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# Functional Regulatory T Cells Accumulate in Aged Hosts and Promote Chronic Infectious Disease Reactivation[1]

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## Abstract

Declines in immune function are well described in the elderly, and are considered to contribute significantly to disease burden in this population. Regulatory T cells ($T_{regs}$), a $CD4^+$ T cell subset usually characterized by high CD25 expression, control the intensity of immune responses, both in rodents and humans. However, because CD25 expression does not define all $T_{regs}$, especially in aged hosts, we characterized $T_{regs}$ by expression of FOXP3, a transcription factor crucial for $T_{reg}$ differentiation and function. The proportion of $FOXP3^+CD4^+$ $T_{regs}$ increased in the blood of the elderly and the lymphoid tissues of aged mice. The expression of functional markers, such as CTLA-4 and GITR, was either preserved or increased on $FOXP3^+$ $T_{regs}$ from aged hosts, depending on the tissue analyzed. *In vitro* depletion of peripheral $T_{regs}$ from elderly humans improves effector T cell responses in most subjects. Importantly, $T_{regs}$ from old FoxP3-GFP knock-in mice were suppressive, exhibiting a higher level of suppression per cell than young $T_{regs}$. The increased proportion of $T_{regs}$ in aged mice was associated with the spontaneous reactivation of chronic *Leishmania major* infection in old mice, likely because old $T_{regs}$ efficiently suppressed the production of IFN-gamma by effector T cells. Finally, *in vivo* depletion of $T_{regs}$ in old mice attenuated disease severity. Accumulation of functional $T_{regs}$ in aged hosts could therefore play an important role in the frequent reactivation of chronic infections that occurs in aging. Manipulation of $T_{reg}$ numbers and/or activity may be envisioned to enhance control of infectious diseases in this fragile population.

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## Introduction

During aging, the integrity of the immune system progressively declines. In particular, the ability to fight off infections is decreased, as evidenced by increased numbers of infections, more severe symptoms, prolonged duration and poorer diagnosis (reviewed in (1-4)). Furthermore, reactivation of chronic infections occurs at a higher frequency in aged humans and mice (5,6). These dysfunctions arise from alterations in every component of the immune system (7-10), but the most consistent and significant alterations are seen in the T lymphocyte compartment (11,12), particularly within $CD4^+$ T cells (8,13-15).

$CD4^+$ regulatory T cells ($T_{regs}$) maintain self-tolerance in the periphery (16-18) and play a role in the control of autoimmunity and tumor immunity (18-20). They have been shown to decrease the level of activation, proliferation and cytokine production of effector T cells ($T_{effs}$) in mice and humans (21-24), as well as control the immune function of dendritic cells (DCs) (25,26). $T_{regs}$ were first characterized by their expression of the IL-2Rα chain (CD25) (16). Additional molecules have been associated to $T_{reg}$ function, such as cytotoxic T lymphocyte associated antigen (CTLA)-4 (27) and the glucocorticoid-induced tumor necrosis factor receptor (GITR) (28). More recently, the transcriptional factor FoxP3 (Forkhead box P3) has been shown to play a crucial role in many aspects of murine $T_{reg}$ biology, namely their differentiation, function and maintenance (29-32). In humans, FOXP3 is also crucial for $T_{reg}$ function, as evidenced by the acquisition of $T_{reg}$ activity following *de novo* FOXP3 expression in non-$T_{regs}$ (33).

Previous studies have shown increased numbers of $CD25^+CD4^+$ $T_{regs}$ in the periphery of aged Balb/c (34,35) or C57BL/6 mice (36). Similar increases were also reported in the peripheral blood of elderly people (37-40). Although, FOXP3 expression has recently been used to assess the proportion of $T_{regs}$ in aged humans (41), it remains unclear whether $T_{regs}$ maintain their suppressive activity in aged hosts. Indeed, some studies show maintenance of suppressive activity of $T_{regs}$ in aged mice (34,36) and elderly people (37,39), whereas some studies reported decreased $T_{reg}$-mediated suppression in aged mice (35) and humans (42).

During the acute phase of the infection by *L. major*, activation of $T_{effs}$ leads to the development of a small cutaneous lesion that heals spontaneously after few weeks (43). We have previously shown that during the chronic phase of the infection a high number of both $T_{effs}$ ($CD4^+CD25^-$ T cells, producing IFN-γ) and $T_{regs}$ accumulate at sites of infection (44). A tight equilibrium between the two populations is responsible for the parasite persistence at the site of inoculation (44). Importantly, changes in the $T_{reg}$:$T_{eff}$ balance at the local site induces parasite multiplication and subsequently reappearance of the lesion (45)

In this report, we show in that: (i) $FoxP3^+$ $T_{regs}$ accumulate in aged mice and elderly humans; (ii) $T_{regs}$ from aged mice and elderly humans are functional; and (iii) depletion of $T_{regs}$ *in vitro* and/or *in vivo* increases Teff responses. Together, these data suggest that $T_{reg}$ accumulation in aged hosts contributes to the immune suppression associated with aging.

## Materials and Methods

### Human subjects

Healthy elderly individuals (≥ 70-year old) were recruited in a retirement community in the Cincinnati area. People in the upper third of functional status and with two or less comorbidities were eligible for enrollment. Enrolled individuals were not receiving immunosuppressive medication and had no chronic infection, known malignancy or cognitive impairment. Volunteers with mild chronic conditions not thought to affect immune function were not excluded. Young healthy donors (≤ 30-year old) were recruited at Cincinnati Children's Hospital Medical Center with the same exclusion criteria as those used in the recruitment of

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elderly subjects. All subjects provided written informed consent to protocols approved by the corresponding Institutional Review Boards.

## Mice

6-8-week old C57BL/6 mice were purchased from Charles River (Wilmington, MA) or Taconic. 20-month old C57BL/6 mice were purchased from Harlan (Chicago, IL) through the National Institute on Aging contract. FoxP3-GFP knock-in C57BL/6 reporter mice were obtained from Dr. M. Oukka, Harvard Medical School, Cambridge, MA (46). Mice were maintained at Children's Hospital Research Foundation Animal Facility or NIH animal house facility under pathogen-free conditions. All experiments on mice were performed in accordance with institutional guidelines (Cincinnati Children's Hospital Medical Center and National Institute of Allergy and Infectious Diseases).

## Cell preparation

For humans, blood samples (40 ml) were collected on sodium heparin. Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation on Ficoll Paque™ Plus (GE Healthcare, Piscataway, NJ) within 4 hours of sample collection and frozen in fetal calf serum (FCS) with 10% dimethyl sulphoxide (DMSO; SIGMA, St. Louis, MO).

For mice, single cell suspensions were prepared from spleen, peripheral (retromaxillar and popliteal) lymph nodes (pLNs) and mesenteric LNs (mLNs). Blood was collected on heparin. Erythrocytes were lyzed by incubating the spleen or blood cells with 1 ml ACK (Cambrex, Walkersville, MD) for 2 min, on ice.

## Phenotypic analysis of cells

Thawed human PBMCs were incubated on ice for 5 min with human IgG (SIGMA) to block Fc receptors and stained for 30 min for cell surface markers with a combination of the following antibodies: anti-CD3-PerCP-Cy5.5 (clone SK7), anti-CD4-Pacific Blue or PE-Cy7 (clone RPA-T4), anti-CD25-APC (clone M-A251), anti-CD69-APC-Cy7 (clone FN50), anti-CCR5-PE-Cy7 (clone 2D7/CCR5), anti-CCR7-PE-Cy7 (clone 3D12), anti-integrin α4-APC (clone 9F10), anti-integrin β7-PE (clone FIB504) from BD Pharmingen (San Diego, CA); anti-CD27-APC-Cy7 (clone O323) and anti-integrin β1-PE (clone MEM-101A) from eBioscience (San Diego, CA); anti-GITR-PE (clone 110416) and anti-TGFβRII-PE (clone 25508) from R&D System (Minneapolis, MN); anti-CD127-PE (clone IM1980) from Immunotech (Marseille, France); anti-CD45RA-Pacific Blue (clone MEM-56) and anti-CXCR4-APC (clone 44717) from Caltag (Burlingame, CA). For intracellular stainings, the eBioscience's protocol for FOXP3 staining and the following antibodies were used: anti-FOXP3-FITC (clone PCH101) and anti-CTLA-4-PE (clone 14D3) from eBioscience; anti-PD-1 (clone MIH4) and anti-Granzyme A-PE (clone CB9) from BD; anti-Granzyme B-APC (clone GB12) from Caltag. 200,000 events/sample were collected on a LSRII™ cytometer using the FACSDiva™ software (BD Biosciences). The $CD25^{hi}$, $FOXP3^{+}$, $GITR^{+}$ and $CTLA\text{-}4^{+}$ gates were determined in relation to the staining on non-$CD4^{+}$ T cells. The gate for Granzyme B, CD127, CD45RA and CD27 expression was set up according to the biphasic distribution of this marker. For all the other markers, the appropriate isotype-matched control antibodies were used to define the positive gates within the $CD4^{+}CD3^{+}$ T cells.

Freshly isolated or cultured murine cells were incubated with 24G2 cell line culture supernatant to block Fc receptors. Cells were stained for 20 min on ice with the following fluorochrome-conjugated antibodies: anti-CD4-PE-Cy5 (clone RM4-5), anti-TCR-βchain-APC-Alexa Fluor 750 (clone H57-597), anti-CD25-APC (clone PC61.5), anti- CD69-PE (clone H1.2F3), anti-CD103-FITC (clone 2E7), anti-CD27-APC (clone LG.7F9), anti-CCR7-PE (clone 4B12), anti-GITR-APC (clone DTA-1) and anti-PD-1- FITC (clone J43). The isotype controls used were

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rat $IgG_1$ (clone R3-34), rat $IgG_{2a}$ (clone eBR2a), rat $IgG_{2b}$ (clone KLH/G2b-1-2), and hamster IgG (clone Ha4/8). Cells were washed with PBS, 1% FCS, then stained with anti-FoxP3-Pacific Blue (clone FJK-16s) and anti-CTLA-4-PE (clone UC10-4F10-11) using the FoxP3 staining set reagents and protocol from eBioscience. All antibodies and controls were purchased from BD Pharmingen, eBioscience or BioLegend (San Diego, CA). Cell acquisition was performed on a FACSCalibur™ cytometer using the CellQuest Pro™ software, or a LSRII™ cytometer using the FACSDiva™ software (BD Biosciences). Analysis was performed after gating on $CD4^+$TCR-$\beta^+$ cells, using CellQuest Pro™ software or FlowJo™ software (Tree Star, Inc., Ashland, OR).

### Human $CD4^+$ T cell stimulation

Monocytes were isolated from PBMCs by positive selection using CD14 MicroBeads according to the manufacturer's instruction (Miltenyi Biotec, Auburn, CA), achieving a purity of > 87%, as determined by flow cytometry. $CD4^+$ T cells (> 85% pure) were obtained by negative selection of $CD14^-$ cells using $CD4^+$ T cell isolation kit II according to manufacturer's instruction (Miltenyi Biotec). $CD4^+$ T cells were depleted of $CD25^+$ cells using CD25 MicroBeads (Miltenyi Biotec). As depletion of $CD25^+$ cells by MicroBeads induces non-specific cell loss in the magnetic column, we controlled for that loss in the total $CD4^+$ T cell fraction by using irrelevant MicroBeads (CD14 MicroBeads) according to manufacturer's instructions. Passage of the $CD4^+$ fraction through those CD14 MicroBeads did not change the percentage of $CD25^+$ or $FOXP3^+CD4^+$ T cells (results not shown).

