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Induction of Regulatory T-cells from Memory T-cells Is Perturbed During Acute Exacerbation of Multiple Sclerosis

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Abstract

Regulatory T-cells (Tregs) are vital for maintaining immunological self-tolerance, and the transcription factor FOXP3 is considered critical for their development and function. Peripheral Treg induction may significantly contribute to the total Treg pool in healthy adults, and this pathway may be enhanced in thymic-deficient conditions like multiple sclerosis (MS). Here, we evaluated iTreg formation from memory versus naïve $CD4^+CD25^-$ T-cell precursors. We report the novel finding that memory T-cells readily expressed CD25 and FOXP3, and demonstrated significantly greater suppressive function. Additionally, the $CD25^-FOXP3^-$ fraction of stimulated memory T-cells also displayed robust suppression not observed in naïve counterparts or *ex vivo* resting ($CD25^-$) T-cells. This regulatory population was present in both healthy subjects and clinically-quiescent MS patients, but was specifically deficient during disease exacerbation. These studies indicate that iTreg development and function are precursor dependent. Furthermore, MS quiescence appears to correlate with restoration of suppressive function in memory-derived $CD4^+CD25^-FOXP3^-$ iTregs.

Keywords

Multiple sclerosis; Immune regulation; FOXP3; T cells; Memory; Naive

INTRODUCTION

T-cell regulation is a critical aspect of immune homeostasis and several mechanisms exist to prevent untoward immune activation, including positive and negative thymic selection,

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anatomic sequestration of antigens, and stringent requirements for immune activation in the periphery [1–3]. Regulatory T-cells (Tregs) comprise yet an additional layer of immune regulation vital towards maintaining self-tolerance [4], and two types of $CD4^+$ Tregs are appreciated: thymus-derived "natural" Tregs (nTregs) and peripherally-generated "induced" Tregs (iTregs). In healthy adults, peripheral Treg induction may significantly contribute to the total Treg pool [5], and this pathway may be further enhanced in nTreg-deficient conditions such as multiple sclerosis (MS), an immune-mediated disease of the central nervous system [6, 7].

The identification of FOXP3 as a master controller of Treg development and function initially provided a reliable phenotype with which to uniquely identify nTregs. However, subsequent observations of transient FOXP3 induction in activated $CD4^+CD25^-$ T-cells complicated the perception of FOXP3 as a distinctive identifier of Treg populations. Many studies have since described additional markers towards discriminating between thymic-derived nTregs and peripherally-induced $CD4^+FOXP3^+$ T-cells, however, all markers to date are found to be expressed by both regulatory populations, including CTLA-4 [8], GITR [9], CD127 [10], and more recently, HELIOS [11, 12]. Despite the lack of unique identifiers, iTregs remain a highly-studied population. The transient FOXP3 expression typical of *in vitro* generated iTregs fuels skepticism regarding their functional ability and biological relevance [13, 14]. Though we, and others, have reported activation-induced FOXP3 expression in peripheral T-cells, regulatory ability is not consistently observed [15–17]. We postulate that discordance between these observations may arise from differences in starting populations, as most protocols evaluate iTreg development solely from enriched naïve $CD4^+CD25^-$ T-cells [18].

MATERIALS AND METHODS

Cell preparation and bead sorting

Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll Paque (GE Healthcare Biosciences, Pittsburgh, PA) density gradient centrifugation. "Untouched" $CD4^+CD25^-$ T-cells were selected via CD4 negative selection and CD25 depletion using the appropriate sorting kits and AutoMACS separator (Miltenyi Biotec, Auburn, CA). $CD4^+CD25^-$ T-cells' purity was routinely > 95% by flow cytometric analysis, and further sub-sorted into memory and naïve enriched fractions using CD45RO selection kits (Miltenyi). $CD45RO^+$ and $CD45RO^-$ fractions were routinely >75% and >95% enriched, respectively, per flow cytometric evaluation. Antigen presenting cells comprised CD3-depleted or CD4-depleted PBMCs irradiated with 3500 rads. Aliquots of autologous $CD4^+CD25^-$ T-cells (responders) and antigen presenting cells (APCs) were frozen for use in future suppression assays at later time points.

