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Expression of 3G11 epitope defines subpopulations of regulatory T cells with different suppressive potency

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Abstract

3G11, a sialylated carbohydrate epitope on the disialoganglioside molecule, is expressed predominantly on the surface of mouse CD4⁺ T cells. Our previous studies suggested that lack of the 3G11 molecule could be a new cell surface marker for regulatory CD4⁺ T cells. In the present study, we explore the relationship between 3G11⁺ and CD25⁺ T cells, a well-defined, naturally occurring regulatory T cell population. We found that a large proportion of CD25⁺CD4⁺ T cells lack expression of 3G11 and that more 3G11⁺CD4⁺ T cells express Foxp3 compared to the 3G11⁺CD4⁺ population. Based on 3G11 and CD25 expression we sorted four CD4⁺ T cell subpopulations and tested their phenotypes. Among four CD25/3G11-related CD4⁺ T cell subpopulations, CD25⁺3G11⁺ T cells expressed the highest levels of Foxp3 and IL-10 and most efficiently inhibited mitogenic and antigen-specific immune responses *in vitro* and clinical EAE *in vivo*, while CD25⁺3G11⁺ T cells produced a higher level of proinflammatory cytokines and enhanced autoimmune responses. Thus, among CD4⁺CD25⁺ T cells, CD25⁺3G11⁺ T cells represent a more effective Treg subpopulation than CD25⁺3G11⁺ T cells.

Keywords

Regulatory T cells; Surface marker; Autoimmunity

1. INTRODUCTION

Regulatory T cells (Tregs) play an important role in the maintenance of immune tolerance and prevention of autoimmunity. This has clearly been shown in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS) (Martin R et al., 1995), where depletion of Treg cells led to susceptibility to EAE of normally resistant mouse strains (Zhang X et al., 2004 and Reddy J et al., 2004). Two major subsets of Treg cells, naturally occurring (nTregs) and induced Treg cells (iTregs), differ in their origin and the mechanism of suppression. nTregs originate in the thymus, represent 5-10% of peripheral CD4⁺ T cells, and can be identified by the constitutive expression of CD25 (O'Garra A et al., 2004). The forkhead/winged helix transcription factor Foxp3 is thought to

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program the development and function of CD4⁺CD25⁺ Tregs and so far is the most unambiguous marker available to identify nTregs (Shevach EM, 2002). Cell-cell contact is the major mechanism of immunoregulation by these Treg cells, although immunoregulatory cytokines may also be involved (Shevach EM, 2002, Sakaguchi S et al., 2008, Liang S. et al., 2005, Lohr J et al., 2006 and Vieira PL et al., 2004). iTregs are generated in the periphery and they primarily act through the release of anti-inflammatory cytokines IL-10 or TGF- β (Rubtsov YP et al., 2008, Buer J et al., 1998, Miller C et al., 1999, Groux H et al., 1997, Barrat FJ et al., 2002 and Askenasy N et al., 2008).

3G11 is a sialylated carbohydrate epitope present on the disialoganglioside, which is predominantly expressed on CD4⁺ T cells. 3G11⁺ T cells stimulated by mitogen produced a large amount of IL-2, while the 3G11⁻ population did not (Hayakawa K et al., 1988, Dittrich F et al., 1994 and Ernst DN et al., 1990). Recently, we have found that tolerance in EAE mice induced by intravenous (i.v.) injection of myelin basic protein (MBP) peptide is associated with the loss of the 3G11 molecule from the surface of CD4⁺ T cells (Zhang GX et al., 2006). These 3G11⁻CD4⁺ T cells produce low levels of IL-2, high levels of IL-10, and suppress MBP-reactive Th1 responses. Furthermore, injection of these T cells into immunized mice significantly inhibited clinical EAE. Taken together, these data suggest that 3G11⁻ T cells have regulatory function.

In the present study, we further characterized 3G11⁻CD4⁺ T cell population and explored the correlation between immunoregulatory function of 3G11⁻ T cells and well defined CD4⁺CD25⁺ Treg population. Our data demonstrate that the lack of 3G11 expression in combination with CD25 expression (CD25⁺3G11⁻) identifies a more effective Treg subpopulation than CD25⁺3G11⁺ T cells.

2. MATERIALS AND METHODS

2.1. Mice and reagents

Female C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice transgenic for a TCR specific for MOG₃₅₋₅₅ were the generous gift of Dr. Kuchroo (Harvard University) (Bettelli E et al., 2003). All mice were housed in the Thomas Jefferson University animal care facilities. All work was performed in accordance with Thomas Jefferson University guidelines for animal use and care. Monoclonal anti-mouse 3G11 IgM antibody (gift of Dr. M. Greene, University of Pennsylvania) was prepared as described (Maier CC et al., 1998).

