Cutting Edge: CD47 Controls the In Vivo Proliferation and Homeostasis of Peripheral CD4+CD25+Foxp3+ Regulatory T Cells That Express CD103

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Peripheral CD103+ Foxp3+ regulatory T cells (Tregs) can develop both from conventional naive T cells upon cognate Ag delivery under tolerogenic conditions and from thymically derived, expanded/differentiated natural Tregs. We here show that CD47 expression, a marker of self on hematopoietic cells, selectively regulates CD103+ Foxp3+ Treg homeostasis at the steady state. First, the proportion of effector/memory-like (CD44highCD62Llow) CD103+ Foxp3+ Tregs rapidly augmented with age in CD47-deficient mice (CD47−/−) as compared with age-matched control littermates. Yet, the percentage of quiescent (CD44low CD62Lhigh) CD103− Foxp3+ Tregs remained stable. Second, the increased proliferation rate (BrdU incorporation) observed within the CD47−/−Foxp3+ Treg subpopulation was restricted to those Tregs expressing CD103. Third, CD47−/− Tregs maintained a normal suppressive function in vitro and in vivo and their increased proportion in old mice led to a decline of Ag-specific T cell responses. Thus, sustained CD47 expression throughout life is critical to avoid an excessive expansion of CD103+ Tregs that may overwhelmingly inhibit Ag-specific T cell responses. The Journal of Immunology, 2008, 181: 5204–5208.

Cutting Edge: CD47 Controls the In Vivo Proliferation and Homeostasis of Peripheral CD4+CD25+Foxp3+ Regulatory T Cells That Express CD103

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CD4+CD25+Foxp3+ regulatory T cells (Tregs) are generated early in life in the thymus. Expression of Foxp3 is dispensable for Treg development but absolutely required for its function. Once in the periphery, Tregs play a crucial role in the control of organ-specific autoimmune diseases by inhibiting the function of undesired autoreactive T cells that escaped central tolerance (1–3). Tregs are comprised of two subsets with distinct phenotypes and homeostasis, the quiescent (CD25+CD44lowCD62LhighFoxp3+) and cycling (CD25+CD44high CD62LlowFoxp3+) Tregs; the suppressive function of both subsets appears quite similar (4). The latter subset was identified as a group of effector memory-like Tregs. They accumulate in secondary lymphoid organs (SLO) of ~70-wk-old mice and thus may contribute to age-associated immune deficiency (5, 6). However, the pool size of quiescent Tregs remains stable in unmanipulated mice throughout their lifetimes. The capacity of CD62Lhigh Tregs to enter the lymph nodes (LNs) through high endothelial venules suggests that they mainly act in SLO to inhibit the activation and proliferation of naive T cells. Notably, suppression of T cell-mediated colitis by Tregs in lymphopenic mice occurs mainly in draining mesenteric LNs (7). In contrast, Tregs also express receptors for inflammatory chemokines (CCR4, CCR9, and CXCR3), integrins (α4β7), and tissue-homing receptors like CD103 (2, 8). Indeed, it was demonstrated that CD103+ Tregs are attracted and retained in inflamed tissues where they may exert their suppressive function (9, 10). Microarray analysis revealed that functional compartmentalization of Tregs is linked to the expression of different phenotypes, with Tregs found in tissues expressing CD103, IL-10, IL-2R, and CCR5 (10). Nowadays, the peripheral origin of Tregs is well established. The adaptive Tregs appear to be involved in the down-regulation of the immune response to both self and nonself Ags such as microbes, tumor Ags, and alloantigens (11). TGF-β in the presence of a low dose of Ag induces the peripheral conversion of naive CD25−CD4+Foxp3− T cells into bona fide Tregs expressing both Foxp3 and CD103 (12, 13). In fact, TGF-β directly controls the expression of Foxp3, the maintenance of peripheral Tregs, and their activation and proliferation status as well as their suppressive function (14).

CD47 is a marker of self that is ubiquitously expressed and thus exerts pleiotropic effects, notably in the immune system (15–17). CD47 ligation on human cord blood mononuclear cells induces naive T cell anergy and promotes the generation of peripheral CD103− Foxp3+ Tregs that are suppressive (18, 19). In this study we sought to evaluate the in vivo impact of CD47...
deficiency on peripheral Treg homeostasis, activation status, and ability to proliferate under steady-state conditions. We found that CD47 negatively regulated the in vivo proliferation and expansion of the Foxp3+ Treg cell subset that expresses CD103 under steady-state conditions without compromising their suppressive functions. As such, we propose that sustained CD47 expression participates in CD103+ Treg homeostasis throughout adult life until senescence.

