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CD47\textsuperscript{high} Expression on CD4 Effectors Identifies Functional Long-Lived Memory T Cell Progenitors

Vu Quang Van,* Marianne Raymond,* Nobuyasu Baba,* Manuel Rubio,* Keiko Wakahara,* Santos A. Susin,‡,§ and Marika Sarfati*

T cell memory is the hallmark of adaptive immunity. Central questions are to determine which cells among proliferating effector T cells will live beyond the crash of the immune response (IR) and develop into functional memory T cells. CD47, considered as a marker of self, is implicated in cell death, cell elimination, and in the inflammatory response. We report in this article that CD47 expression was transiently regulated on Ag-specific CD4 T cells, that is, from CD47\textsuperscript{high} to CD47\textsuperscript{low} to CD47\textsuperscript{high}, during the course of the in vivo IR. Specifically, CD47\textsuperscript{high} status marked central memory CD4 T cell precursors at an early time point of the IR. By contrast, cytokine production was a functional attribute restricted to CD47\textsuperscript{high}, but not CD47\textsuperscript{low}, polyclonal effector CD4 T cells during recall responses in an experimental model of chronic airway inflammatory disease. Passive transfer of CD47\textsuperscript{high}, but not CD47\textsuperscript{low}, CD4 T cells in nonlymphopenic naive mice generated long-lived memory T cells capable of anamnestic responses. We conclude that CD47\textsuperscript{high} status on CD4 T cells identifies functional long-lived memory T cell progenitors. The Journal of Immunology, 2012, 188: 4249–4255.

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Abbreviations used in this article: BAL, bronchoalveolar lavage; BMDC, bone marrow-derived dendritic cell; CD44, CD44 tumor necrosis factor receptor; CD62L, P-selectin glycoprotein ligand-1; CD127, IL-7 receptor; DC, dendritic cell; IFN-\(\gamma\), interferon-\(\gamma\); IL-12, interleukin-12; IONO, ionomycin; IR, immune response; i.t., intratracheal; LN, lymph node; mLN, mediastinal LN; T<sub>CM</sub>, central memory T (cell); T<sub>EM</sub>, effector memory T (cell); T<sub>MDR</sub>, memory T cell receptor; T<sub>RM</sub>, effector memory T (cell); T<sub>TEM</sub>, effector memory T (cell); WT, wild type.

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by DCs (10, 11), IL-12 responsiveness (12), and Th1 differentiation (13, 14), whereas it promotes the differentiation of regulatory T cells under neutral conditions (15, 16). Because inflammatory context negatively regulates memory T cell development (17), CD47 may control the generation of memory T cells.

In this report, we asked whether surface CD47 was implicated in memory CD4 T cell differentiation program. Our findings indicate that CD47 is transiently regulated on Ag-specific T cells. CD47 status marks CD4 effector cells that commit to long-lasting memory at an early time point of the IR.

Materials and Methods

Animals

CD47−/− 129s/veg mice were backcrossed onto CD47+/+ BALB/c mice for 16–18 generations. Mice expressing the DO11.10 TCR transgene, which is specific for the peptide residues 323–339 of chicken OVA, were purchased from Charles River Laboratories and backcrossed into CD47+/+ mice. BALB/c CD45.1 congenic mice were purchased from Jackson Laboratories. Female mice 6–10 wk-old were used in all experimental protocols as approved by the Canadian Council on Animal Care and maintained under specific pathogen-free conditions.

Flow cytometry for phenotypic analysis

mAbs against mouse Ags: CD4 (RM4-5), TCR (DO11.10) (KJ126), CD127 (SB/199), CD62L (MEL-14), CCR3 (83101; R&D Systems), Ly-6G(1A8), CD3 (145-2C11), CD45R (RA3-6B2), CD45.1 (A20), IL-13 (eBio13A; eBioscience), IL-17 (TcII-18h10.1), CD45.2 (104), Bcl-2 (BCL/10C4), and Fas (Jo2). CD47 expression was revealed with mu-signaling regulatory protein α-Fc (18). All staining was done in FACS buffer (PBS supplemented with 2% FCS, 2 mM EDTA, and 0.1% sodium azide at 4˚C for 30 min). Abs were purchased from BioLegend unless otherwise indicated.