2.2. Flow cytometry

mAbs to mouse CD25, CD4, CD8, CD62L, CD44, CD45RB, CD154 and CD103 were purchased from BD Bioscience. FITC-labeled rat-anti-mouse-IgM mAb and PE-labeled anti-Foxp3 mAb were purchased from eBioscience. For immunostaining, MNCs were resuspended in the staining buffer (PBS, 1% FCS, 0.02% NaN₃) and incubated with antibody for 30 min at 4°C. For 3G11 staining, splenocytes were incubated with anti-3G11 mAb, washed, and then incubated with rat-anti-mouse-IgM mAb. Intracellular staining was performed for cytokine production and Foxp3 expression. Cells that had been stained for surface markers were fixed and permeabilized using Cytofix/Cytoperm system (BD Bioscience). After permeabilization, cells were stained with mAbs for 30 min at 4°C. Data were collected by using FACSAria and analyzed by FACSDiva.

2.3. Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) Assays

CFSE was obtained from Invitrogen. Purified T cell populations of interest were washed and resuspended in PBS containing 5 μ M CFSE. After incubation for 3-5 min at room

temperature, 1/5th volume of fetal bovine serum was added for 30 seconds; labeled cells were then washed and used in proliferation assays. CD4⁺ T cells, CD4⁺3G11⁺ and CD4⁺3G11⁻ T cells (1×10⁶ cells/ml) were cultured in the presence or absence of 1.0 µg/ml anti-CD3 mAb. After 72 hours of culture, stained cells were analyzed using FACSaria (Becton Dickinson).

2.4. Co-culture experiments for naturally occurring 3G11⁻ MOG-reactive T cells

3G11⁺CD4⁺ and 3G11⁻CD4⁺ T cells were purified from splenocytes of MOG TCR transgenic mice. Unseparated splenocytes of MOG-transgenic mice were co-cultured in triplicate (1×10⁶/ml) with purified T cell subpopulations at a ratio of 5:1 (unseparated splenocytes : purified T cells), in the presence of MOG₃₅₋₅₅ (20 µg/ml). After 60 h of incubation, proliferative response was determined by [³H]-methylthymidine incorporation. After Three days of culturing, supernatants were harvested and IL-10 levels were detected by CBA-Flex assay (BD Bioscience). To determine whether the suppression induced by 3G11⁻CD4⁺ T cells was antigen-specific, the plates were pre-coated with anti-CD3 mAb. Purified CD4⁺ T cells (1×10⁶/ml) from naïve C57BL/6 mice as effector cells were co-cultured with 3G11⁻ T cells of MOG-transgenic mice (B6 background) at a 1:1 ratio. Proliferative response was determined by [³H]-methylthymidine incorporation.

2.5. Purification of T cell subpopulations

Naïve female mice were sacrificed at 7 weeks of age. Splenocytes of naïve C57BL/6 mice were harvested and stained with anti-3G11, anti-CD25 and anti-CD4 mAbs. CD4⁺ T cells were gated and expression of CD25 and 3G11 of these cells is determined with FACSaria. To separate CD4⁺ CD25⁺3G11⁻ and other subpopulations, cells were stained with anti-CD4, anti-CD25, and anti-3G11 Abs and sorted by FACSaria sorter. The purity of resulting populations was confirmed by FACSaria analysis (all over 95%).

2.6. Proliferative responses of CD25⁺3G11⁻ T cells in trans-well co-culture system

Purified CD4⁺CD25⁺3G11⁻ T cells from C57BL/6 mice were cultured (1 × 10⁶/ml) in triplicate with 1.0 µg/ml anti-CD3 mAb. Wells without antibody served as control. After 60 h of incubation, the cells were pulsed for 12 h with 1 µCi of ³H-methylthymidine (specific activity 42 Ci/mmol). Thymidine incorporation was measured by a scintillation counter.

To determine the potential immunosuppressive effects of the four 3G11^{+/-} and CD25^{+/-} T cell populations, these cells were co-cultured at a ratio of 1:1 with anti-CD3 mAb stimulated CD25⁻CD4⁺ T cells in triplicate (both at 1×10⁶/ml). Proliferative responses were determined by ³H-methylthymidine incorporation.

For the transwell co-cultures, CD25⁻CD4⁺ T cells were plated in lower wells and the same number (1×10⁶/ml) of four 3G11- and CD25-related T cell populations were plated in upper wells. Cells were stimulated with 1.0 µg/ml anti-CD3. Proliferative responses of lower wells were determined by ³H-methylthymidine incorporation. Mixed cells were cultured in parallel in regular wells.