$T_{reg}$-depleted $CD4^+$ and total $CD4^+$ fractions were labeled for 5 min at room temperature with 0.625 µM of carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, OR) in PBS. $5 \times 10^5$ CFSE-labeled T cells were cultured with $2 \times 10^5$ $CD14^+$ monocytes in RPMI supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 292 µg/ml L-glutamine (Invitrogen, Gibco®, Carlsbad, CA) and 10% FCS, and stimulated or not with phytohemagglutinin (PHA; 2 µg/ml; SIGMA) at 37°C in 5% $CO_2$. After 3 days of stimulation, the recovered cells were incubated on ice for 5 min with human IgG to block Fc receptors and were stained 30 min with anti-CD4-PE (clone RPAT4; BD), anti-CD3-PerCP-Cy5.5, anti-CD69-APC (clone FN50; BD) and anti-CD95- Pacific Blue (clone DX2; Caltag). After washes and formaldehyde fixation, up to 50,000 events/sample were collected on a LSR-II™ cytometer with the FACSDiva™ software. Data were analyzed by using FlowJo™ software.

### In vitro T cell suppression assay

$GFP^-CD4^+$ ($T_{effs}$) and $GFP^+CD4^+$ ($T_{regs}$) cells were sorted from spleens or pLNs of FoxP3-GFP knock-in C57BL/6 mice as described below for $CD25^{hi}CD4^+$ and $CD25^-CD4^+$ T cells. $5 \times 10^4$ $T_{effs}$ were stimulated with 0.5 µg/ml anti-CD3 (clone 145-2C11, BD Biosciences) in the presence of $1 \times 10^5$ $CD90^+$ cell-depleted and 3,000-rad γ-irradiated spleen cells. Cultures were set up in triplicate, in 96-well U-bottom plates, with different ratio of $T_{regs}$ to $T_{effs}$. 48 hours later, 1 µCi [$^3$H]-thymidine was added into each well for 22 hours. Plates were harvested and radioactivity measured on a Wallac Trilux MicroBeta scintillation counter.

### L. major infection

Promastigotes (metacyclics) of *L. major* clone V1 (MHOM/IL/80/Friedlin) were isolated as previously described (47). 8-10-week old mice were infected in the ear dermis with $10^3$ *L. major* metacyclic promastigotes using a 27½ G needle in a volume of 10 µl.

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### In vitro restimulation assay of lymphocytes from L. major-infected mice

A single-cell suspension was prepared from the retromaxillar LNs of *L. major*-infected mice as described (43). $CD4^+$ T cells were pre-enriched by negative selection using magnetic beads ($CD4^+$ T cell isolation kit; Miltenyi Biotec). $CD25^{hi}CD4^+$ and $CD25^-CD4^+$ T cells were then purified using a FACSVantage® cell sorter as previously described (21). The T cell subsets were > 98% pure as analyzed by flow cytometry. Isolated cells were labeled for 5 min at room temperature with 1.25 μM CFSE in PBS. $5 \times 10^4$ T cells were incubated with $1.4 \times 10^5$ bone marrow-derived dendritic cells (BMDCs) in 200 μl RPMI containing 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamicin, 55 μM 2-mercaptoethanol, 10% FCS, as previously described (48). BMDCs were previously incubated overnight with or without *L. major* metacyclics (parasites:BMDCs ratio = 5:1) and washed before culture with T cells. After 4 days of stimulation at 37°C in 5% $CO_2$, cells were analyzed by flow cytometry (see above) and culture supernatants were collected for cytokine assays (see below).

### $CD25^+$ T cell depletion

Mice were injected with 1 mg anti-CD25 (clone PC6C1; American Type Culture Collection) or isotype control (clone A1101-1) antibodies, as described before (45). Antibodies were produced using serum-free medium (BD Biosciences) and a CELLine™ device (BD Biosciences) according to the manufacturer's instructions. Antibodies were purified by protein G affinity chromatography (Pierce Chemical Co.). The efficiency of depletion was shown to be > 80%.

### Cytokine assays

Mouse IFN-γ, IL-2, IL-10, and GM-CSF were quantified in culture supernatants using the DuoSet Enzyme Linked Immunosorbent Assay (ELISA) system (R&D Systems). Alternatively, a multiplex assay (Linco Research, St. Charles, MO) was used following the manufacturer's protocol.

### Statistical analysis

Statistical analysis was performed using Prism (GraphPad Software Inc., San Diego, CA). For mice, group comparisons were made using the two-group Student's *t*-test. For human, the distribution of the values in the different groups was tested by Shapiro-Wilk normality test. If the distribution was normal, group comparisons were made using the two-group Student's *t*-test. If the distribution was not normal, a Box-Cox transformation was made and transformed values were tested again for their normality. If the distribution became normal after transformation, a two-group Student's *t*-test was made; otherwise, group comparisons were made using a Mann-Whitney test. A $p$ value < 0.05 was considered statistically significant. The Box-Cox transformation and the subsequent statistical analyses were conducted using SAS v9.

## Results

### The proportion of circulating $T_{regs}$ is increased in healthy elderly subjects

FOXP3 has been identified as the most specific marker of $T_{regs}$ in humans (32). We therefore investigated whether the number of $FOXP3^+CD4^+$ T cells was altered in elderly individuals (≥ 70-year old) compared with young adults (≤ 30-year old). Healthy donors were enrolled, applying criteria detailed in the Materials and Methods. Mean age of the elderly and young subjects was 82.8 ± 7.2 and 25.8 ± 2.7-year old, respectively.

The proportion of $FOXP3^+$ cells was significantly increased within $CD4^+$ T cells from elderly compared to young subjects (5.8 ± 0.4% versus 4.4 ± 0.4%; $p = 0.03$, unpaired *t*-test; N = 16/

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group; Fig. 1A and B). Because the level of FOXP3 per cell is an important factor determining $T_{reg}$ activity (49), we evaluated the mean fluorescence intensities (MFI) of FOXP3 staining, which were similar in elderly and young donors (Fig. 1C). High level of CD25 expression is also described as a characteristic feature of $T_{regs}$. The number of $CD25^{hi}CD4^{+}$ T cells was determined using a stringent gate to define $CD25^{hi}$ expression (Fig. 1A). The proportion of $CD25^{hi}$ cells in the $CD4^{+}$ T cell population was not significantly different in elderly and young subjects, although there was a trend towards increased proportion in the elderly (Fig. 1A and B; $p = 0.1$, Mann-Whitney test). $FOXP3^{+}CD4^{+}$ T cells were more frequent than $CD25^{hi}CD4^{+}$ T cells, in both young and elderly subjects (Fig. 1A and B; both $p < 0.001$). It has been recently described that $T_{regs}$ expressed low levels of the IL-7 receptor, CD127, and that this expression pattern better defines $T_{regs}$ in humans (50, 51). We therefore analyzed the percentage of $CD25^{+}CD127^{lo}CD4^{+}$ T cells and found an increased percentage of those cells in the elderly (6.5 ± 0.8% versus 3.9 ± 0.4% in elderly and young subjects, respectively; $p = 0.01$, *t*-test). Thus, whether assessed by FOXP3 or CD25, CD127 expression, the frequency of $T_{regs}$ is significantly increased in elderly humans.

### $T_{regs}$ from elderly and young subjects express the same functional markers

We then assessed the expression of markers on $FOXP3^{+}CD4^{+}$ T cells that have been previously associated with $T_{reg}$ phenotype and function (52), such as CD25, CTLA- 4 and GITR. Expression of CD25, CTLA-4 and GITR by $FOXP3^{+}CD4^{+}$ $T_{regs}$ was similar in young and elderly subjects (Table I; all $p > 0.05$). In both groups of subjects, $FOXP3^{+}CD4^{+}$ $T_{regs}$ expressed also higher levels of CTLA-4 than FOXP3-$CD4^{+}$ T cells (~ 40% versus 4%; $p < 0.0001$) (Table I). Similar data were obtained when $CD25^{hi}$ expression was analyzed (Table I). GITR expression was very low in all groups (< 7%), but $FOXP3^{+}CD4^{+}$ $T_{regs}$ expressed significantly higher levels than $FOXP3^{-}CD4^{+}$ T cells, in both groups of subjects (Table I). We also analyzed the proportion of naïve $T_{regs}$ based on their expression of CD45RA. The proportion of naïve $T_{regs}$ was lower in young individuals than the proportion of naïve $T_{effs}$, as previously reported (53). This proportion was further decreased in elderly individuals, although the difference did not reach statistical significance ($p = 0.09$; Table I). We also analyzed the expression of several molecules which have been reported to correlate with regulatory activity in human or mice $T_{regs}$, such as CD27 (54-56), PD-1 (57,58), TGFβRII (59), Granzymes A and B (60-62). CD27 expression by $FOXP3^{+}CD4^{+}$ $T_{regs}$ was similar in young and elderly subjects (Table I), in agreement with a recent publication (41). PD-1, TGFβRII and Granzyme A/B expression by $T_{regs}$ was also similar between young and old $T_{regs}$ (Table I).

FOXP3, CD25, GITR and CTLA-4 are not only markers for $T_{regs}$, but also are transiently upregulated on human non-$T_{regs}$ upon activation (22,63,64). Therefore, we investigated whether the increased percentage of $FOXP3^{+}CD4^{+}$ T cells in elderly subjects reflects increased numbers of activated T cells. To do that, we looked at the expression of CD69 by $FOXP3^{+}$ cells (65). Low CD69 expression (< 5%) was observed in $FOXP3^{+}CD4^{+}$ $T_{regs}$ from both young and elderly, and was similar between the 2 groups (Table I). Together, these data indicate that the phenotype of circulating $FOXP3^{+}CD4^{+}$ $T_{regs}$ is similar between young and elderly subjects, as assessed by multiple markers. Moreover, it appears unlikely that the increased proportion of $FOXP3^{+}CD4^{+}$ T cells in elderly individuals reflect recent activation, because it was not associated with the expression of a classical activation marker.

### The ratio of expression ($T_{effs}$ versus $T_{regs}$) of homing markers is similar between old and young subjects

The increased proportion of $T_{regs}$ in the blood from elderly subjects could be the consequence of their altered homing to tissues. To address this issue, we analyzed the expression by $T_{regs}$ and $T_{effs}$ of several T cell homing markers, namely CCR7, which mediates T cell entry into

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secondary lymphoid organs (66), α4β1 integrin, CXCR4 and CCR5, which allows T cell migration to inflamed tissues, and the gut-associated α4β7 integrin (67-72).

$FOXP3^+CD4^+$ $T_{regs}$ expressed similar levels of CCR5, CCR7 and α4β1 in young and old subjects (Table I), as recently reported for CCR7 (41). In contrast, $FOXP3^+CD4^+$ $T_{regs}$ expressed lower levels of α4β7 in aged subjects compared to those in young subjects (Table I). However, the same change in expression pattern was observed in $T_{effs}$ from old subjects compared with young $T_{effs}$ (Table I). Indeed, when the ratio of expression ($T_{effs}$ versus $T_{regs}$) was calculated for homing markers, no difference was found between old and young subjects (Table I). Only CXCR4 expression was different, with a specific decrease on old $T_{regs}$. Interestingly, CXCR4 has been associated with $T_{reg}$ migration and maintenance in the bone marrow (71). All together, these data suggest that the increased proportion of circulating $T_{regs}$ in the elderly is not likely due to a selective dysregulation of $T_{reg}$ homing to tissues, although we cannot rule out a role for decreased CXCR4 in the retention of $T_{regs}$ in the blood.

## The proportion of $T_{regs}$ is increased in aged mice

Further determination of $T_{reg}$ dynamics in humans being difficult due to the obvious restricted access to tissues, we pursued our analysis through the characterization of $T_{reg}$ markers in multiple lymphoid organs from aged (≥ 20-month old) and young adult C57BL/6 mice (≤ 3-month old). In all tissues, the proportion of $FoxP3^+CD4^+TCR^+$ cells was significantly higher in aged mice compared to young mice (Fig. 2A). FoxP3 level per cell was identical in cells from aged and young mice (Fig. 2B). In contrast, the proportion of circulating $FoxP3^+CD4^+TCR^+$ cell was the same in the blood of aged and young mice (Fig. 2A). When the proportion of $CD25^{hi}CD4^+TCR^+$ cells was analyzed, we found a significant accumulation of these cells in peripheral (pLNs) and mesenteric lymph nodes (mLNs), but not in the spleen or the blood, of aged mice compared to young mice (Fig. 2C). The proportion of $CD25^{hi}CD4^+TCR^+$ cells was always lower than the proportion of $FoxP3^+CD4^+TCR^+$ cells in all tissues, in young and in aged mice (all $p < 0.05$). Furthermore, the proportion of $T_{regs}$ expressing CD103, a specific marker for natural $T_{regs}$ (73,74), was increased in aged mice (Fig. 2D).