Materials and Methods

Mice

BALB/c CD47-deficient (CD47−/−) mice were obtained from Dr. P.A. Oldenborg, Umeå University, Umeå, Sweden. BALB/c and DO11.10 mice were purchased from Charles River and The Jackson Laboratory, respectively. DO11.10 CD47-deficient mice (CD47−/− transgenic (Tg)) were generated by backcrossing DO11.10 into BALB/c CD47−/− mice. All mice are maintained under specific pathogen-free conditions at the Centre Hospitalier de l’Université de Montréal Research Center. Eight- to 52-wk-old mice were used in all experimental protocols as approved by the Canadian Council on Animal Care.

Cell preparation and flow cytometry

Single-cell suspensions were harvested from different organs as described (8, 16). For cell surface staining, cells were incubated with the appropriate FITC-, PE-, PerCP- and allophycocyanin-labeled appropriate Abs on ice for 30 min at 4°C. The following Abs were used: CD4 (RM4-5), CD25 (PC61.5.3 or 7D4), CD44 (IM7, 8.1), CD103(M290), or the KJ1.26 clonotypic mAb. Intracellular staining with anti-Foxp3 or anti-CTLA-4 (UC10-4F10-11) mAbs was performed as described (19). All Abs were purchased from BD Biosciences except for anti-Foxp3 from eBioscience and KJ1.26 from Caltag Laboratories.

Evaluation of cell cycling by BrdU incorporation

Two milligrams per mouse of BrdU (Sigma) were injected i.p. and added to the drinking water (0.8 mg/ml). Seven days later, spleens and LN were harvested and stained for different regulatory T cell subsets and the cells were then fixed, permeabilized (BD Cytofix/Cytoperm), and treated with DNase I (Sigma-Aldrich) for 1 h at 37°C before staining with anti-BrdU-FTTC (BD Biosciences).

In vitro and in vivo assays

CD4+CD25+ (Treg) and CD4+CD25− (effector) cells were purified by negative selection followed by positive selection from the spleens of CD47−/− or CD47−/− mice by using the EasySep kit (StemCell Technologies). For APC, T cell-depleted splenocytes from CD47−/− mice were treated with mitomycin C (30 μg/ml) at 37°C for 30 min.

For the in vitro assay, effector cell (50 × 103) were cocultured in 96-well flat-bottom plates with decreasing numbers of Tregs in the presence of APC (50 × 103) and 1 μg/ml anti-CD3 (145-2C11; BD Biosciences) in RPMI 1640 supplemented with 10% FCS. [3H]Thymidine incorporation was measured after 72 h. In some experiments, effector cells were labeled with CFSE (Invitrogen) and 72 h later CFSE dilution was evaluated by flow cytometry.

For the in vivo assays, 0.5 × 106 CFSE-labeled effector cells isolated from DO11.10 mice were co-injected with 1 × 106 Treg isolated from CD47−/− or CD47−/− Tg 24 h before s.c. administration of 10 μg of OVA peptide (323–339; Cedarlane Laboratories). After 3 days, cell division was assessed by CFSE dilution in draining LN. In some experiments, mice were treated or not with CD25 mAb (P6/1) for 3 days prior adoptive transfer of 1 × 106 CD47+/− Tg and i.v. immunization with an OVA peptide. Recovery of KJ1–26+ Tg cells was evaluated in the spleen after 7 days.

Autoantibody

Sera from >50-wk-old mice were assessed for anti-DNA autoantibodies by using an ELISA kit from Alpha Diagnostic International.

Statistical analysis

Student’s t test; *p < 0.05; **p < 0.01.