Bone marrow-derived DCs

Bone marrow cell suspensions were obtained from femurs and tibias of BALB/c mice, and cultured in culture medium (RPMI 1640; Wisent) supplemented with 5% FBS and 20 ng/ml GM-CSF (PeproTech) for 13 d. The medium was refreshed every 3 d. Bone marrow-derived DCs (BMDCs) were pulsed with 100 µg/ml OVA (Grade V, Sigma) (BMDC-OVA) and with or without LPS (1 µg/ml; Sigma) on day 12 of the culture.

Adoptive transfer experiments

BALB/c mice were passively transferred (i.v.) with 2 × 10^6 or 5 × 10^3 CFSE-labeled CD4 T cells isolated from CD47−/− DO11.10 (transgenic [Tg]) mice. After 1 d, mice were immunized either s.c. with OVA protein pulsed on day 12 with 20 ng/ml OVA (Grade V, Sigma) (BMDC-OVA) and with or without LPS (1 µg/ml; Sigma) on day 12 of the culture.

Cytokine production

For lung explants, lungs were cut into small pieces and cultured for 24 h in RPMI 1640 culture medium (19). IL-4, IL-6, IL-13, IL-17, CCL11/eotaxin (R&D Systems), and thymic stromal lymphopoietin (eBioscience) were measured in lung culture supernatants by ELISA. IL-13 and IL-17 produced by guest on April 14, 2017 http://www.jimmunol.org/ Downloaded from

Results

CD47 expression is transiently downregulated on Ag-specific CD4 T cells during a primary IR in vivo

We first asked whether CD47 expression was regulated on Ag-specific CD4 T cells during a primary IR. To address this, we investigated CD47 expression on Ag-specific T cells after immunization. Naive hosts were passively transferred with OVA-specific DO11.10 Tg CD4 T cells and immunized with OVA protein plus adjuvant or a DEC205-OVA hybrid Ab, which elicits immunity or tolerance, respectively (20) (Supplemental Fig. 1). Downregulation of CD47 expression (hereafter referred to as CD47low) was observed on the surface of activated Tg CD47−/− CD4 T cells in wild type (WT), irrespective of the mode and the route of immunization, when compared with basal expression (hereafter referred to as CD47high) seen on unstimulated (day 0) Tg CD4 T cells (Fig. 1A, 1B). However, CD47low status differed from lack of expression seen on CD47-deficient (CD47−/−) T cells. The decrease in CD47 expression preceded the peak of Tg T cell proliferation (Fig. 1C). At the nadir of CD47 expression on Tg T cells (day 6), mice were challenged in the opposite footpad with Ag (OVA-CFA) to track the Tg T cell fate. All retracted Tg T cells in the draining LNs were CD47high (Supplemental Fig. 2). This suggested that a CD47high status was either maintained on a few Tg T cells that had proliferated and/or that Tg CD47low T cells had reacquired a CD47high phenotype in vivo. To distinguish between these two possibilities, we tracked cell division of activated Tg T cells in relation to CD47 expression. A significant proportion of highly divided Tg T cells displayed a CD47high status in response to an immunogenic, but not a tolerogenic, immunization (Fig. 1D). Coinjection of CD40 mAb together with DEC205-OVA Ab, which breaks tolerance and allows Tg T cell proliferation (20), correlated with the upregulation of CD47 on a significant proportion of the dividing Tg T cells (Fig. 1E). We next demonstrated that purified Tg CFSElowCD47low T cells passively transferred into naive mice continued proliferating in the spleen in the absence of Ag challenge, and that a significant proportion of the divided cells converted to CD47high status (Fig. 1F). Therefore, we conclude that CD47 status is transiently modulated in response to TCR stimulation during a primary IR in vivo.
CD47high status marks early TCM precursors in primary response in vivo