We also analyzed the expression by $FoxP3^+CD4^+$ T cells of markers associated with $T_{reg}$ function. $FoxP3^+CD4^+TCR^+$ $T_{regs}$ express high levels of GITR, CTLA-4 and PD-1 in both groups of animals, with a trend towards higher expression in aged mice, depending on the tissue analyzed (Table II and Fig. 2D). The activation marker CD69 was more expressed by old $FoxP3^+$ cells than young $FoxP3^+CD4^+TCR^+$ cells, and that in all analyzed tissues (Table II and Fig. 2D). However, there was a trend towards decreased CD69 expression on circulating $T_{regs}$ from aged mice ($p = 0.07$, Table II). The trend towards decreased proportion of CD27 and CCR7 expression in old $T_{regs}$ (Table II) also suggests increased $T_{reg}$ differentiation in aged mice.

## $T_{reg}$ depletion increases in vitro $T_{eff}$ function in the elderly

Our phenotypic data show increased proportion of cells with $T_{reg}$ characteristics in aged humans and mice. However, because $FOXP3^+$ or $CD25^{hi}$ cells are not always functionally suppressive, we further characterized $T_{reg}$ function in elderly humans by analyzing the effect of $T_{reg}$ depletion on CD4 function. Because of its intracellular localization, FOXP3 expression cannot be used to deplete cells. However, the number of $FOXP3^+$ cells was significantly reduced following the depletion of $CD25^+$ cells from total $CD4^+$ T cells and no significant difference was observed between individuals (Fig. 3A). In absence of stimulation, $CD4^+$ T cells, with or without $T_{regs}$, did not proliferate or express activation markers (data not shown). After 3 days of PHA stimulation, $T_{reg}$- depleted cells proliferated better than total $CD4^+$ T cells (not depleted of $T_{regs}$) in 4 of the 7 tested individuals (group A: subjects □, Δ, ∇, +; Fig. 3B).

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In contrast, in 3 individuals, $T_{reg}$ depletion did not result in increased proliferation after PHA stimulation (group B: subjects ◊, ○, ×; Fig. 3B).

Because proliferation does not recapitulate overall CD4 T cell function, particularly for memory cells, we also analyzed other read-outs of T cell activation, such as expression of the activation markers CD69 and CD95 (Fig. 3C). PHA stimulation induced CD69 and CD95 up-regulation and cytokine production in all PHA-stimulated cultures, compared with unstimulated cultures (data not shown). In 3/4 samples of the group A, CD69 and CD95 expression both increased following $T_{reg}$ depletion (Fig. 3C). In the 4$^{th}$ subject from that group (subject +), $T_{reg}$ depletion led to decreased CD69 expression, but stable and high CD95 expression (Fig. 3C). Of note, this individual exhibited the highest level of proliferation, before and after $T_{reg}$ depletion. In group B individuals, depletion did not change CD95 expression and increased CD69 expression in only one individual (subject ○) (Fig. 3C). Depletion of $T_{regs}$ therefore increased activation and/or proliferation of $CD4^+$ $T_{effs}$ in 5/7 elderly after 3 days of culture with PHA. Of note, this percentage of responders is similar to that reported in young individuals, in whom $T_{reg}$ depletion led to increased proliferation in response to PHA in 4/6 individuals (75). These data suggest that $T_{regs}$ in elderly humans are functional in most individuals and may be able to inhibit $T_{eff}$ responses *in vitro*.

## $T_{regs}$ from old mice suppress anti-CD3-induced proliferation of $T_{effs}$

To more clearly delineate the functional activity of $FoxP3^+$ cells during aging, we sorted $FoxP3^+$ T cells from pLNs of aged (15- to 18-month old) or young (2- to 4-month old) FoxP3-GFP knock-in C57BL/6 mice. $T_{effs}$ ($CD4^+GFP^-$ T cells) were sorted from pLNs of 2-month old mice, to eliminate the confounding effect of decreased responsiveness of aged $T_{effs}$ (reviewed by (76,77)). $T_{regs}$ from pLNs of aged mice were more suppressive on a per cell basis than those from young mice, suppressing 80% of anti-CD3-stimulated $T_{eff}$ proliferation at a $T_{eff}$:$T_{reg}$ ratio of 1:1 in comparison to the 54% suppression achieved by young $T_{regs}$ (Fig. 4). At a $T_{eff}$:$T_{reg}$ ratio of 10:1, 20% suppression was observed with old $T_{regs}$, compared to the 3% induced by young $T_{regs}$ (Fig. 4). Interestingly, $T_{effs}$ from aged mice could be inhibited equally by $T_{regs}$ from old and young mice (data not shown).

## L. major infection spontaneously reactivates in aged mice

We next reasoned that, if $T_{reg}$ function and proportion were increased with age, it would interfere with the ability of the aged hosts to control chronic infections. To test this hypothesis, we used the *L. major* model, in which $T_{regs}$ have been shown to play a major role in lesion reactivation (44,45). After inoculation of $10^3$ metacyclic promastigotes of *L. major* into the ear dermis, young C57BL/6 mice develop a small lesion that resolves spontaneously within 12 weeks post-inoculation, although a few viable parasites persist in the site of the former lesion and in the draining LNs (44). 8 weeks post-healing, 5% of the infected mice exhibited clinical signs of lesion reactivation (Fig. 5). Importantly, spontaneous reactivation increased with aging, until 75% of the mice had reactivated at 24 months post-healing (Fig. 5), suggesting increased $T_{reg}$ activity in aged *L. major*-infected mice.

We previously reported that $T_{regs}$ from *L. major*-infected young mice are able to respond specifically to *L. major* (78). Therefore, we analyzed whether *L. major* specific $T_{reg}$ activity was detectable at the time when spontaneous disease reactivation occurs. $CD25^+CD4^+$ $T_{regs}$ were purified from draining LNs of aged mice or young mice, which had all been infected when they were 2-month old, and were restimulated *in vitro* with bone marrow-derived dendritic cells (BMDCs), infected or not with *L. major* metacyclic promastigotes. In aged mice, extensive proliferation of $T_{regs}$ was detected in response to both uninfected and infected BMDCs (Fig. 6A). The fact that old $T_{regs}$ from infected mice proliferate in presence of uninfected BMDCs may reflect their higher state of *in vivo* activation, a finding in agreement

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with their higher expression of several activation markers on $T_{regs}$ from non-infected mice (Table II and Fig. 2). However, the lower MFI observed in the culture with infected BMDCs suggests a more sustained proliferation of $T_{regs}$ in this condition. $T_{regs}$ from aged mice also produced cytokines in response to infected BMDCs, although at a lower level than young $T_{regs}$ (Fig. 6B). Taken together, these results suggest that the $T_{reg}$ activation level is higher in aged mice, potentially through antigen-independent pathways, but the proportion of *L. major* specific $T_{regs}$ and/or their per cell basis capacity to respond is lower. There are several potential explanations for these results. First, as antigen load increases, more $T_{regs}$ are stimulated by *L. major* antigen *in vivo* and continue to proliferate when they are removed from the animal, seemingly non-specifically. Second, $T_{regs}$ that are not specific for *L. major* are expanded *in vivo*, recognize and respond to endogenous antigens displayed on BM-DCs. Third, the proliferation of aged $T_{regs}$ in response to uninfected BM-DCs has nothing to do with antigen recognition by $T_{regs}$ but could be due to soluble factors released from BMDCs that cause $T_{regs}$ to proliferate. Without *L. major* specific MHC class II tetramer, it is difficult to address the frequency of *L. major* specific $T_{regs}$, how this frequency changes with age, and whether exogenous/endogenous antigens versus soluble factors drive $T_{reg}$ proliferation.

$CD25^{-}CD4^{+}$ $T_{effs}$ from aged mice produced IL-2 and GM-CSF after stimulation with infected BMDCs, albeit at a lower level than those from young mice, and a similar amount of IL-10 (Fig. 7A). Importantly, the co-culture of $T_{effs}$ and $T_{regs}$ clearly decreased the production of both IL-2 and GM-CSF in aged mice, as well as in young mice (Fig. 7A), confirming the maintained suppressive capacity of $T_{regs}$ in aged mice and their potential to suppress protective immune responses in aged *L. major*-infected mice. Increased IL-10 production was observed in the $T_{reg}$:$T_{eff}$ co-cultures, likely due to the IL- 10 production by $T_{regs}$, but levels were similar between aged and young T cells (Fig. 7A). Because $T_{reg}$ purification on the basis of CD25 expression may lead to their contamination by $T_{effs}$, we confirmed those data by purifying $T_{regs}$ from old FoxP3-GFP knock-in mice that had been infected by *L. major* when they were young. As shown in Fig. 7B, significant suppression of IFN-γ production by $T_{effs}$ was achieved when increasing numbers of $T_{regs}$ were added to $T_{eff}$ culture stimulated with *L. major*-infected BMDCs. No major effect was observed for IL-10 production (data not shown).

## $T_{regs}$ play a critical role in L. major reactivation in aged mice

To directly address the role played by $T_{regs}$ in *L. major* reactivation in old mice, we treated old *L. major*-infected mice (> 48-week old, which had been infected when they were young) with anti-CD25 Ab or isotype control, following the experimental protocol that we had previously used (45). As shown in Fig. 8, *in vivo* depletion of $T_{regs}$ in the anti-CD25-treated group significantly increased IFN-γ production by the T cells purified from the infection site and draining LNs. As expected, anti-CD25 treatment significantly increased IFN-γ production in young infected mice (Fig. 8B). However, IL-10 production did not change.

## $T_{regs}$ play a critical role in the exacerbation of L. major infection in aged mice

Another important and related question is whether the increased proportion of functionally suppressive $T_{regs}$ in aged hosts plays a role in the increased disease severity observed for multiple infections in such aged hosts. We have previously shown that lesion size is a direct indication of parasite growth. As shown in Fig. 9A, primary infection with *L. major* induces a more severe course of disease in old mice, compared with young mice. Indeed, lesions appeared 3 weeks post-infection in both groups, but tended to be larger in old animals already at 4 weeks post-infection. Disease severity was clearly apparent at all the following time points. Moreover, 1 out of the 3 old infected animals lost the infected ear at 7 weeks post-infection, through an acute necrotic process, at a time when lesions were starting to heal in young animals. To determine whether the age-related increased proportion of $T_{regs}$ had played a major role in such an exacerbated pathological process, we treated old mice (> 43-week old) with anti-CD25 Ab

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or isotypematched Ab control at the time they were infected with *L. major* parasites, and then twice a week. Infection outcome was then monitored by measuring the lesion size. As shown in Fig. 9B, treatment with anti-CD25 Ab significantly reduced the lesion size at 4 and 6 weeks, suggesting the importance of $T_{regs}$ in the increased severity of *L. major* infection in aged animals.

## Discussion

In this study, we investigated the proportion, phenotype and suppressive function of $T_{regs}$ in aged mice and humans. Our study shows increased proportion of $FoxP3^+CD4^+$ T cells in tissues of aged C57BL/6 mice as well as in the blood of healthy elderly humans. Importantly, our *in vitro* and *in vivo* data strongly support our hypothesis that $T_{regs}$ inhibit immune responses in aged hosts, contributing to reactivation of chronic infectious diseases.

Our data show a significantly increased proportion of $FoxP3^+CD4^+$ T cells in multiple lymphoid tissues from aged C57BL/6 mice, compared to young mice, as already reported in aged Balb/c mice (34). Increased proportion of $CD25^{hi}CD4^+$ T cells in the lymphoid tissues of aged animals was also found in these animals, in agreement with other studies (35,36). Interestingly, the proportion of $CD25^{hi}CD4^+$ T cells is always lower than that of $FoxP3^+CD4^+$ T cells in both aged and young hosts, supporting the notion that CD25 expression defines only a portion of $T_{regs}$ (79). FoxP3 expression clearly plays a crucial role in the maintenance of $T_{reg}$ activity, its ablation in adult mice leading to catastrophic autoimmune diseases (80). In contrast, $CD25^{-/-}$ mice exhibit reduced numbers of $FoxP3^+$ cells but those cells are fully able to suppress *in vitro* (81,82).