Treg induction

All activations were conducted in a total of 1 mL H5 media (RPMI 1640 media containing 10% human serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 1 mg/mL L-glutamine) contained within 5 mL FACS tubes. For each stimulus, cells were incubated for 5 days at 37°C in 5% CO2. After the 5 day incubation, dead cells were removed from cultures

using removal kit (Miltenyi-Biotec). For experiments comparing equal numbers of $CD25^+FOXP3^+$ induced cells, putative iTregs were selected by sorting for $CD25^+$ cells (Miltenyi-Biotec). For experiments comparing memory versus naïve $CD4^+$ T-cells, each precursor population was independently activated in parallel. Mixed Lymphocyte Reaction (MLR): 1.0×10^6 $CD4^+CD25^-$ T-cells were stimulated using 1.0×10^6 irradiated allogeneic APCs. Anti-CD3 stimulation: 1.0×10^6 $CD4^+CD25^-$ T-cells and 1.0×10^6 irradiated APCs were co-incubated with immobilized anti-CD3 (OKT3, eBiosciences, San Diego, CA). Anti-CD3 was immobilized onto FACS tube bottoms via 30 minutes incubation at 37°C, using 500 μL of a 2 μg/mL antibody solution. Combined anti-CD3 anti-CD28 stimulation: 1.0×10^6 cells T-cells were co-incubated with immobilized anti-CD3 (eBiosciences) and anti-CD28 (eBiosciences). Anti-CD3 and anti-CD28 antibodies were coated onto FACS tube bottoms via 30 minutes incubation at 37°C, using 500 μL of a 2 μg/mL antibody solution.

Flow cytometric suppression assay

Freshly thawed $CD4^+CD25^-$ T-cells (responders) and autologous APCs were stained with CFSE tracking dye (Invitrogen Molecular Probes, Eugene, OR) as described [17]. APCs were stained with PKH26 tracking dye (Sigma-Aldrich, St. Louis, MO) tracking dye, which was performed per manufacturer's protocol. Putative iTregs (suppressors) were stained with Cell Tracker Red CMTPX (Invitrogen). Briefly, suppressors were suspended at 1.0×10^6 cells/mL and incubated for 15 min at 37°C with 700 nM CMTPX. The 1:1 suppressor to responder ratio utilized 1.0×10^5 suppressors, 1.0×10^5 responders and 1.0×10^5 APC, in a total of 300 μL H5 media in 96 well plates. For additional ratios, responders and APC and responder numbers were held constant while suppressors were serially diluted. All ratios (except the non-stimulated control) were then stimulated with 1 μg/mL αCD3 for 5 days. Proliferation was calculated via flow-cytometric measurement of CFSE-dilution, from which CMTPX stained suppressors and PKH stained APC were gated out during analysis. A responder index was calculated using control condition of no iTregs added (full response) and no stimulus provided (background, no response). This responder index was then utilized to calculate percent suppression [19].

Patient inclusion criteria

Clinically stable (n=4) and acute exacerbation patients (n=5) were treatment-naïve at time of recruitment and clinically defined as relapsing remitting MS (RRMS) subtype according to established McDonald criteria. These subjects were a subset of the same cohort described in our previously published studies [20] with an age range of 24–50 years (mean 36) and M/F distribution of 2/7. Exclusion criteria consisted of other autoimmune, immunosuppressive, or neurodegenerative conditions, corticosteroid treatment within last 3 months or any history of disease-modifying immunomodulatory therapy. Pregnant patients, HIV^+ individuals or patients with malignancies were also excluded from recruitment. For clinically quiescent patients, an acute relapse (clinically defined) was also an exclusion criterion. Patients with exacerbation were recruited during an active clinical episode/relapse before the administration of steroids. This ranged between 2 to 40 days from the initiation of the clinical relapse. All studies were approved by the Institutional Review Board.