We therefore asked whether differential CD47 expression on Ag-specific CD4 T cells could discriminate effector and memory T cells, and thus was linked to the generation of memory CD4 T cells in vivo. Phenotypic analysis revealed that Tg CFSElowCD47low T cells displayed an effector phenotype (CD44highCD127lowCD62Llow) compared with the Tg CFSElowCD47high T cells, which expressed a central memory (CD44highCD127highCD62Lhigh) phenotype, indicating that the transition from effector to memory cells can be detected at an early time point postimmunization (Fig. 2A). Notably, passive transfer of physiologically relevant number of Tg precursors (5 × 10^3) produced lower numbers of effector cells at day 6, with the same proportion of CD47high (Fig. 2B) and similar phenotype (Fig. 2C) as those starting from a large Tg precursor number (2 × 10^6). Kinetics indicated that the proportion of CD127+ and CD62Lhigh cells gradually increased within the CD47high and, to a lesser extent, in CD47low Tg T cell populations (Fig. 2D). These results suggested that the change to CD47high status preceded the upregulation of CD127, a receptor required for T cell survival. We therefore examined whether de novo CD47 expression was required and necessary for CD127 expression. Naive CD47-/- and CD47+/+ T cells displayed similar levels of CD127 expression, which was downregulated at day 6 postimmunization in CD47-/- and CD47+/+ mice (Fig. 2E), demonstrating that CD127 expression was not controlled by CD47.

To allow recovery of sufficient numbers of Ag-specific T cells to perform phenotypic analysis at a later time point, we immunized mice with BMDC-OVA as previously reported (2). On day 35, virtually all of the CD44high Tg T cells displayed the CD47high status and a TCM phenotype in LNs (Fig. 2F), indicating that survivors of the contraction phase have converted to CD47high cells. Notably, CD47-/- Tg T cells transferred into CD47-/- mice displayed an effector, rather than TCM phenotype, at this later time point, supporting the concept that enhanced cell survival, T-bet expression, and Th1 responses as seen in CD47-deficient mice (13, 21) correlate with impaired CD4 TCM differentiation.

Taken together, our data demonstrate that the presence of CD47, and more particularly the conversion to CD47high status, distinguishes effector and early TCM precursors in primary response.

Polyclonal CD47high T cells are the main source of cytokines in secondary response

After a second and third immunization, Ag-specific memory CD4 T cells rapidly generate large numbers of effector cells, some of which will survive the crash of a recall response and give rise to long-lived memory T cells. Because long-lived memory T cells emerge from cytokine-expressing effector T cell progenitors (7), we next asked which of the CD47high or CD47low subpopulation was the source of cytokine production in secondary response. The study of long-term memory responses in vivo has historically relied on the transfer of Tg T cells, a method that does not necessarily reflect the behavior of endogenous Ag-specific T cells during physiologic IR. We thus followed polyclonal secondary IR in BALB/c mice that had been immunized and challenged with BMDC-OVA i.t., an experimental model we recently developed that leads to Th17/Th2-associated severe airway inflammation (19). The frequency and absolute numbers of polyclonal secondary effector CD44high CD4 T cells that expressed a CD47high phenotype...
progressively augmented in the mLNs after repeated airway challenge (Fig. 3A) and further increased after a resting period of 4 wk (Fig. 3B). At an early time point (24 h after the last challenge), ∼60% of CD47high T cells within the CD44high CD4 T cell population expressed an effector (CD127low) phenotype, whereas at a late time point (4 wk) the majority of the CD47high T cells had acquired a memory (CD127high) phenotype (Fig. 3B). The CD44highCD47high T cells displayed more of the antiapoptotic molecule Bcl-2 than CD44highCD47low T cells, but had a similar level of Fas expression (Fig. 3C). We next found that 90% of the CD44highCD47high T cells produced cytokines (IL-13 and/or IL-17), as compared with 25% of the CD44highCD47low T cells (Fig. 4A). Frequency of cytokine-producing CD44high T cells significantly correlated with the mean fluorescence intensity of cell surface CD47 and was not linked to CD127 expression (Fig. 4B, 4C, left panels). IL-13 and IL-17 were mainly produced by the CD47high T cells, independently of their expression of CD127 (Fig. 4B, 4C, right panels). This demonstrates that the CD47high CD4 T cell subtype represents the major source of cytokine production after Ag challenge in polyclonal response.