Increased proportion of $FOXP3^+CD4^+$ T cells was also found in the blood of healthy elderly humans. There was a trend towards increased proportion of $CD25^{hi}CD4^+$ T cells in the blood of elderly donors, although the difference was not significant. It has recently been shown that low level of the IL-7 receptor, CD127, increases the specificity of $T_{reg}$ characterization for human cells (50,51). Significantly increased proportion of $CD25^+CD127^{lo}CD4^+$ T cells was also found in elderly humans. Previous studies using $CD25^{hi}$ expression to characterize $T_{regs}$ have reported similar (53,83) or increased $T_{reg}$ proportions (37-40) in elderly individuals. Discordances between studies may arise from differences in the phenotyping techniques and/or the characteristics of the studied populations (e.g. mean age, health status, or criteria of exclusion). In mice, the proportion of $T_{regs}$ in the blood was not increased; in contrast, we observed an increased proportion of circulating $FOXP3^+CD4^+$ $T_{regs}$ in elderly humans compared to young subjects. Differences between mice and humans could reflect the fact that FoxP3 is less specific of the $T_{reg}$ lineage in humans than in mice, because its expression is transitorily induced in $T_{effs}$ following TCR activation (64). However, the low level of CD69 expression on $FoxP3^+$ cells argues against that hypothesis. Alternatively, increased triggering of the $T_{reg}$ compartment in humans may come from the higher level of stimulation of the immune system exerted by constant exposure to pathogens. Of note, we have found that, in young adults, the proportion of $FoxP3^+CD4^+$ T cells was higher in tonsils than in blood (approximately 10% versus 4%, unpublished data), similar to the murine data. Study of tissue $T_{regs}$ in elderly humans has not yet been undertaken and will be essential to clarify this issue.

An important question raised by our data is how $T_{regs}$ accumulate with age. Circulating $T_{regs}$ in elderly humans do not express specifically altered patterns of homing receptors, suggesting that defective tissue homing is not likely to explain their increased proportion, which is confirmed by the fact that increased $T_{reg}$ proportion was found in all lymphoid tissues in aged mice. Peripheral $T_{regs}$ could derive either from $T_{regs}$ that have developed in the thymus or converted non-$T_{regs}$. In aged mice, the total number of $CD25^+CD4^+$ single positive (SP) thymocytes decreased following the reduction in thymocyte numbers, although the percentage

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of $CD25^+$ cells increased in $CD4^+$ SP thymocytes (35), suggesting a reduced thymic $T_{reg}$ input in those mice. $T_{regs}$ in aged hosts are mostly memory cells with a highly differentiated phenotype as shown in this study and by others (35,41,79,84,85). These data suggest that $T_{regs}$ in elderly might come from thymic-derived $T_{regs}$ that have proliferated in the periphery. Few data are available on $T_{reg}$ *in vivo* turnover, particularly in aging. In young mice, the $CD25^+CD4^+$ $T_{reg}$ population is composed of two subsets with distinct homeostasis (86), one subset exhibiting a rapid proliferation rate, whereas the other subset did not divide, but was long-lived. These findings suggest that, although $T_{regs}$ can proliferate *in vivo*, resting thymic-derived $T_{regs}$ may also persist for long time *in vivo* and this population may participate in the maintenance of peripheral $T_{reg}$ numbers. In healthy humans, young and elderly alike, $CD45RO^+$ $T_{regs}$ had a rapid doubling time compared with those of memory or naïve $CD4^+$ T cells (40). However, human $CD45RO^+$ $T_{regs}$ exhibited short telomere length -in both young and elderly individuals-, and did not upregulate telomerase after activation (40,87), raising the question of whether peripheral proliferation of thymic-derived $T_{regs}$ is sufficient to maintain the increased proportion of $T_{regs}$ seen in aged hosts.

$T_{regs}$ might also be generated from CD25-$CD4^+$ T cells in the periphery. In both mice and humans, transforming growth factor-β (TGF-β) induces $CD25^-FOXP3^-CD4^+$ T cells to become $FOXP3^+$ $T_{regs}$ (reviewed by (88)), although the stability of such conversion, as well as the requirement for other molecules than TGF-β, is still debated (89-93). The state of activation of DCs, as well as the proportion of different DC subsets (mDC versus pDC), also plays a role in both $T_{reg}$ conversion and peripheral expansion (25,26). Interestingly, DC subsets and maturation levels are changed during aging (94,95), and this could play a role in $T_{reg}$ accumulation in elderly individuals. However, the contribution of such converted cells to the pool of circulating $T_{regs}$ remains an open question, since no marker(s) have been found that distinguish between natural $FoxP3^+$ $T_{regs}$ and induced $FoxP3^+$ $T_{regs}$. Of note, our published data support the idea that no neo-generation of $T_{regs}$ occur during *L. major* infection (78).

An extensive phenotypic characterization of $T_{regs}$ (defined as $FoxP3^+CD4^+$ T cells) was performed in the old mice. Expression of CD25 by $T_{regs}$ from aged animals was lower than in their young counterparts, a result in agreement with previous studies (34,36). The underlying mechanisms have not been completely elucidated, and could include the loss of CD25 expression by $FoxP3^+CD4^+$ $T_{regs}$. In support of that argument, *in vivo* expanded $T_{regs}$ become $CD25^{low}$ while maintaining similar levels of other $T_{reg}$-associated molecules (96). Similarly, CD25 turnover may be increased in aging, as suggested by increased soluble CD25 levels in the serum of aged humans (97,98). In contrast to decreased CD25 expression, expression of other markers associated with $T_{reg}$ function such as GITR, CTLA-4 or PD-1, was maintained or increased on $FoxP3^+CD4^+$ $T_{regs}$ from aged mice, depending on the tissue analyzed. The activation marker CD69 was also more expressed by old $FoxP3^+CD4^+$ $T_{regs}$ than young $T_{regs}$, and that in all analyzed tissues except in the blood in which the frequency of $CD69^+$ $T_{regs}$ tended to decrease, suggesting an accumulation of activated $T_{regs}$ in tissues in aged mice. Similarly, we found a trend towards decreased proportion of CD27 and CCR7 expression in old $T_{regs}$ (Table II), suggesting increased $T_{reg}$ differentiation. Taken together, those data suggest that $T_{regs}$ are more activated in tissues from aged mice than in young mice.

We also extensively characterized circulating $T_{regs}$ in healthy elderly humans, in comparison to young $T_{regs}$, and did not find major age-related differences in the expression of molecules that have previously been associated with $T_{reg}$ function, such as CD27, PD-1, TGFβRII or Granzymes (A and B). Similar to our data in old mice, there was a trend towards decreased expression of CD25 on $FoxP3^+CD4^+$ $T_{regs}$ in elderly humans. Low and constant expression of CD69 was found on both young and old human blood $T_{regs}$, in contrast to its decreased expression in circulating $T_{regs}$ of aged mice, suggesting a difference in activation patterns between species.

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Because phenotype does not recapitulate functional activity, we analyzed $T_{reg}$-mediated suppression during aging. Interpretation of suppression assays in aging mice can be confounded by the fact that CD25 expression is not accurate to recapitulate FoxP3 expression (and $T_{reg}$ activity) in aged mice (35,36,79). Therefore, to circumvent this caveat, we used FoxP3-GFP knock-in mice, and clearly show that $FoxP3^+$ cells from aged mice have a greater *in vitro* suppressive activity on a per cell basis than their young counterparts. Old $T_{regs}$ were also able to suppress $T_{effs}$ from aged mice. The use of FoxP3-GFP cells in our study may explain the difference between our results and previous studies that showed similar or decreased suppressive activity of old $CD25^{hi}$ $T_{regs}$ compared to young $T_{regs}$. As we show that many of the $FoxP3^+$ cells in aged mice are $CD25^{lo}$, the previous studies sorting old $T_{regs}$ based on CD25 expression may have been by contamination of the effector population with $FoxP3^+CD25^-$ $T_{regs}$. In addition, $CD25^+$ $T_{regs}$ from aged chronically *L. major*-infected mice also maintained their responsiveness to *L. major* antigens and their ability to suppress IFN-γproduction by $T_{effs}$ in response to *L. major*-infected DCs. In elderly humans, $T_{regs}$ appear functional, their *in vitro* depletion leading to increased $CD4^+$ T cell function in most individuals, although the functional consequence of such depletion was modest. Several factors could have contributed to such a result, including the expected heterogeneity between human subjects, the use of old autologous $T_{effs}$ and the fact that human $T_{regs}$ could not be depleted on the basis of their FOXP3 expression.

Increased proportion of functional $T_{regs}$ in aging may translate into dampened immune responses in aged hosts. Strongly supporting this hypothesis, spontaneous reactivation of *L. major* lesions, which we have previously shown to result from $T_{reg}$ accumulation in the chronic infectious site (44,45), occurred in the majority of aged infected mice. We also demonstrate a direct role of $T_{regs}$ in such reactivation, because *in vivo* depletion of $T_{regs}$ in old mice, infected young, significantly increased the production of the effector cytokine IFN-γ by the $T_{effs}$ purified from the infection site and draining LNs. Furthermore, our experiments show that $T_{regs}$ play a critical role in the increased disease severity of *L. major* infection in old mice. Taken together, our data strongly suggest that the age-associated $T_{reg}$ accumulation is likely to play a major role in the increased severity of chronic infections, as well as the reactivation of chronic infections, in aged mice and humans. Our data in an infectious model are thus in agreement with the recent study showing that $T_{reg}$ accumulation in aged mice plays a crucial role in inhibiting the activation of anti-tumor responses (34).

How $T_{regs}$ act is still unclear and we have not formally ruled out that the mechanisms mediating such suppression may be different in old $T_{regs}$. Expression of activation markers by old tissue $T_{regs}$ was increased, whereas, on a per a cell basis, old $T_{regs}$ produced less IL-10, which has been shown to play a role in $T_{reg}$-mediated suppression in the *L. major* model (44). $T_{regs}$ from old murine lymphoid tissues were clearly suppressive both *in vivo* and *in vitro*, although the mechanisms by which old $T_{regs}$ suppress will need to be further investigated in the future.

An alternative explanation of increased severity and frequency of infectious diseases in aged hosts could be the decreased capacity of aged $T_{effs}$ to proliferate and produce cytokines. Indeed, profound, diverse alterations in TCR-mediated activation have been described in T cells from aged mice and humans (99,100). Accordingly, decreased cytokine production by old $T_{effs}$, including in response to *L. major* antigens, was observed in both our study (Fig. 6) and previous studies (36,79). Similarly, in 3/7 old individuals, depletion of CD25 resulted in partial or absent improvement of CD4 T cell function, suggesting intrinsic decreased responsiveness of the $T_{eff}$ subset in these individuals. However, when old memory TCR Tg $CD4^+$ T cells were transferred into young animals, they function as well as their young counterparts (101) suggesting that antigenic-specific protective effector mechanisms are retained in old hosts. In support of that hypothesis, purified $CD4^+$ $T_{effs}$ from old animals produced large amounts of the effector cytokine IFN-γ after restimulation with *L. major*-antigens, which was blocked by

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co-culture with $T_{regs}$. Furthermore, $T_{reg}$ depletion *in vivo* allowed for better control of *L. major*, suggesting that aged $T_{effs}$ had retained some function in such experimental conditions. Of note, in the model of infection with high doses of *L. major*, which is less sensitive to $T_{reg}$ regulation than our model, old mice controlled infection as well as young mice (102), further suggesting that $T_{eff}$ function is not completely abolished in old animals.

Taken together, our findings suggest that decreased T cell responsiveness in aged hosts results from both intrinsic defects and an altered balance between stimulatory/regulatory mechanisms, the exact contribution of each mechanism likely to be variable depending on the context (infectious diseases/auto-immunity/cancer). Manipulation of $T_{reg}$ numbers and/or activity may therefore be critical to enhance immune responses in the aged, and may be envisioned to enhance control of chronic infectious diseases, as well as vaccine efficiency, in this fragile population.