Results and Discussion

CD47 negatively regulates activated CD103+ Treg cell homeostasis

Because of thymic involution at puberty, it appears unlikely, especially in humans, that long-lived thymic emigrants would be sufficient to maintain the pool of functional Tregs during aging. Compared with young individuals (<35 years old), old individuals have an increased number of CD45RO+ Tregs and decreased numbers of naive CD45RA+ Tregs (20). At least two mechanisms have been proposed to explain the maintenance of CD45RO+ Tregs throughout life. First, Tregs in adults might originate from the thymic-derived Tregs that are continuously proliferating to replenish the pool of peripheral Tregs. Hence, activated CD45RO+ Tregs that maintain their regulatory function in vitro have been described in the mouse and humans (4, 21). Second, TGF-β or low Ag exposure induces peripheral Treg conversion from naive or rapidly proliferating memory CD4 T cells (12, 13). CD47 ligation promotes Treg generation, albeit via a TGF-β-independent pathway (19). In this study, we first evaluated the proportion and accumulation of Tregs in nonmanipulated CD47−/− mice. The analysis of the thymus, spleen, and LN of young mice (<10 wk old) did not reveal any differences in the frequency of CD25+ cells among CD4 T cells, indicating a normal Treg development in the absence of CD47 (Fig. 1A and data not shown). However, we noticed a gradual and significant increase during aging in the percentage of peripheral CD4 CD25+ T cells in the SLOs of CD47−/− mice as compared with aged-matched control littermates (Fig 1A). More specifically, CD47 deficiency led to drastic augmentation in the proportion of CD4+CD25+ T cells (Fig. 1B). Rather, the percentage of resting (CD44low) CD4+CD25+ T cells remained comparable and stable (~10%) in both strains of mice until senescence. We next examined the nature of the two CD25+CD4+ T cell subsets and showed that the majority of them expressed Foxp3 (~90%) with similar intensity (Fig. 2A). These data demonstrated that CD4+CD25+ T cells indeed belonged to Treg subtypes that included quiescent and effector/memory-like activated Tregs. Note that the two strains of mice displayed a comparable proportion (~3%) of Foxp3+CD25− and Foxp3+CD25+CD4+ T cells (Fig. 2A, right panels). A more detailed phenotypic analysis of the activated T cell subset revealed a selective augmentation in the frequency of Foxp3+ T cells that expressed CD103 (from ~5 to 15%) in the LN of...
CD47−/− mice (Fig. 2B, left panels). In contrast, the proportion of CD103+Foxp3+ Tregs remained quite stable. CD47 deficiency resulted in a similar increase in the percentage of activated Tregs coexpressing Foxp3+/CD103+/ICOS+/CD103− and CTLA-4+/CD103− in the spleens (not shown).

Members of CD103+Foxp3+CD4+ T cell subpopulation have the particularity of being retained in inflamed tissues where they are likely to exert their suppressive function to control inflammatory or pathogen-specific T cell responses (8–10, 22). CCR4 and CCR7 control CD103+ Treg migration into inflamed tissues (8, 23). However, the CD103+ Treg cell compartment is enriched in skin and lung tissues even in the absence of any inflammatory response (8). We found an increased percentage of Foxp3+ Tregs that expressed CD103 in CD47−/− lung tissues at steady state (Fig. 2B, right panels). In contrast to CD47−/− tissues, Treg cell accumulation is impaired in CCR4−/− tissues at steady state.

Taken together, our results demonstrate that under steady-state conditions CD47 ablation leads to a gradual and selective augmentation during aging in the proportion of activated Foxp3+CD4+ T cells that express CD103.

**CD47 inhibits the proliferation and accumulation of CD4+CD103+ Tregs in vivo**

We next showed that the percentage of cycling CD4+CD25+ Tregs that incorporated BrdU was increased in CD47−/− mice (Fig. 3A). This largely reflected the augmented proliferation of CD44hiCD25+CD4+ T cells (Fig. 3B) and, more specifically, that of CD103− Treg cells (Fig. 3, C and D) that significantly accumulated in CD47−/− LN (Fig. 3E). Studies that analyzed the TCR excision circle content reveal that CD103+ Tregs, as opposed to CD103− Tregs, undergo excessive cell proliferation, corroborating our previous findings (22). Although the percentage of BrdU+ Tregs lacking CD103 remained constant (~12%) (Fig. 3C), their absolute number was decreased. This may be linked to the significant reduction in the CD4+ T cell compartment seen in old CD47−/− mice (Fig. 3E). Of interest, CD47 expressed by T cells or as part of the environment controls T cell death. CD47 mediates caspase-independent necrosis-like cell death on T and B cells (24), and murine-activated T cells lacking CD47 appear more resistant to Fas-mediated apoptosis (25). We did not find any evidence for dysregulated Treg cell apoptosis in CD47−/− mice at steady state (not shown).

The mechanisms that regulate the selective expansion of activated CD25+ Tregs expressing CD103 in CD47−/− SLO and tissues at steady state are yet to be elucidated. It was hypothesized that CD103+ Treg cells may expand in response to antigenic stimulation (for instance, to commensal flora at steady state) (4). CD11b+CD103+ dendritic cells and TGF-β are critically involved in the conversion to adaptive Treg cells that express CD103, especially in the intestine and mesenteric LNs (26, 27). However, the proportion of the CD103+ dendritic cells was unaltered in the mesenteric LN of 50-wk-old mice (data not shown). Furthermore, our unpublished observations indicate no difference in basal TGF-β expression in the spleen, nor in the TGF-β production by Tregs in the two strains of mice.