Antibody staining, data, and statistical analysis

Multicolor phenotypic panels were established using different combinations of CD3-Pacific Blue, CD4-PE-Cy5, CD25-APC, CD45RA-FITC and CD45RO-PE (BD Biosciences, San Jose, CA). FOXP3-Alexa700 (clone PCH101) was used for intracellular staining (eBiosciences). Stained cells were re-suspended and acquired in 1% paraformaldehyde. Flow cytometric data was acquired on a 4-laser, 19-parameter BD LSR II using FACS Diva software (Becton Dickinson). CFSE, CMTPX, and PKH were detected in the FITC, PE-Texas-Red, and PE-Green channels, respectively. FCS 3.0 files were analyzed using FlowJo software (TreeStar, Ashland, OR). Unless indicated otherwise, all statistical methods utilized the two-tailed student t-test and calculated using the Prism 5 statistical software (Graphpad Software, La Jolla, CA).

RESULTS AND DISCUSSION

Various types of Treg subtypes are now well recognized including thymic derived-nTregs, peripherally induced-iTregs, including Tr1 regulatory cells [21, 22], Th3 cells [23, 24] and $CD8^+$ Tregs [19, 20, 25, 26]. Impaired immunosuppression by iTregs, including Tr1 cells or induced $CD8^+$ Tregs has been associated with MS [27, 28]. *In vitro*-generated, activation-induced iTregs have remained controversial due to inconsistencies in their suppressive potential. To address the disparity observed in suppressive function of induced $CD4^+$ T-cells, we developed a novel flow cytometric suppression assay wherein each cellular population (APC, responder T-cell, and putative iTreg) was stained with uniquely separable tracking dyes prior to incubation (Suppl Fig 1a). This approach permitted sensitive exclusion of APCs and putative iTregs when quantifying suppression of responder cell proliferation (gating strategy in Suppl Fig 1b). We validated this assay by duplicating our prior observations using this new method and confirmed that $CD4^+CD25^-$ T-cells from healthy adults exhibited activation-induced expression of CD25/FOXP3 (Suppl Fig 2). We also demonstrated robust suppressive function that was comparable to *ex vivo*-derived “natural” Tregs (Suppl Fig 3). We further showed that this suppressive phenotype was not explained by competition for APC, media nutrients or IL-2, as supplementation with these factors did not significantly influence suppression (Suppl Fig 4). In contrast, when the source of T-cells was cord blood (predominantly naïve T-cells), we did not observe regulatory function despite the presence of stimulus-induced CD25 and FOXP3 expressing cells (Suppl Fig 5).

These initial observations indicated that naïve T-cells have reduced ability to generate functional iTregs. We addressed this hypothesis by independently evaluating memory ($CD45RO^+$) and naïve ($CD45RO^-$) $CD4^+CD25^-$ T-cell fractions derived from healthy adults (Fig 1a,b). We observed that the memory T-cell pool generated a significantly greater proportion of $CD25^+$ and $FOXP3^+$ cells than corresponding naïve T-cells in response to all tested stimuli, including MLR, anti-CD3, and combined anti-CD3 + anti-CD28 (Fig 1c,d,e). When equivalent numbers of total cells from the memory and naïve activation cultures were evaluated for suppressive function, we found that memory-derived cultures had significantly higher suppressive ability compared to naïve cultures (Fig 1f).

It is well appreciated that memory T-cells possess lowered thresholds for cellular activation [29, 30], which may explain the greater frequency of $CD25^+FOXP3^+$ T-cells in memory

cultures (Fig 1c–e). To control for the greater Treg frequency in memory cultures, we next isolated equivalent numbers of memory and naïve-derived $CD25^+FOXP3^+$ T-cells through CD25 magnetic bead selection. The resulting populations exhibited comparable mean fluorescent intensities for both CD25 and FOXP3 (Suppl Fig 6). When these "normalized" populations were assayed, iTregs generated from MLR- and anti-CD3-stimulated $CD45RO^+$ memory precursors displayed significantly greater regulatory function (Fig 2). It is worth noting that although the combined anti-CD3/anti-CD28 condition failed to achieve statistical significance, stimulated naïve $CD4^+$ T-cells exhibited suppressive function in only 50% of experiments, corresponding with others reports of poor iTreg generation using the same stimulus [31]. In contrast, memory cells easily attained suppressive function following combined anti-CD3/anti-CD28 stimulus in every experimental replicate (Fig 2). Collectively, these data indicate that memory $CD4^+CD25^-$ T-cells can induce greater numbers of $FOXP3^+/CD25^+$ T-cells, with significantly higher suppressive ability. To our knowledge, these data provide the first evidence of a precursor-dependent bias in peripheral iTreg generation, which may resolve conflicting reports of iTreg origin and function in healthy adults [18, 32].