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## Abbreviations

$T_{regs}$, regulatory T cells
$T_{effs}$, effector T cells
*L. major*, *Leishmania major*
FoxP3, Forkhead box P3
CTLA-4, Cytotoxic T Lymphocyte Associated antigen 4
GITR, Glucocorticoid-Induced Tumor necrosis factor Receptor
MFI, Mean Fluorescence Intensity
PHA, phytohemagglutinin
LNs, lymph nodes
pLNs, peripheral LNs (retromaxillar and popliteal)
mLNs, mesenteric LNs
PBMCs, Peripheral Blood Mononuclear Cells
FCS, Fetal Calf Serum
DMSO, Dimethyl sulphoxide
CFSE, Carboxyfluorescein diacetate, succinimidyl ester
ELISA, Enzyme Linked Immunosorbent Assay
DCs, Dendritic cells
BMDCs, Bone Marrowderived DCs
pDCs, plasmacytoid DCs
mDCs, myeloid DCs
TGF-β, Transforming Growth Factor-β

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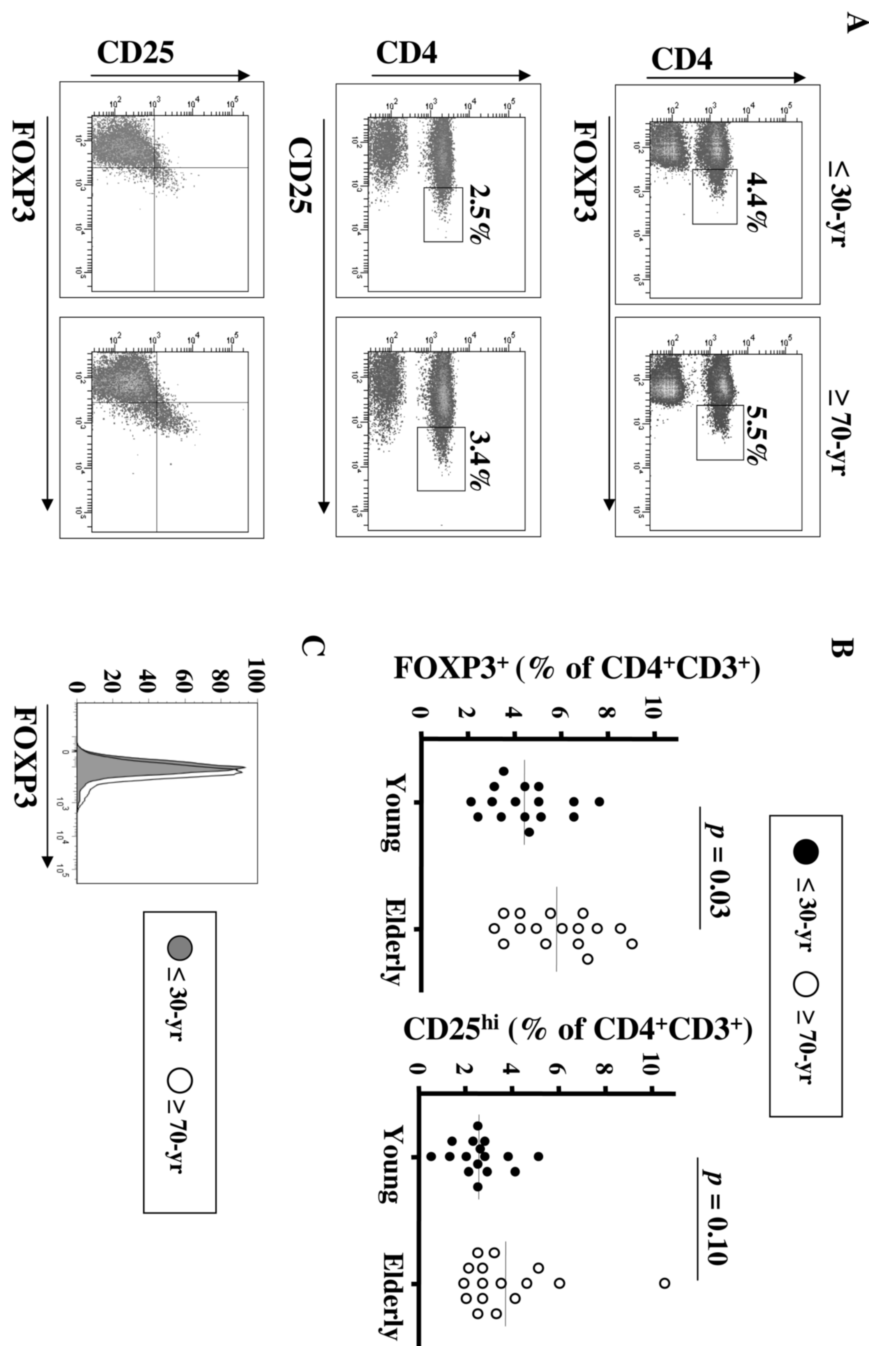


**FIGURE 1. $T_{reg}$ frequency is increased in the blood of elderly individuals**

$T_{reg}$ frequency was analyzed in PBMCs from 16 young (≤ 30-year old) and 16 elderly (≥ 70-year old) subjects. For FOXP3 staining, $CD4^{-}CD3^{+}$ cells were used as negative control to determine the positivity threshold in $CD4^{+}CD3^{+}$ T cells. High expression of CD25 in $CD4^{+}$ T cells was determined based on the absence of $CD25^{hi}$ cells within the CD3- cells. (A) Representative expression of FOXP3 and CD25 in young and elderly subjects. The percentages of $FOXP3^{+}CD4^{+}$ and $CD25^{hi}CD4^{+}$ cells in gated $CD3^{+}$ T cells in a representative young and an elderly subject are shown in the upper and middle plot respectively. Expression of FOXP3 and CD25 in gated $CD4^{+}CD3^{+}$ T cells in a representative young and an elderly subject is shown in the lower plot. (B) Percentages of $FOXP3^{+}$ and $CD25^{hi}CD4^{+}CD3^{+}$ T cells in young and

elderly subjects. Horizontal lines represent the mean values for each group. (C) Representative FOXP3 expression in gated $CD4^+CD3^+$ T cells from a 23-year old (filled line) and a 72-year old (unfilled line) donor.

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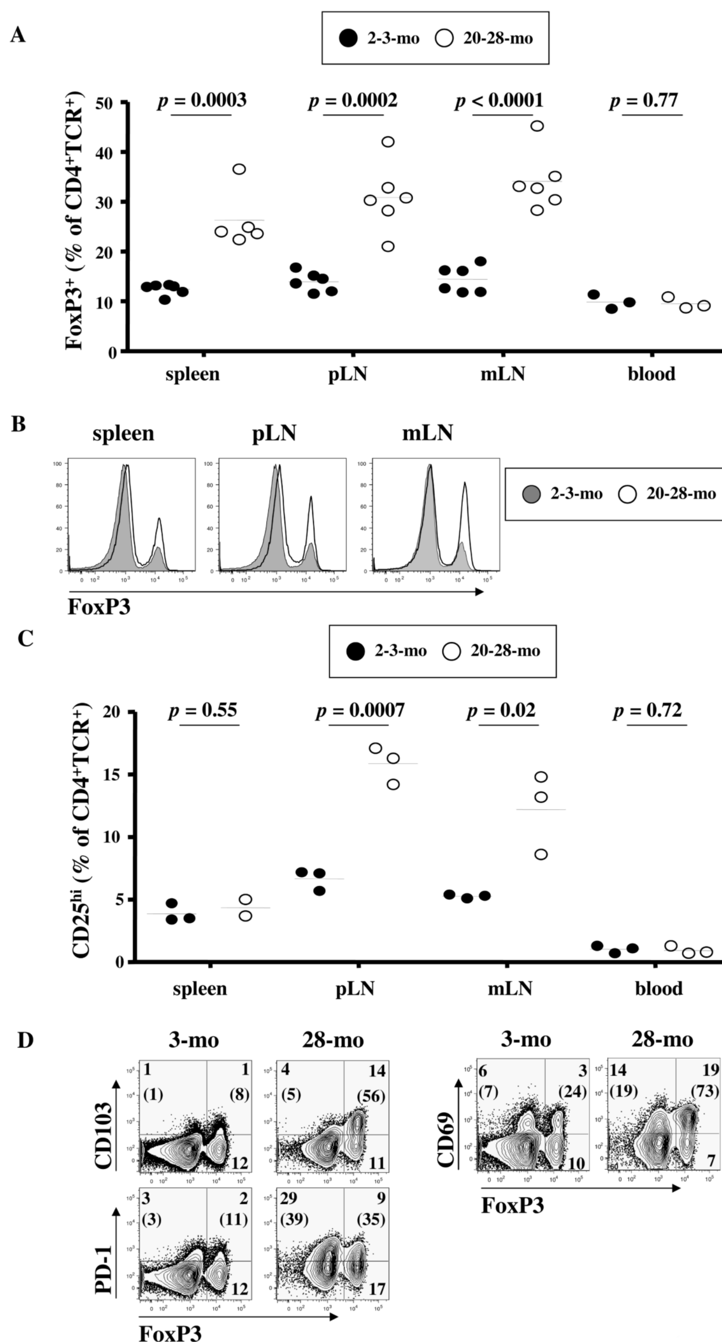


**FIGURE 2. Increased proportion of $T_{regs}$ in aged mice**

Single cell suspensions from spleens, peripheral (pLNs), mesenteric (mLNs) lymph nodes and blood were first stained for the surface markers CD4, TCR, CD25, CD69, CD103 and PD-1, followed by staining for the intracellular markers FoxP3. Flow cytometry analysis on gated $CD4^+TCR^+$ cells is shown. (A) Percentages of $FoxP3^+$ cells in the $CD4^+TCR^+$ cell populations from 2-3- month old (closed circles) or 20-28-month old (open circles) mice. Horizontal lines represent the mean values for each group. (B) Representative overlay of Foxp3 expression in spleen, pLNs and mLNs cells from a 3-month (filled line) and a 28-month (unfilled line) old mouse. (C) Percentages of $CD25^{hi}$ cells in the $CD4^+TCR^+$ cell populations from 2-3-month old (closed circles) or 20-28-month old (open circles) mice. Horizontal lines represent the mean

values for each group. (D) Expression of FoxP3 with CD69, CD103 and PD-1 is shown for splenic cells of a 3-month (left panel) or 28-month (right panel) old mouse, representative of 6 mice each. Values represent the percentages of each population in the indicated quadrant. Values in parenthesis are the percentages of gated FoxP3- (left) or $FoxP3^+$ (right) cells that are positive for the Y axis marker.