**TGF-β inhibits Treg proliferation and at the same time is required for the survival of peripheral Treg cells** (14). In contrast to CD47−/− mice, lack of TGF-β signaling in T cells increases the absolute number of CD4+ T cells in the spleen and LN that leads to a heavy cellular infiltration in multiple tissues and loss of B cell tolerance, as shown by high amounts of DNA autoantibodies (14). We noticed a significant increase in the serum production of DNA autoantibodies in >50-wk-old CD47−/− mice as compared with age-matched related BALB/c mice (Fig. 3F). Despite increased frequency of Ag-experienced effectors T cells after 28 wk in LN (Fig. 2A), we did not observe any cellular infiltration in liver, lung, pancreas, or kidney in old CD47−/− mice (data not shown).
When cocultured with CD4 T cells, Tregs suppressed as well as CD47+/+ Tregs the in vitro proliferation of effector cells expressing or not expressing CD47 (Fig. 4A). We next examined the ability of CD47−/− Tregs to inhibit Ag-specific naive T cell activation in vitro and in vivo in CD47-deficient hosts. CD47−/− and CD47+/+ Tregs from DO11.10 TCR Tg mice abrogated in vitro proliferation of CFSE-labeled effector Tg T cells (Fig. 4B). Also, CFSE-labeled Tg T cells were adoptively transferred alone or together with CD47−/− or CD47+/+ Treg Tg into CD47−/− recipients 1 day before s.c. immunization with OVA peptide. Recovery of CFSE-labeled KJ1–26+ cells in spleen is shown after 7 days. Data are presented as means ± SD of three mice per group (C) and ± SEM of six mice per group (D).

**CD47 expression on Treg does not regulate their function in vitro and in vivo**

CD47 is considered as a marker of self on immune and nonimmune cells, preventing their clearance by macrophages through the delivery of a negative signal via signal regulatory protein (SIRP)-α (28). This precludes the in vivo studies of Treg function in the form of an adoptive transfer of Treg lacking or not lacking CD47 together with pathogenic T cells into naïve BALB/c scid mice. Therefore, we first evaluated Treg function in vitro. When cocultured with CD4+CD25− effector T cells, CD47−/− Tregs suppressed as well as CD47+/+ Tregs the in vitro proliferation of effector cells expressing or not expressing CD47 (Fig. 4A). We next examined the ability of CD47−/− Tregs to inhibit Ag-specific naive T cell activation in vitro and in vivo in CD47-deficient hosts. CD47−/− and CD47+/+ Tregs from DO11.10 TCR Tg mice abrogated in vitro proliferation of CFSE-labeled effector Tg T cells (Fig. 4B). Also, CFSE-labeled Tg T cells were adoptively transferred alone or together with CD47−/− or CD47+/+ Treg Tg into CD47−/− recipients 1 day before s.c. immunization with OVA peptide in the absence of adjuvant. The results depicted in Fig. 4C demonstrate that CD47−/− Tg Tregs remained functional in vivo, as they strongly impaired naïve T cell proliferation in response to soluble Ag. Finally, we examined the possible in vivo biological consequence of an altered proportion of functional Tregs in the spleens of old CD47−/− mice. We found a significant decline in Ag-specific T cell responses in CD47−/− hosts that was restored in mice pretreated with CD25 mAb, a process known to deplete/inactivate Tregs (Fig. 4D).

We thus conclude that Tregs lacking CD47 are functional in vivo in SLOs of aged mice, corroborating their sustained level of Foxp3 (Fig. 2A), the expression of which is required to maintain Treg function (1).

**Concluding remarks**

A tight regulation of number and function of Foxp3+ Tregs is crucial throughout life to avoid the development of fatal autoimmune disorders. On the contrary, excessive Treg numbers may dampen immune responses and thus be deleterious to the host. Two consequences of Treg expansion are enhanced tumor emergence and pathogen survival. Hence, in some cases pathogen persistence is beneficial because it is mandatory to the establishment of long-term protective immunity (11). We show in this study that maintenance of CD47 expression throughout life negatively controls the turnover and expansion of a particular subpopulation of activated Foxp3+ Tregs that express CD103. We thus predict that sustained CD47 expression will endow the host with the capacity to prevent an excessive accumulation of Tregs that may overwhelmingly inhibit pathogen or tumor-specific CTL responses. By contrast, lowering CD47 expression may be beneficial to control organ-specific autoimmunity, because it leads to increased proliferation and accumulation of functional CD103+ Treg cells. When enforced in a cell-type specific manner, decreased CD47 expression will result in cell elimination. This is best illustrated in nonimmune RBCs expressing low (semenct) or no CD47 (Rh null) that are readily cleared from the circulation, the consequence of which may be the development of autoimmune hemolytic anemia (28).

Taken together, we propose that sustained CD47 expression is required throughout the adult life until senescence to maintain peripheral CD103 Foxp3+ Treg homeostasis, albeit dispensable for their function.

**Disclosures**

The authors have no financial conflict of interest.

**References**