**Polycional CD47high T cells are the progenitors of long-lived memory T cells**

We further investigated whether secondary effector CD47high T cells, which we identified as the cytokine-producing cells, were the precursors of long-lived memory CD4 T cells capable of anamnestic responses. To address this key issue, we transferred naive congenic CD45.1 hosts with secondary CD44highCD45.2 effectors purified on the basis of CD47 status (high or low). After a resting period of 4 wk, congenic mice were nebulized on 4 consecutive days with OVA. An increased proportion and absolute number of transferred CD47high as compared with CD47lowCD4 T cells were retraced in the mLNs and in the lungs of congenic hosts (Fig. 5A). Congenic CD45.2 effectors produced IL-13 (Fig. 5B), which was exclusively expressed by CD47high T cells (Fig. 5C). We next showed that passive transfer of polyclonal secondary CD44highCD47high T cells, as opposed to CD44highCD47low or CD44low naive T cells, elicited the recruitment of innate inflammatory cells and lymphocytes in the BAL fluid (Fig. 5D) and lungs (Fig. 5E). The airway inflammatory response occurred concomitantly with the release of type 2 cytokines and IL-17 in lung explants, which matched the cytokine profile of the transferred CD47high T cells (Figs. 4, 5F). The release of eotaxin by epithelial cells likely reflected the increase in IL-13 production by effector CD47high T cells (22). The latter elicited neither thymic stromal lymphopoietin, IL-6, IL-25, nor IL-33 release, which are usually triggered by epithelial damage provoked by exposure to an environmental Ag. We conclude that polyclonal cytokine-expressing CD47high CD4 effectors comprised the progenitors of long-lived functional memory T cells.
Discussion

A central question is how memory T cell development is regulated to confer long-lived, Ag-specific protection against repeated challenges with invading pathogens, whereas maintaining tolerance to self-Ag. This process is key to the development of vaccine strategies and improved therapeutic interventions for chronic infections and autoimmune diseases (23). During a primary IR, pathogen-specific naive CD4 T cells replicate and generate large numbers of effector T cells. Upon clearance of the infection, the majority of effectors undergo cell death, whereas a small number of precommitted memory T cell precursors survive the contraction phase and persist after the resolution of the primary IR. Memory T cells remain quiescent until rapid reactivation is required in response to a recall Ag challenge (3). The elimination of unwanted T cells is an important process in the resolution of inflammation, because uncontrolled responses result in collateral tissue damage and chronic inflammatory disorders caused by memory T cells targeted against innocuous or self-Ag. However, the precise mechanisms that govern the generation of long-lived memory CD4 T cells are poorly understood (24).

