and increased further only following prolonged in vitro stimulation (23). Although it is currently unclear whether the effector Th subsets correspond to the memory Th subsets, it is conceivable that the divergence of the CD49b- and CD49b+ memory Th subsets is initiated at the effector stage. In support of this idea, it has been recently suggested that the heterogeneous expression of progressive differentiation markers in influenza-specific memory Th cells, directly reflects the heterogeneity of the effector response (36). Interestingly, induction of CD49b expression in effector Th cells was observed in Th1, but not Th2, cells following prolonged in vitro stimulation (23), which is in agreement with the enhanced proinflammatory activity of the CD49b+ memory Th subset, characteristic of the Th1 response. However, our data further argue that expression of CD49b is not a universal feature of Th1 differentiation. Instead, CD49b expression defines a distinct subset or branch of Th1 cells, associated with a distinctive pattern of cytokine production and helper activity, which strongly suggests further functional dichotomy within the Th1 subset.

Expression of CD49b was used in this study as a marker to distinguish between two subsets of memory Th cells with different functional properties. Whether this expression has any functional significance in the activation and subsequent function of memory Th cells is currently unclear. Collagen receptors on T cells have been implicated in T cell migration into and through peripheral tissues, their ability to induce inflammation at these sites and also protection against acute viral infection (33, 37). However, most of these effects were mediated by the collagen receptor VLA-1, instead of VLA-2, expressed in CD8+ memory T cells (33, 37). These findings are supported by the fact that, in contrast to the transient expression of VLA-2, expression of VLA-1 is permanently induced in the majority of CD8+ memory T cells (31–33). The migration pattern of resting memory Th cells seems to be largely independent of CD49b expression, as the relative representation of CD49b- and CD49b+ memory Th cells is similar between lymphoid and most extralymphoid tissues. However, CD49b+ memory Th cells were highly enriched in the bone marrow, a primary lymphoid organ. Although the reasons for preferential homing, survival and/or proliferation of CD49b+ memory Th cells in the bone marrow are unknown, it may be related to a unique combination of collagen and laminin isoform expression by bone marrow stromal cells (38, 39). CD49b in T cells has also been suggested to function as a costimulatory molecule, enhancing TCR-mediated signals (23, 40). However, a costimulatory function of CD49b alone could not account for the functional differences between CD49b- and CD49b+ memory Th cells, because at least some of them were qualitative and they were also apparent when pharmacological activation of purified T cells was used. Thus, the functional impact of CD49b expression in memory Th cells remains to be determined. Nevertheless, CD49b can be used to predict the functional properties of memory Th cells, because its expression is associated with a distinct type of helper activity. CD49b+ memory Th cells display a more potent proinflammatory phenotype, both in vitro and in vivo, compared with their CD49b- counterparts. Of note, the increased proinflammatory activity of the CD49b- subset seems to be independent of IFN-γ, as the production of this cytokine was comparable between the two subsets. Instead, the observed functional difference could be sufficiently explained by a combination of increased TNF-α production by the CD49b+ subset and IL-10 production exclusively by the CD49b- subset. However, additional experiments will be needed to determine the extent to which each of these cytokines contributes to the functional behavior of the two subsets. Lastly, although preferential migration of CD49b+ memory Th cells into inflamed tissues, following antigenic stimulation, could also contribute to their enhanced proinflammatory activity in vivo, increased migration of this subset into influenza-infected lungs was not observed in our preliminary experiments.

Our observation that different memory Th cell subsets, defined by CD49b expression, can be associated with distinct immunological outcomes reveals a new target for preventive and therapeutic interventions. The marked ability of CD49b+ memory Th cells to mediate macrophage activation and subsequent killing of intracellular bacteria is a desirable property in certain types of infection, such as infection with *Mycobacterium tuberculosis*. Although memory Th cells respond faster to *M. tuberculosis* infection and control it at a lower level than their naive Th precursors, they nevertheless fail to clear the infection (41). Thus, in the case of *M. tuberculosis*, immunization regimes aiming at induction of the CD49b+ subset of memory Th cells specifically, may provide better protection against infection. In contrast to infection with intracellular bacteria, protection against many viral infections relies
heavily on the presence of neutralizing Abs. In acute viral infection, such as with influenza A virus, memory Th cells provide indispensable help for neutralizing Ab production, but can also mediate immunopathology (42). Importantly, these two opposing properties of memory Th cells seem to segregate with CD49b expression. The CD49b+ memory Th subset provides help for neutralizing Ab production that is comparable to the CD49b− one and, at the same time, mediates minimal immunopathology. Therefore, in the case of infection with influenza or other acute respiratory viruses, inhibition of CD49b− or specific induction of CD49b+ virus-specific memory Th cells by immunization would be more protective against infection. Further insight into the cellular and molecular events that influence the commitment to or development of these subsets will ultimately benefit vaccination strategies.

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Disclosures

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