Down-regulation of activation-induced FOXP3 has been cited as further evidence of iTreg instability [32]. However, it is possible that the transcriptional program conferring regulatory function persists after down-regulation, permitting iTregs to remain suppressive in the absence of FOXP3. Certainly, several other established $CD4^+$ regulatory populations exhibit regulatory function independent of FOXP3, such as IL-10 secreting T_{r1} cells or TGF-β secreting T_h3 cells [33]. It is similarly possible that a subset of iTregs may bypass the FOXP3 pathway altogether. To determine if regulatory ability develops in $CD4^+FOXP3^-$ T-cells in a precursor-dependent manner, we evaluated post-stimulus memory- and naïve-derived $CD25^-$ T-cells for iTreg function (Fig 3a). Our results demonstrate that memory-derived $CD4^+CD25^-$ T-cells displayed significant suppressive ability compared to their naïve counterparts (Fig 3b). Importantly, resting *ex-vivo* memory $CD4^+CD25^-FOXP3^-$ T-cells failed to show suppression (Fig 3c). These results indicate that prior stimulation was required for the induction of these cells and suggest that derivation of FOXP3-negative iTregs may also be precursor- (memory) dependent.

Collectively, these data support a model in which the combined circulating Treg pool develops from significant contributions by both nTregs and iTregs [34]. Upon encountering their cognate antigen, circulating memory $CD4^+$ T-cells may preferentially cycle to a $FOXP3^+$ transitional state. Given their reduced activation requirements, the transient regulatory state may function as a rate-limiting "waiting period" imposed on memory $CD4^+$ T-cells before they are licensed to initiate an immune response. Alternatively, the regulatory state may represent a form of feedback inhibition during which memory-derived iTregs suppress the activation of later-arriving effectors. This model is compatible with recent observations of formed memory T-cells in antigen-inexperienced individuals [35], suggesting that relevant iTreg populations can be established prior to cognate antigen exposure. Our findings are also consistent with emerging data revealing Tregs co-express signature transcription factors of their target populations. For example, T-bet expressing Tregs reportedly excel at suppressing Th^1 responses [36], and similar couplings are demonstrated for IRF/Th^2 and STAT-3/IL-17 [37]. In the same vein, iTregs generated from

Th-polarized $CD4^+$ T-cells in the same inflammatory cytokine milieu may excel at suppressing fellow Th-effectors. After coming to rest, these memory-derived iTregs may persist as FOXP3-downregulated cells that induce the FOXP3-driven regulatory program on repeat activation or maintain suppressive function independent of FOXP3 expression.

We next evaluated this model in the context of multiple sclerosis. Altered FOXP3 induction and Treg frequency have both been implicated as contributing factors in MS pathogenesis, but there is no consensus on an exact regulatory deficiency [19, 38–40]. We assessed whether there was a deficiency of induced Treg function in MS patients compared to healthy subjects. As we had previously observed dramatic differences in immune regulation during acute exacerbation of disease compared to clinically quiescent MS [19, 20], we recruited cross-sectional cohorts of patients during an acute relapse versus those in clinical remission. All patients were treatment naïve at the time of blood draw (see Methods). Anti-CD3-stimulated memory- and naïve-derived iTregs were generated from all patients, and the resulting $CD25^+$ and $CD25^-$ fractions were evaluated for suppression as described herein.

iTreg populations from clinically stable patients displayed suppressive ability that was comparable to that of healthy adults. In these same cohorts, the $CD25^+$ and $CD25^-$ fractions of memory-derived iTregs demonstrated significantly greater suppressive ability compared to naïve counterparts (Fig 4a). There was no significant suppression demonstrated in the stimulated CD4+ naïve fraction from any cohort (Fig 4b). Surprisingly, we also found no significant difference in the suppressive function of memory derived $CD25^+FOXP3^+$ iTregs between healthy, quiescent, and acute exacerbation cohorts, indicating that $CD4^+CD25^+FOXP3^+$ Treg induction from memory precursors remains unperturbed throughout the course of relapsing-remitting MS. In contrast, though memory-derived $CD4^+CD25^-FOXP3^-$ iTregs were comparably suppressive in healthy controls and clinically quiescent MS patients, this population was significantly deficient during acute MS exacerbation (Fig 4b).