We showed in this study that the status of CD47 expression (CD47\textsuperscript{high} or CD47\textsuperscript{low}) was linked to the development of memory CD4 T cells. We identified polyclonal effector CD47\textsuperscript{high} T cells as the main IL-17- and IL-13-producing cells and demonstrated that they gave rise to long-lived memory T cells that caused airway inflammation after their adoptive transfer in nonlymphopenic naive hosts. Our data corroborate the findings of Harrington et al. (7), who show that memory CD4 T cells emerge from effector T cells that express \textit{ifng} gene once in their life. Although our results support the linear model of memory T cell differentiation, they also favor the concept that effector T cell fate is decided at an early time point after Ag encounter. Hence CD47\textsuperscript{low} and CD47\textsuperscript{high} status discriminated effector and memory CD4\textsuperscript{+} T cells at day 6.
postimmunization, irrespective of the route and the mode of Ag delivery. These observations may be taken as an indication that transient change in CD47 expression may occur across different type of immunizations in vivo.

Once CD47^{high} T cells have escaped the contraction of the IR, they must survive to generate long-term memory. Several evidences indicate that Bcl-2 family members are involved in this process (24, 25). Memory T cells express antiapoptotic Bcl-2 molecules, which inhibit the formation of apoptosis-inducing complexes consisting of Bim and Bak. CD47^{high} T cells expressed higher Bcl-2 when compared with CD47^{low} T cells. The survival of memory cells also critically depends on IL-7R (CD127) signaling, which activates STAT5 and Akt that limit Bim (26, 27). CD127 expression progressively increased within CD47^{high} Tg T cell populations, indicating that conversion to CD47^{high} status preceded the upregulation of CD127 expression. We further demonstrated that CD47 expression was not required for CD127 expression. In fact, overexpression of CD127 does not influence the pool of memory cells (28).

Environmental and molecular factors control the generation of memory T cells. T-bet expression and inflammatory conditions negatively regulate memory T cell development. In the context of inflammation, increased T-bet expression favors the expansion of CD8 effector cells at the expense of memory cell development and alters cell recirculation (17, 29, 30). Also, equal proportions of the two memory CD4 T cell subsets are generated after Listeria monocytogenes infection in WT mice: the T-bet^{high} cells that lack CCR7 and rapidly produce IFN-γ, and thus resemble Th1 effector memory cells; and the T-bet^{low} cells that express CCR7 and produce none of the lineage-defining cytokines, and therefore resemble TCM (25, 31). Lack of CD47 expression enhances T-bet expression on CD4 T cells as well as exacerbates and sustains T cell-mediated contact hypersensitivity response, which correlates with an enhanced type 1 IR (13, 32). Therefore, the lower frequency of TCM seen in CD47^{-/-} hosts in the absence of lymphosplenomegaly may be best explained by increased T-bet combined with decreased production of IL-7 in CD47^{-/-} when compared with WT hosts (33).

Finally, we showed that polyclonal T cells, which have survived the contraction phase, maintained their CD47^{high} status after repetitive challenge in vivo. Repeated injection of inflammatory DCs i.t. drives triple IL-17+IL-4+IL-13+ cells that coexpress Gata-3 and ROR_γt in lymphoid and lung tissues, which correlates with the development of a severe IgE-independent corticosteroid-resistant airway inflammation (19). Our findings demonstrated that IL-17/IL-13–producing polyclonal CD47^{high} T cells represented the progenitors of pathogenic memory T cells, which elicited a Th17/Th2 cytokine profile in lung tissues. Hence passive transfer of