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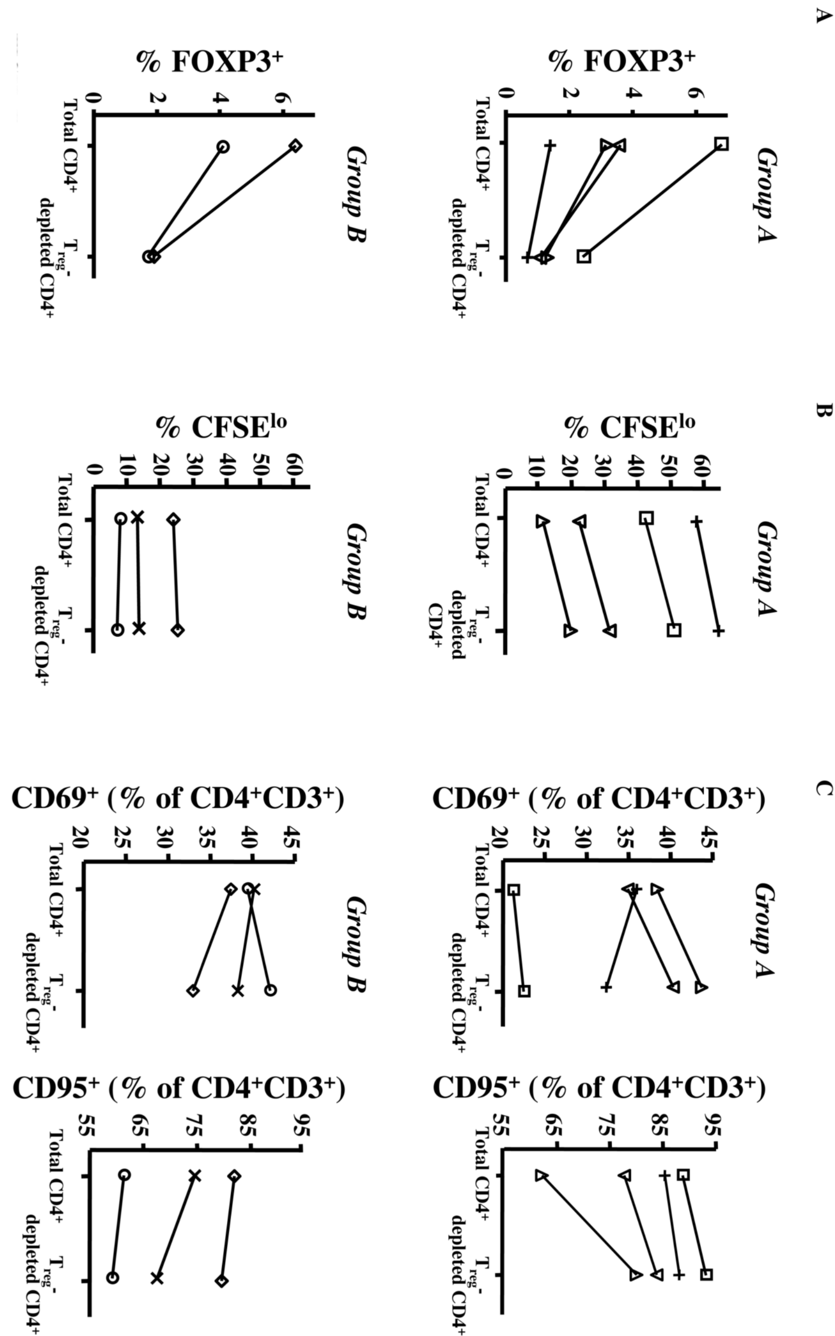


**FIGURE 3. Depletion of $CD25^{hi}CD4^{+}$ T cells from the blood of elderly individuals increased CD4 T cell function**

5 × 105 CFSE-labeled total $CD4^{+}$ or $T_{reg}$-depleted $CD4^{+}$ ($CD25-CD4^{+}$) T cells from 7 elderly individuals (≥ 70-year old) were cultured with 2 × $10^{5}$ autologous $CD14^{+}$ monocytes and 2 μg/ml PHA for 3 days. (A) Percentage of $FOXP3^{+}$ cells before $CD25^{hi}$ depletion (Total $CD4^{+}$, left) and after $CD25^{hi}$ depletion ($T_{reg}$-depleted $CD4^{+}$, right). The values from the same subject are linked by a line. Group A is composed of the 4 individuals in whom $T_{reg}$ depletion led to increased proliferation, whereas group B comprised the 3 individuals who exhibited no increase in proliferation after PHA stimulation. The percentage of $FoxP3^{+}$ cells following CD25 depletion was determined in all subjects, except subject X. (B) CFSE dilution and (C)



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the expression of CD69 and CD95 markers were analyzed by flow cytometry on gated $CD4^+CD3^+$ T cells. Percentages of dividing cells (CFSElow, corresponding to cells that have divided at least once), in total or $T_{reg}$-depleted $CD4^+$ T cells, are shown in (B). Percentages of $CD69^+$ and $CD95^+$ cells, in total or $T_{reg}$-depleted $CD4^+$ T cells, are shown in (C). Each individual is represented by the same symbol in panels (A), (B) and (C).

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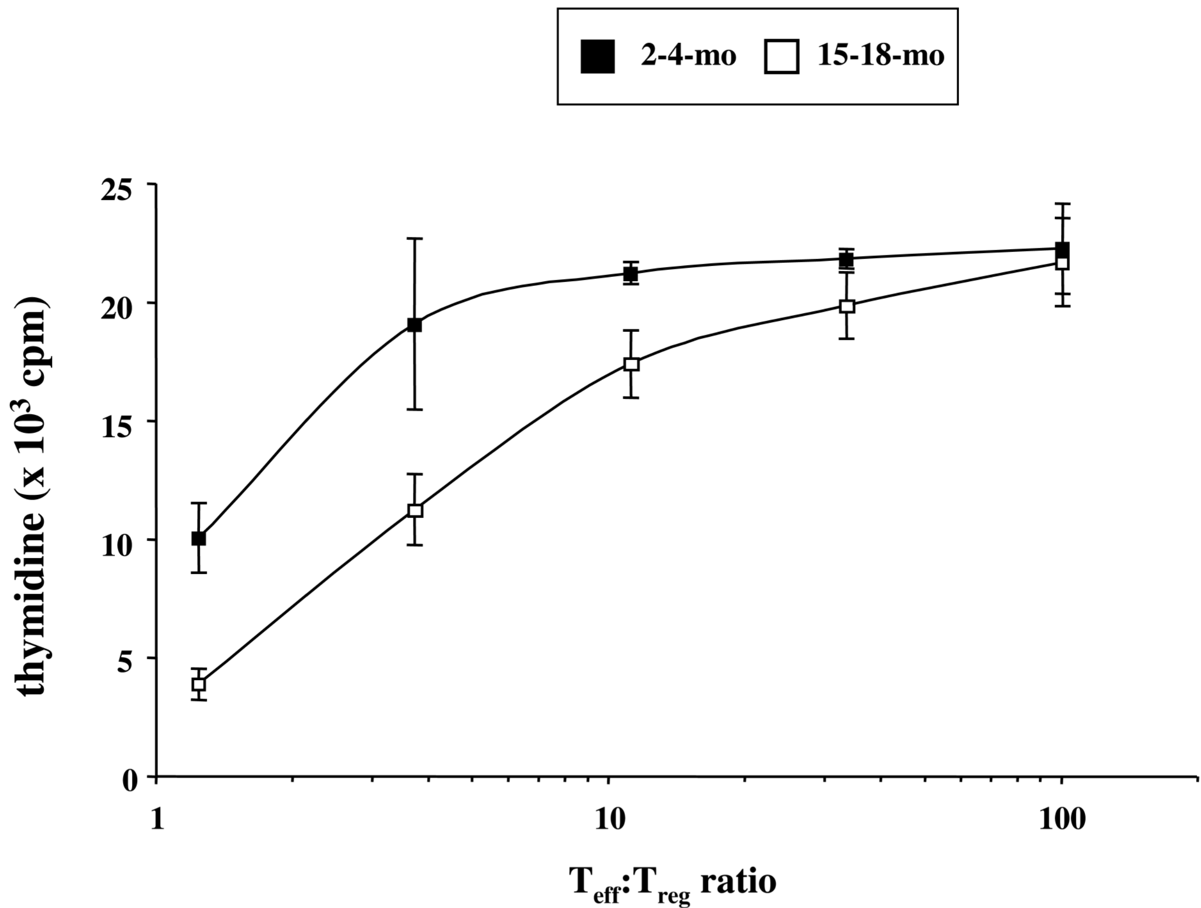


**FIGURE 4. FoxP3$^+$CD4$^+$ T cell suppressive function is intact in aged mice**

$5 \times 10^4$ GFP$^-$CD4$^+$ T cells ($T_{effs}$) were sorted from LNs of 2-4-month old FoxP3-GFP knock-in C57BL/6 mice, and stimulated in triplicate with 0.5 μg/ml anti-CD3 and $1 \times 10^5$ irradiated T cell-depleted spleen cells from the same mice. GFP$^+$CD4$^+$ T cells ($T_{regs}$) were sorted from LNs of 2-4-month (closed squares) or 15-18-month (open squares) old mice and co-cultured with $T_{effs}$ at different $T_{eff}$:$T_{reg}$ ratios, ranging from 1.3:1 to 101:1. Proliferation was measured by thymidine incorporation in the last 22 hours of a 3-day culture. In the absence of $T_{regs}$, 21,800 ± 2,075 cpm were counted.

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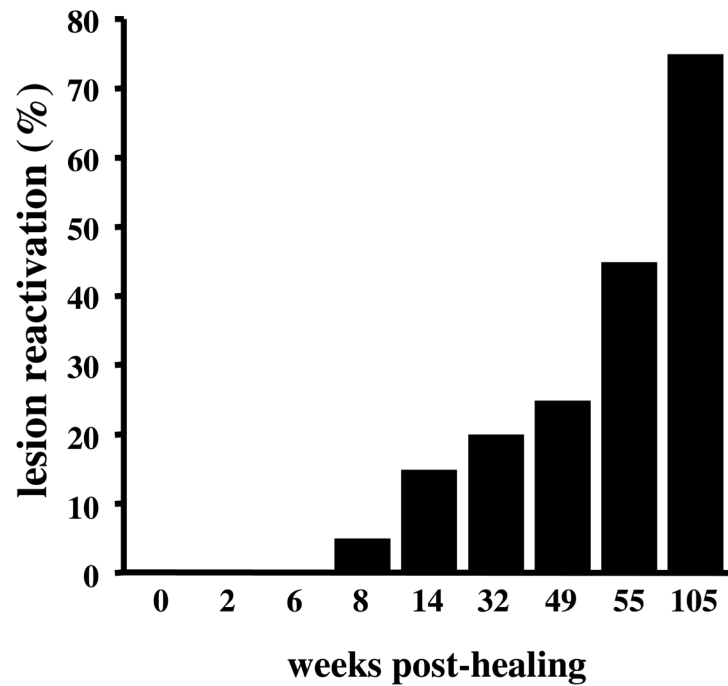


**FIGURE 5. *L. major* spontaneously reactivates in aged mice**

8-week old C57BL/6 mice (n = 20) were inoculated in the ear dermis with $10^3$ *L. major* metacyclic promastigotes. After the lesions were resolved 12 weeks later, mice were monitored for clinical signs of ear swelling and inflammation indicating a reactivation of the lesions.

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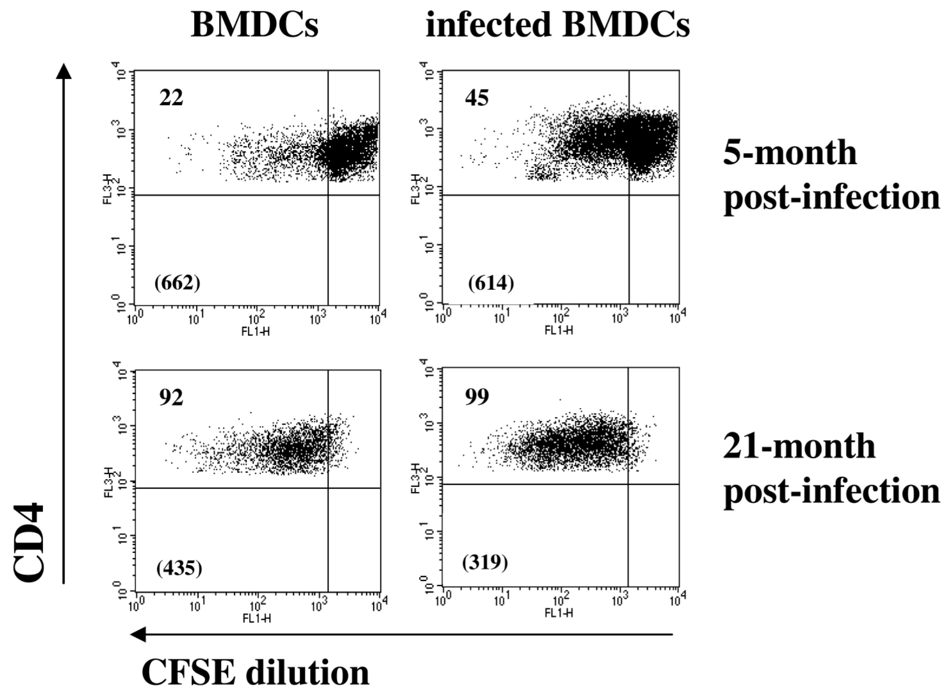