To our knowledge, a specific disruption in peripheral Treg induction from memory $CD4^+$ T-cells has not been described. Overall, our studies make three novel observations: First, we resolve the apparent controversy of FOXP3/CD25 expression vs. suppressive ability of human T-cells, showing that this functionality is unique to the memory T-cell compartment. Second, we describe a novel population of CD25−/FOXP3− Tregs derived exclusively from stimulated memory T-cells. Finally, we show that this population has critical clinical significance through its functional correlation to clinical quiescence in MS patients, demonstrating a previously unappreciated mode of regulatory dysfunction in MS. It is tempting to speculate that clinical improvement from exacerbation in MS correlates with restoration of $CD4^+CD25^-$ iTregs. While several FDA-approved MS disease modifiers reportedly function through induction of phenotypically similar regulatory cells [41, 42], the revelation and further characterization of this novel population may lead to new avenues of immunologic targeting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Memory (but not naïve) CD4+CD25^{-} T-cells have greater capacity to generate iTregs
- Memory-derived CD4+CD25−FOXP3− T-cells also possess suppressive ability
- Memory-derived CD4+CD25− FOXP3− iTregs are deficient during acute relapse of MS

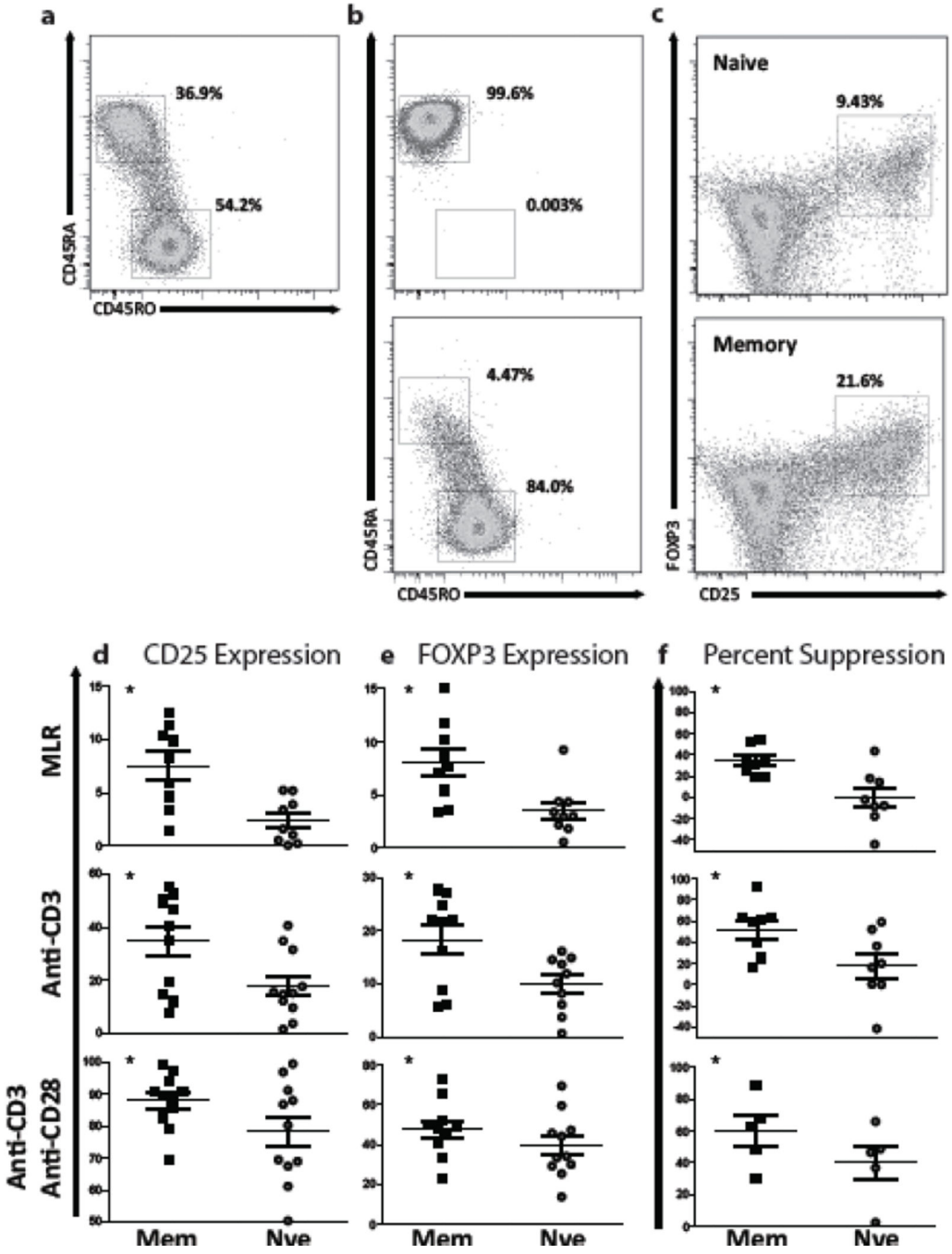

Figure 1. CD4$^+$ memory T-cell cultures demonstrate higher frequency of activation-induced CD25$^+$FOXP3$^+$ T-cells with significantly greater suppressor ability