FIGURE 5. Polyclonal CD47^{high} CD4 effectors are the progenitors of long-lived memory T cells. (A-F) BMDC-OVA were injected i.t. (3×) into CD45.2 mice. CD47^{high} or CD47^{low} CD44^{hi} CD4 T cells were isolated from CD45.2 mLNs 24 h after last Ag challenge and transferred into naive CD45.1 hosts. For some experiments, CD44^{low} CD45.2 CD4 T cells were transferred into congenic hosts. Four weeks later, recipient mice were challenged with an aerosol of OVA protein for 4 consecutive days. BAL, lungs, and mLNs were analyzed by flow cytometry 24 h after the last nebulization. (A) Frequency of CD45.2 T cells in mLNs and lungs. (B) Intracellular IL-13 production in mLNs and lung cells restimulated with OVA protein for 72 h and PMA-IONO for the last 5 h. (C) IL-13 production in relation to CD47 expression in ex vivo isolated mLNs. (D) Recruitment of lymphocytes, neutrophils, and eosinophils in BAL. (E) Percentage of eosinophils and neutrophils in the lungs. (F) Cytokine release was measured in the supernatants of lung tissue cultures (24 h). Data are representative of four independent experiments (n = 2 mice per experiment). (D and F) Data represent the mean ± SD; Student t test: *p < 0.05, ***p < 0.001.
CD47high but not CD47low polyclonal CD4 T cells induced eosinophila and lung inflammation after 4 wk in nonlymphopenic mice. In support of our observations, adoptive transfer of in vitro-generated Ag-specific Th17 cells initiates neutrophil recruitment in the Airways with no inflammation, whereas cotransfer of Th17 and Th2 cell lines is required to promote a mixed neutrophilic and eosinophilic infiltration and airway hyperreactivity (AHR) (34). Collectively, our data favor the concept that Th1/Th2 double-producing cells, which are found in increased proportion in the circulation of patients with severe asthma (35) and identified as CD47high in this study, are pathogenic and drive airway disease.

In summary, this study demonstrates that CD47high status on CD4 T cells identifies functional long-lived memory T cell precursors at an early time point of the IR. We propose that early memory CD47high precursors represent effectors that have passed two checkpoints (cell death and elimination) before they acquired long-term survival signaling via CD127. Hence CD127 expression is not sufficient for the generation of long-lived memory cells because CD127+ and CD127− cells are similarly able to generate memory T cells (36). The precise molecular mechanisms behind the generation of memory CD4 T cells remain to be clarified. Our results support exploring strategies that enforce a CD47high status on CD4 effectors for development of improved vaccines, whereas promoting CD47low status on auto-aggressive T cells for control of chronic inflammatory disorders.

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Disclosures
The authors have no financial conflicts of interest.

References
**Legends to supplementary figures**

**Fig.S1:** Anti-DEC205-OVA hybrid antibody induces transient Tg T cell proliferation followed by cell elimination.

CFSE-labeled CD4 Tg T cells were adoptively transferred into BALB/c mice. 24 h later, mice were immunized s.c. in the left footpad with anti-DEC205 or isotype control coupled with OVA peptide (5). Draining lymph nodes were harvested and stained with anti-CD4 and anti-TCR (KJ126). A) Dose response and kinetics of anti–DEC205-OVA hybrid Ab–induced Tg T cell proliferation. B) At day 6 or 9, mice were reimmunized in the opposite footpad with OVA peptide emulsified in IFA to track Tg T cell fate. Tg T cells were tolerized at day 6 and eliminated by day 9. Data are representative of 3 independent experiments (n=6 mice per group).

**Fig.S2:** Recruitment of CD47\(^{\text{high}}\) Tg T cells in lymph nodes after Ag challenge.

2X10\(^6\) CD4 Tg T cells were adoptively transferred into BALB/c mice. At day 1, mice were immunized s.c. with OVA-CFA and challenged s.c. with IFA-OVA in the opposite footpad at day 6. After 3 days, CD47 expression was examined on the surface of Tg T cells in draining LNs. The great majority of Tg T cells retraced in LNs were CD47\(^{\text{high}}\). Data are representative of 2 independent experiments (n=4 mice /group).
Supplementary Fig. 1

A. Day 3  Day 5  Day 8  Day 12

500 ng

% Max

1000 ng Isotype CTRL

% Max

CFSE

Day 8

B. Isotype  DEC205

Day 6

0.48  2.5  0.36  0.97

73  24  69  30

Day 9

0.23  3.3  0.09  0.02

75  21  69  30

CD4
Supplementary Fig. 2

6d after first challenge on left footpad

3d after a second challenge on right footpad

CD47

KJ126