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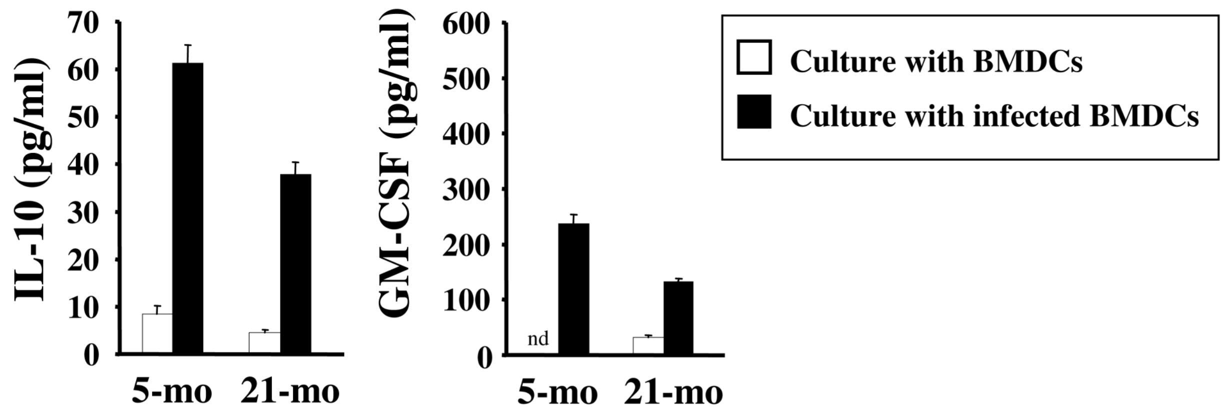


**FIGURE 6. $T_{regs}$ from aged mice respond to *L. major***

8- to 10-week old C57BL/6 mice were inoculated in the ear dermis with *L. major*. 5 or 21 months later, $CD25^+CD4^+$ $T_{regs}$ were purified by FACS from the draining LNs. $5 \times 10^4$ CFSE-labeled T cells were restimulated with $1.4 \times 10^5$ uninfected or *L. major*-infected BMDCs for 4 days. (A) CFSE dilution was analyzed by flow cytometry on gated $CD4^+TCR^+$ cells. Values in top left quadrants are the percentages of $CFSE^{low}$ cells. Values in parenthesis are the CFSE mean fluorescence intensities (MFI) within the $CFSE^{low}$ cells. (B) Cytokines were quantified in $T_{reg}$ cultures with uninfected BMDCs (open bars) or *L. major*-infected BMDCs (black bars). Results are representative of 6 independent experiments. nd, not detected.

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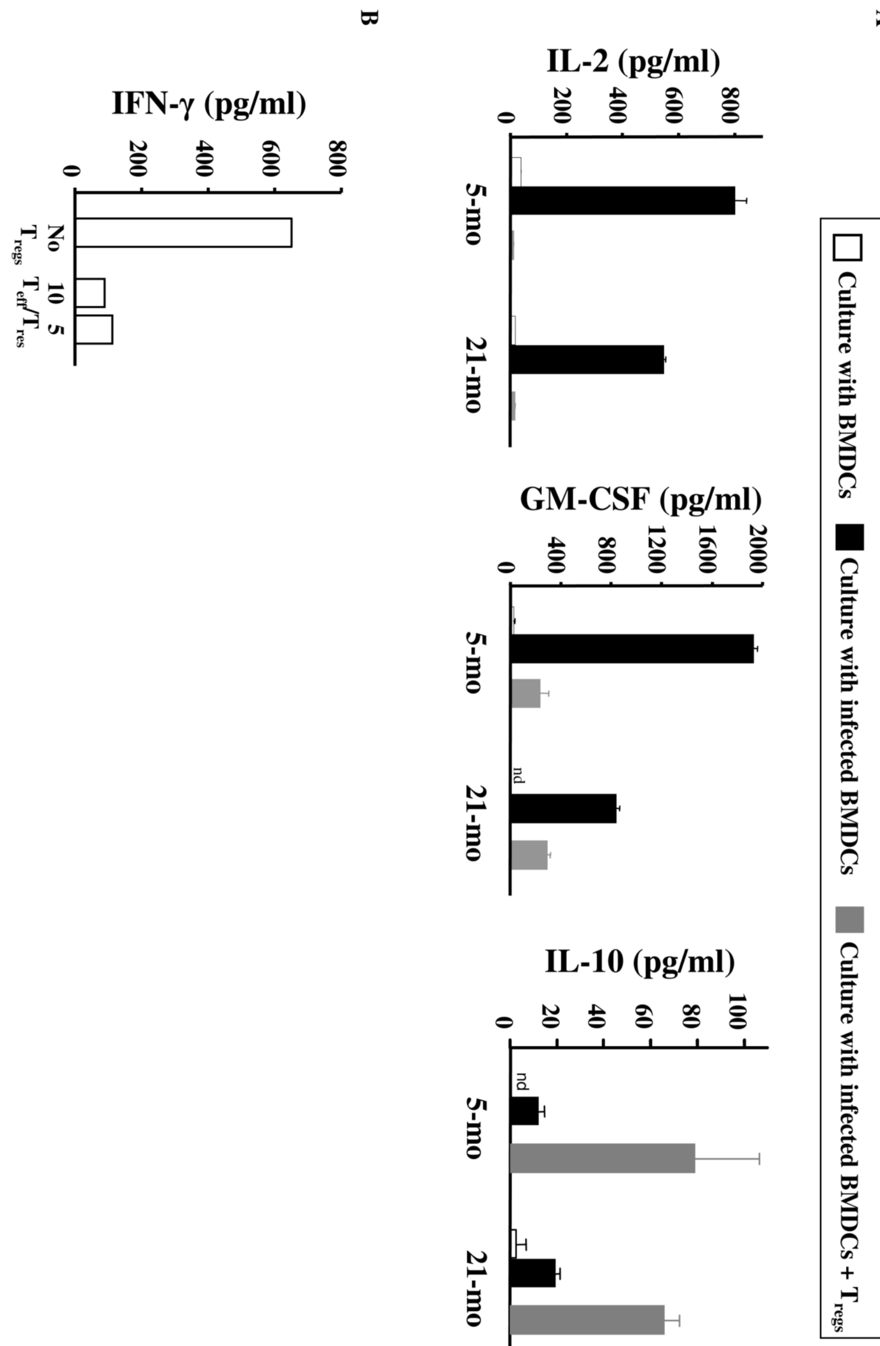


**FIGURE 7. Cytokine production by $T_{effs}$ from *L. major*-infected mice in response to *L. major* antigens is blocked by $T_{regs}$**

(A) 8- to 10-week old C57BL/6 mice were inoculated in the ear dermis with *L. major*. 5 or 21 months later, $CD25^+CD4^+$ ($T_{regs}$) and CD25-$CD4^+$ ($T_{effs}$) were purified from the draining LNs (see Fig. 6). $T_{effs}$ were cultured with uninfected BMDCs (open bars), *L. major*-infected BMDCs (black bars), or cocultured with $T_{regs}$ ($T_{eff}$:$T_{reg}$ ratio of 5:4) and *L. major*-infected BMDCs (grey bars). Cytokines were measured by ELISA. Results are representative of 6 independent experiments. nd, not detected. (B) $FoxP3^+$ $CD4^+$ T cells were sorted from FoxP3-GFP knock-in animals of 57-week old *L. major*-infected mice that were infected when young (9-10-week old). $FoxP3^+$ cells were then mixed at different $T_{eff}$:$T_{reg}$ ratios with $T_{effs}$ ($CD4^+$CD25-CD62L-)

purified from a 22-week old infected mice in presence of *L. major*-infected BMDCs. IFN-_ was measured by ELISA in 4 day-supernatant.

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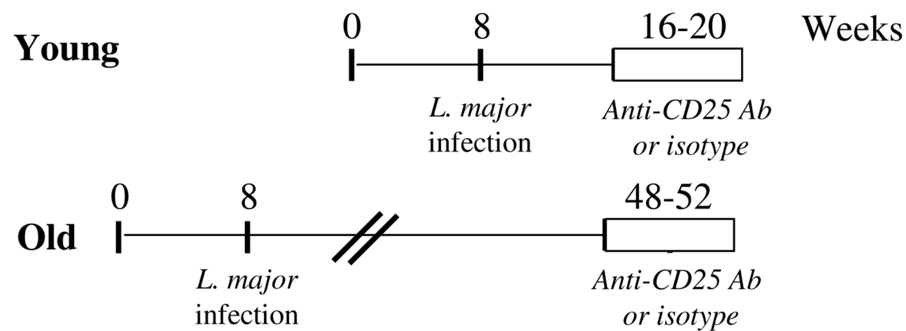


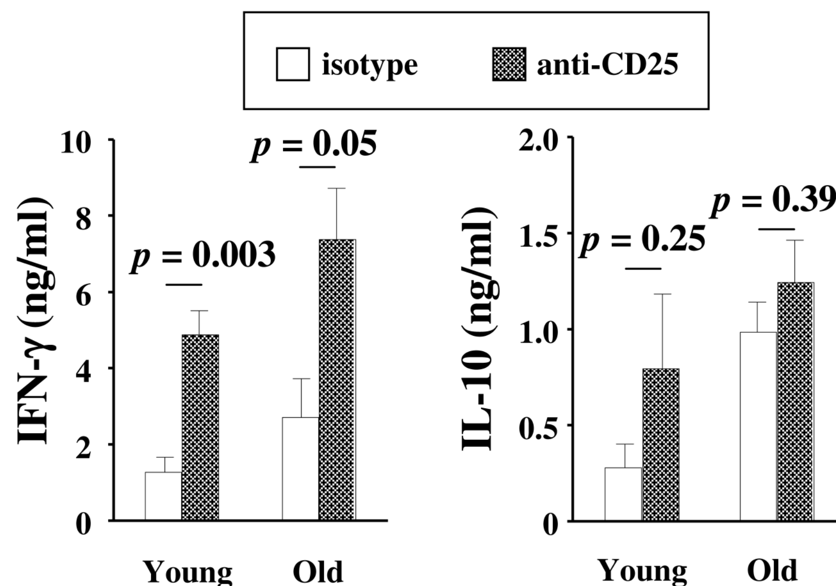


**FIGURE 8.** ***In vivo*** **depletion of $T_{regs}$ in old** ***L. major*****-infected mice increases the production of IFN-γ by $T_{effs}$ at the infection site**

(A) Old *L. major*-infected mice (> 48 week old, which had been infected when they were 8-week old) or young *L. major*-infected mice (16-week old, which had been infected when they were 8-week old) were treated with anti-CD25 Ab or isotype control (N = 4/group) (1 mg for 3 weeks, twice a week). After Ab treatment, mice were sacrificed. T cells were purified from the infection site and draining LNs and restimulated *in vitro* with *L. major*-infected BMDCs. (B) IFN-γ and IL-10 were measured by ELISA in 4-day supernatants. P values correspond to the comparisons between mice treated with isotype control (open symbols) or anti-CD25 Ab (hatched symbols) using *t*-tests.