(**a**) Representative dot plot of memory and naïve T-cell distribution in total CD4$^+$CD25$^-$ T cells derived from PBMCs. CD45RO is depicted on X-axis and CD45RA is depicted on Y-axis.

(b) Representative purity following AutoMACS sorting protocol, which yields enriched fractions of naïve (top) and memory (bottom) CD4$^+$CD25$^-$ T-cells.

(c) Representative dot plots depicting higher frequency of $CD25^+$ and $FOXP3^+$ cells in 5-day allo-stimulated memory cultures compared to naïve cultures. CD25 expression is shown on X-axis and FOXP3 expression is shown on Y-axis. Numbers indicate percentage of CD25+FOXP3+ cells.

(d, e, f) Cumulative data comparing phenotype and function of activated memory (Mem) and naïve (Nve) CD4+ T-cells. Memory and naïve data points are represented as filled squares and open circles, respectively. Precursor cells were separately stimulated using MLR (top row), anti-CD3 (middle row), or combination anti-CD3 anti-CD28 (bottom row) for 5 days. Putative iTregs were then evaluated for CD25 expression (left column), FOXP3 induction (middle column) and suppressive function (right column). % expression or % suppression are plotted on Y-axis. (*$p < 0.05$, two-tailed student t-test).

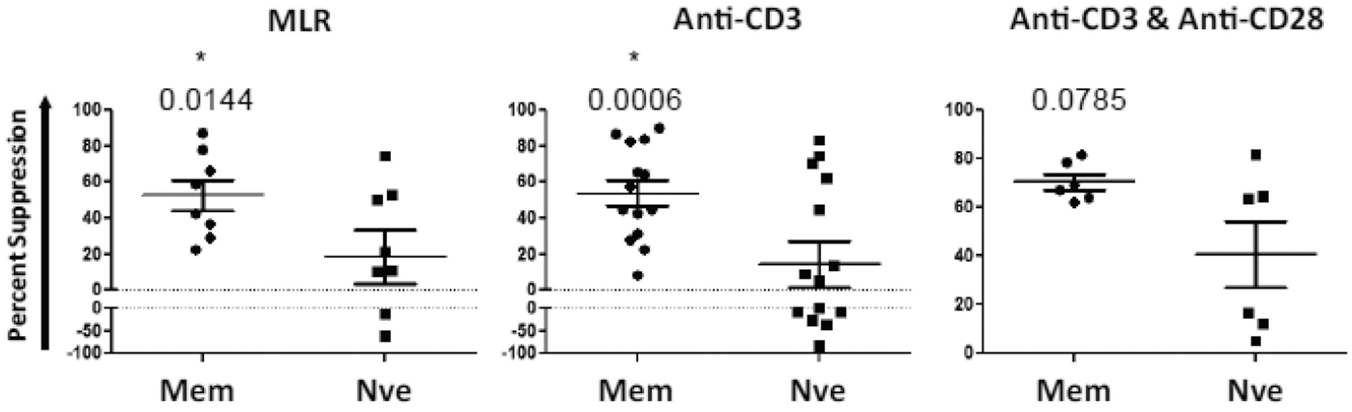

Figure 2. Memory-derived CD25+ T-cells have significantly higher suppressive ability on a per-cell basis

$CD25^{+}$-sorted cells obtained from MLR (left), anti-CD3 (middle) and anti-CD3+anti-CD28 stimulated (right) memory- (Mem) and Naïve- (Nve) cultures and used at equivalent numbers in suppression assays. Percent suppression is shown on Y-axis. Data points from memory and naïve cohorts are shown as circles and squares, respectively. p values noted, two-tailed student t-test.

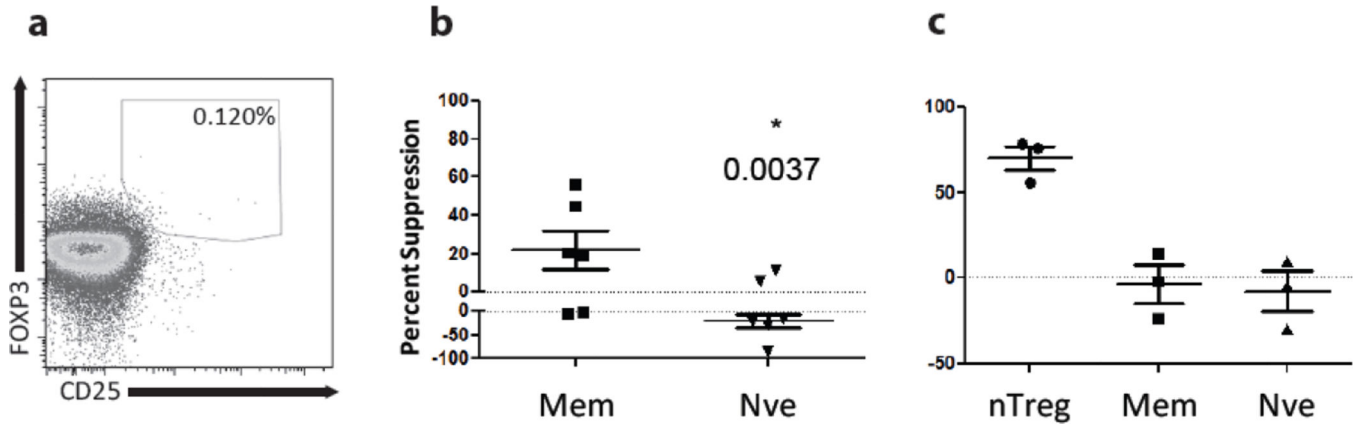

Figure 3. Activation-induced suppressive function in post-stimulus memory-derived $CD4^+CD25^-FOXP3^-$ T-cells

(a) Representative dot plot of CD25-depleted cells post-stimulus sorted cells. CD25 is shown on X-axis and FOXP3 is shown on Y-axis. Sorted population was devoid of $CD25^+FoxP3^+$ cells (<0.2%; as shown in the gate).

(b) Comparison of suppressive function in stimulated $CD4^+CD25^-$ T-cells derived from memory (Mem) vs naïve (Nve) anti-CD3-activated cultures. Percent suppression is shown on Y-axis. (*$p<0.05$, two-tailed student t-test).

(c) Comparison of suppressive function between nTregs and unstimulated ex vivo-derived $CD4^+CD25^-$ memory or naïve T-cells. Percent suppression is shown on Y-axis. Data points from nTregs depicted as circles. All cells tested for suppressive function were sorted ex-vivo from healthy adult PBMCs and tested directly in suppression assays. Only nTregs suppressed responder cell proliferation.