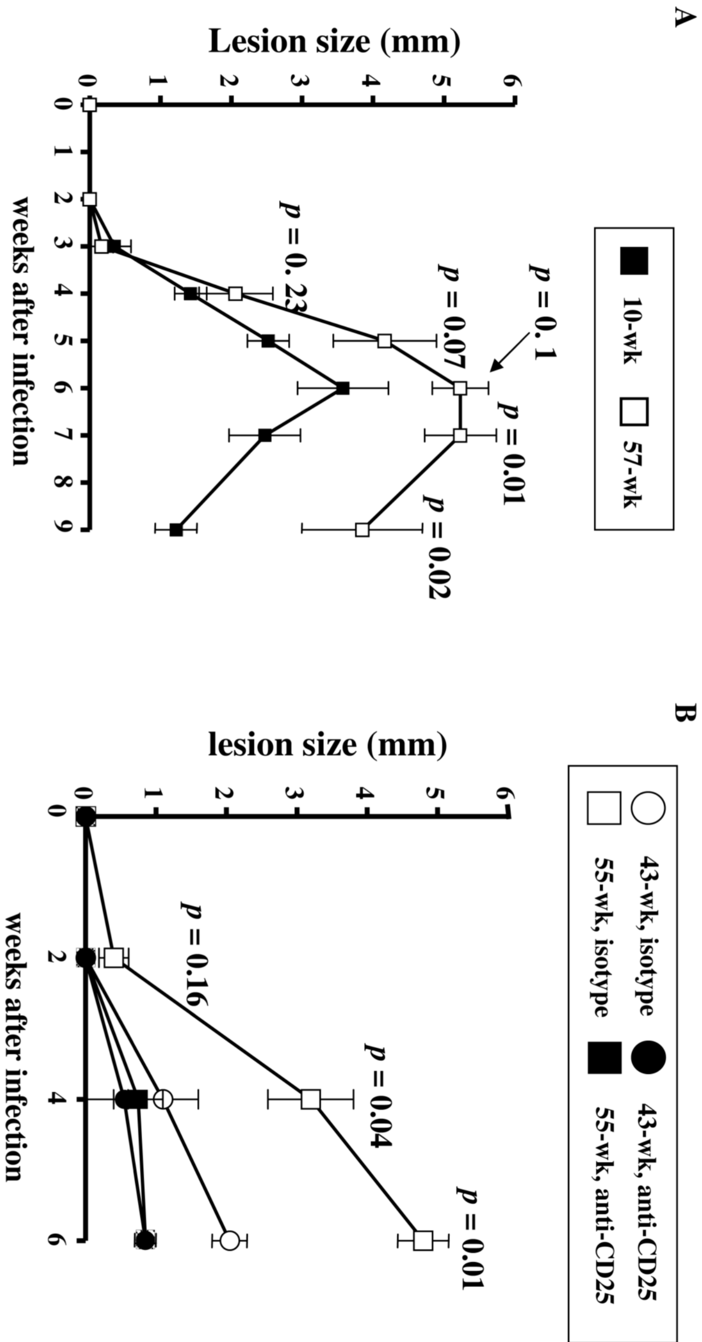


**FIGURE 9. *L. major* causes exacerbated disease in old mice, and $T_{regs}$ contribute to such increased disease severity**

(A) Primary infection with *L. major* parasites: 4 young mice (10-week old) and 3 old C57BL/6 mice (57-week old) were inoculated in the ear dermis with $10^3$ *L. major* metacyclic promastigotes. Lesion size was measured weekly in all animals. Mean (and SD) lesion sizes (mm) are shown for young (black symbols) and old (white symbols) mice. P values indicate differences at each time point (*t*-test). At 7 weeks post-infection, one of the old mice lost an ear through an acute necrotic process. (B) Effect of anti-CD25 Ab on primary *L. major* infection in old mice. Two 43-week old mice (black circles) and two 55-week old mice (black squares) received anti-CD25 treatment (1 mg for 3 weeks, twice a week) at the time of *L. major* infection.



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Three 43- week old mice (white circles) and three 55-week old mice (white squares) received the isotype control Ab following the same regimen. Lesion size (mm) was measured weekly in all animals. P values correspond to the differences in lesion size between anti-CD25- treated and isotype-treated mice, at the indicated time points.

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**Table 1**

Phenotypic characterization of $T_{regs}$ and Teffs in young and elderly subjects[a]

| | Young | | | | | Elderly | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **N**[b] | $T_{reg}$ | $T_{eff}$ | $P$[c] | **ratio**[d] | **N**[b] | $T_{reg}$ | $T_{eff}$ | $P$[c] | **ratio**[d] | $p_{Treg}$[e] | $p_{Teff}$[f] |
| **$CD25^{hi}$** | 16 | 42.5 ± 2.4 | 0.6 ± 0.1 | ** | | 16 | 49.1 ± 2.9 | 1.0 ± 0.3 | ** | | 0.10 | 0.32 |
| **$CD25^{+}$** | 16 | 75.4 ± 1.8 | 8.5 ± 0.8 | ** | | 16 | 78.8 ± 1.9 | 11.8 ± 1.8 | ** | | 0.20 | 0.45 |
| **$CD25^{+}$ $CD127^{lo}$** | 9 | 58.8 ± 4.3 | 2.2 ± 0.2 | ** | | 11 | 69.0 ± 3.1 | 3.4 ± 0.6 | ** | | 0.07 | 0.21 |
| **$CTLA\text{-}4^{+}$** | 16 | 44.6 ± 3.9 | 3.9 ± 0.6 | ** | | 13 | 39.8 ± 3.9 | 3.7 ± 0.4 | ** | | 0.41 | 0.96 |
| **$GITR^{+}$** | 16 | 5.2 ± 0.4 | 1.3 ± 0.1 | ** | | 16 | 6.4 ± 0.7 | 1.7 ± 0.3 | ** | | 0.16 | 0.24 |
| **$CD45RA^{+}$** | 10 | 14.7 ± 2.5 | 39.0 ± 4.3 | ** | 2.65 | 10 | 9.5 ± 1.4 | 21.5 ± 3.0 | * | 2.26 | 0.09 | < 0.01 |
| **$CD27^{+}$** | 16 | 92.1 ± 0.9 | 88.5 ± 1.5 | * | | 13 | 93.3 ± 0.9 | 81.2 ± 4.6 | * | | 0.38 | 0.17 |
| **$PD\text{-}1^{+}$** | 9 | 22.9 ± 7.9 | 21.9 ± 9.2 | ns | | 11 | 22.8 ± 6.8 | 25.1 ± 7.6 | ns | | 0.82 | 0.59 |
| **$TGF\beta RII^{+}$** | 8 | 6.8 ± 2.6 | 2.2 ± 0.6 | ns | | 10 | 4.9 ± 1.1 | 2.0 ± 0.4 | * | | 1.00 | 0.82 |
| **$GranzA^{+}$** | 9 | 3.7 ± 1.1 | 4.1 ± 1.0 | ns | | 9 | 4.6 ± 2.0 | 7.2 ± 3.0 | ns | | 1.00 | 1.00 |
| **$GranzB^{+}$** | 9 | 4.0 ± 1.5 | 4.1 ± 1.3 | ns | | 9 | 4.2 ± 1.6 | 12.6 ± 4.5 | * | | 0.55 | 0.05 |
| **$CD69^{+}$** | 15 | 4.3 ± 0.9 | 2.7 ± 0.4 | ns | | 15 | 4.4 ± 0.9 | 2.4 ± 0.3 | ns | | 0.98 | 0.80 |
| **$CCR5^{+}$** | 9 | 27.5 ± 4.3 | 9.4 ± 1.2 | ** | 0.3 | 11 | 28.6 ± 3.2 | 15.1 ± 1.9 | * | 0.5 | 0.85 | 0.02 |
| **$CCR7^{+}$** | 18 | 29.4 ± 3.6 | 41.0 ± 5.2 | ns | 1.3 | 19 | 38.4 ± 3.9 | 49.8 ± 5.2 | ns | 1.3 | 0.10 | 0.23 |
| **$CXCR4^{+}$** | 9 | 28.2 ± 3.7 | 37.4 ± 3.6 | ns | 1.3 | 11 | 15.8 ± 2.0 | 33.2 ± 2.1 | ** | 2.1 | < 0.01 | 0.31 |
| **$\alpha4^{+}\beta1^{+}$** | 18 | 44.2 ± 2.0 | 65.2 ± 2.6 | ** | 1.4 | 19 | 38.5 ± 2.1 | 63.1 ± 2.2 | ** | 1.6 | 0.06 | 0.54 |
| **$\alpha4^{+}\beta7^{+}$** | 18 | 12.0 ± 1.2 | 41.5 ± 2.4 | ** | 3.5 | 19 | 7.6 ± 0.6 | 29.0 ± 2.5 | ** | 3.8 | 0.02 | 0.001 |

[a] PBMCs were obtained from healthy elderly individuals (≥ 70-year old) and young donors (≤ 30-year old). $T_{regs}$ were defined as FOXP3+CD4+, Teffs were defined as FOXP3- CD4+ T cells. Values represent the mean (± SEM) percentage of positive cells for each marker.

[b] N is the number of tested samples.

[c] $p$ values compare the percentage of $T_{reg}$ and Teff expressing each marker. ** $p<0.0001$; * $p<0.05$; ns: not significant ($p>0.05$).

[d] ratio was calculated as the proportion of $T_{effs}$ expressing each marker divided by the proportion of $T_{regs}$ expressing it.

[e] $pT_{reg}$ values compare the percentage of $T_{regs}$ expressing each marker in young versus elderly subjects.

[f] $pT_{eff}$ values compare the percentage of $T_{effs}$ expressing each marker in young versus elderly subjects.

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**Table 2**

Expression of markers in $FoxP3^{+}CD4^{+}TCR^{+}$ cells from young and aged mice[a]

| Tissue | CD25+ Young | CD25+ Aged | $p^d$ | GITR+ Young | GITR+ Aged | $p^d$ | CTLA-4+ Young | CTLA-4+ Aged | $p^d$ |
|---|---|---|---|---|---|---|---|---|---|
| **Spleen**[b] | 62.6 ± 1.8 | 35.6 ± 4.4 | < 0.01 | 93 ± 1 | 97.5 ± 0.7 | < 0.01 | 51.5 ± 5.1 | 65.6 ± 6.3 | 0.11 |
| **pLN**[b] | 72.6 ± 1.1 | 65.3 ± 5.2 | 0.19 | ND | ND | ND | 55.0 ± 5.2 | 71.8 ± 5.7 | 0.05 |
| **mLN**[b] | 72.0 ± 1.1 | 58.6 ± 4.5 | 0.02 | 93.1 ± 1.5 | 95.5 ± 1.3 | 0.24 | 55.6 ± 5.7 | 76.6 ± 6.2 | 0.03 |
| **Blood**[c] | 37.2 ± 2.7 | 33.2 ± 2.3 | 0.33 | 83.9 ± 5.2 | 94.0 ± 1.2 | 0.13 | 26.7 ± 5.0 | 26.1 ± 1.2 | 0.92 |
| **Tissue** | **CD27+ Young** | **CD27+ Aged** | $p^d$ | **CCR7+ Young** | **CCR7+ Aged** | $p^d$ | **CD69+ Young** | **CD69+ Aged** | $p^d$ |
| **Spleen**[c] | 87.0 ± 1.9 | 70.1 ± 3.7 | 0.02 | 5.2 ± 0.6 | 1.5 ± 0.2 | 0.02 | 14.8 ± 0.5 | 50.2 ± 1.6 | < 0.01 |
| **pLN**[c] | 85.4 ± 1.6 | 75.5 ± 3.5 | 0.06 | 3.7 ± 0.8 | 1.5 ± 0.5 | 0.08 | 20.1 ± 0.9 | 38.4 ± 1.7 | < 0.01 |
| **mLN**[c] | 86.3 ± 0.8 | 73.0 ± 1.3 | < 0.01 | 4.4 ± 0.6 | 2.0 ± 0.5 | 0.03 | 25.8 ± 0.0 | 59.2 ± 5.4 | < 0.01 |
| **Blood**[c] | 27.0 ± 6.6 | 11.2 ± 1.8 | 0.08 | 15.4 ± 7.1 | 3.5 ± 1.2 | 0.18 | 19.2 ± 5.7 | 5.2 ± 1.0 | 0.07 |

[a] Single cell suspensions from spleens, pLNs, mLNs and blood were stained for surface expression of CD4, TCR, CD25, CD27, CD69, CCR7 and GITR, followed by intracellular staining for FoxP3 and CTLA-4. Results are expressed as mean (± SEM) percentages of $FoxP3^{+}CD4^{+}TCR^{+}$ cells expressing each marker. Young mice were 2-3 month old and aged mice were 20-28 month old.

[b] 6 mice/group were analyzed. ND: not determined.

[c] 3 mice/group were analyzed.

[d] $p$ values ($t$-test) compare proportions in young and old mice.