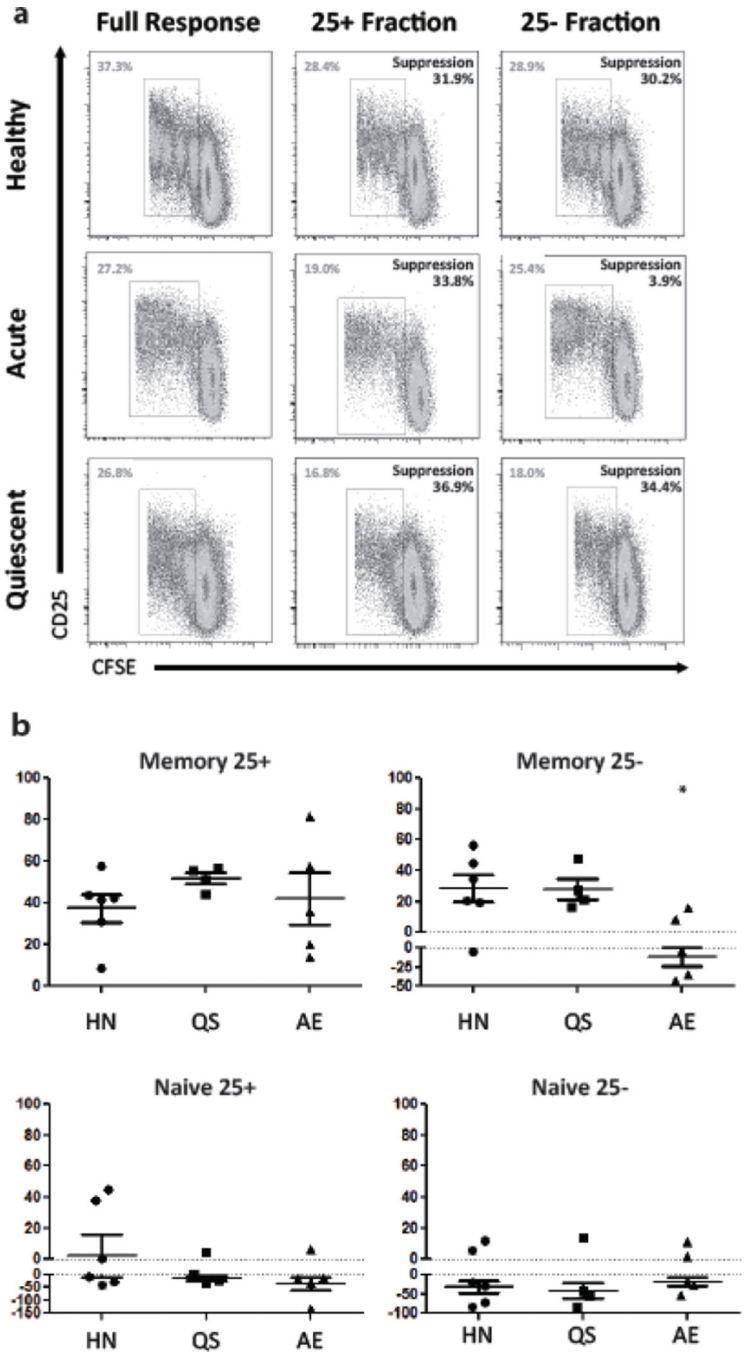

Figure 4. Deficient memory-derived CD4^{+}CD25^{-}FOXP3^{-} iTreg induction during acute exacerbation of multiple sclerosis

(a) Representative dot plots depicting suppression mediated by memory-derived anti-CD3 stimulated CD4^{+}CD25^{+} iTreg (middle row) and memory-derived post-stimulus CD4^{+}CD25^{-} iTregs (right row). CFSE dilution and CD25 expression of responder cells is shown on X-axis and Y-axis, respectively. Left column shows responder proliferation in the absence of suppressor cells (1:0). Percent proliferation is indicated at left-top of each dotplot and calculated percent suppression is shown at right-top in middle and right columns. Healthy

controls (top row) and clinically stable patients (bottom row) demonstrated no significant differences in any of the populations evaluated, however, $CD25^{-}$ iTregs from relapsing MS patients (middle row, right column) were specifically deficient at suppression.

(b) Cumulative data comparing suppressive function of anti-CD3 stimulated iTregs from clinically stable and relapsing MS patients. Percent suppression is shown on the Y-axis. Data from 6 healthy adults, 4 quiescent MS patients, and 5 patients in acute exacerbation are shown as circles, squares, and triangles, respectively. Healthy controls and quiescent patients demonstrated no significant differences in any of the populations evaluated, however, $CD25^{-}$ iTregs from relapsing MS patients were specifically deficient compared to either healthy subjects or quiescent disease (top right panel; $*p < 0.05$, two-tailed student t-test).