To test the involvement of IL-10 in the suppression of different CD25- and 3G11-related T cell subpopulations, these subpopulations were purified from IL-10^{-/-} and wild type mice by FACS sorting. These cells were then co-cultured with anti-CD3 mAb stimulated CD25⁻CD4⁺ T cells of wild type mice at a ratio of 1:1. Proliferative responses were determined by ³H-methylthymidine incorporation.

2.7. Cytokine production

Supernatants from the culture/co-culture system described above were harvested and concentrations of IL-2, IL-4, IL-5, IL-10, IFN- γ , TNF- α and GM-CSF were determined by CBA-Flex assays according to the manufacturer's instructions (BD). Concentrations of the cytokines detected were analyzed and calculated based on standard curves obtained from known concentrations by FCAP Array software (BD).

2.8. Induction of EAE and treatment with 3G11⁻ and CD25⁺ related T cell subpopulations

Female C57BL/6 mice, 8-10 weeks of age, were immunized with 200 μ g MOG₃₅₋₅₅ emulsified in CFA. Pertussis toxin (200 ng/mouse/injection) (List Biological, Campbell, CA) was given i.p. at the time of immunization and 48 h later. EAE was scored daily in a blind fashion as follows (Benson JM et al., 2000): 1, limp tail or waddling gait with tail tonic; 2, waddling gait with limp tail (ataxia); 2.5, ataxia with partial limb paralysis; 3, full paralysis of one limb; 3.5, full paralysis of one limb with partial paralysis of second limb; 4, full paralysis of 2 limbs; 4.5, moribund; and 5, death.

Splenocytes of naïve C57BL/6 mice were stained with anti-CD4, anti-3G11 and anti-CD25 mAbs. CD4⁺CD25⁺3G11⁻, CD4⁺CD25⁺3G11⁺, CD4⁺CD25⁻3G11⁻; CD4⁺CD25⁻3G11⁺ cells were purified by FACS sorting (purity >96%) and injected i.v. into MOG-immunized C57BL/6 mice at the same number (4×10^6 /mouse) on days 0 and 7 p.i. Mice that received PBS-i.v. served as controls.

2.9. Statistics

The Mann-Whitney U test was used for comparison of average clinical scores, and the Student's *t* test for other parameters among different groups. All tests were two-sided.

3. RESULTS

3.1. CD4⁺ 3G11⁻ T cells are highly enriched in Foxp3⁺ Tregs

Expression of Foxp3 is critical for development of Treg cells and Foxp3 is often used as a reliable marker for these cells (Selvaraj RK et al., 2007, Campbell DJ et al., 2007 and Banham AH et al., 2006). To test our hypothesis that 3G11⁻CD4⁺ T cells represent a Treg population, we first examined Foxp3 expression among 3G11⁻ vs. 3G11⁺ CD4⁺ T cells from naïve C57BL/6 mice. We found that, 12.6% of splenic CD4⁺ T cells were 3G11⁻ (Fig. 1A). Only $4.1 \pm 0.5\%$ of 3G11⁺CD4⁺ T cells expressed Foxp3, while $34.9 \pm 1.5\%$ of 3G11⁻CD4⁺ T cells were Foxp3⁺ (Fig. 1B). The difference between these two subpopulations was highly significant ($p < 0.001$). This result shows that 3G11⁻ population is highly enriched in Foxp3⁺ Treg cells.

3.2. Proliferation and cytokine production of CD4⁺3G11⁻ T cells upon TCR stimulation

To test the hypothesis that 3G11⁻ T cells have a suppressive function, while 3G11⁺ T cells are effectors, we isolated these subpopulations from spleen of naïve C57BL/6 mice and labeled them with CFSE. After stimulation with anti-CD3 mAb for 3 days, dilution of CFSE showed that the 3G11⁺ population proliferated more vigorously (53.5%) than 3G11⁻ cells (2.9%) (Fig. 2A). 3G11⁺ T cells secreted significantly higher levels of IL-2 (488 ± 61.2 pg/ml vs. 199.5 ± 18.4 pg/ml) and TNF- α (395.5 ± 36.3 pg/ml vs. 30.7 ± 0.9 pg/ml) than 3G11⁻ T cells; however, 3G11⁻ T cells secreted a large amount of IL-10 (104.0 ± 6.8 pg/ml) while in 3G11⁺ T cell culture supernatants IL-10 was below detection limit (Fig. 2B). The differences in cytokine concentrations were significant (all $p < 0.01$).

To confirm high IL-10 production by 3G11⁺ T cells, intracellular staining of this cytokine was performed in 3G11⁺ and 3G11⁺ cells after anti-CD3 mAb stimulation. As shown in Fig. 2C, the percentage of 3G11⁺ cells (~4.6%) secreting IL-10 was four times higher than 3G11⁺ cells (~1.1%).

3.3. Suppressive function of naturally occurring CD4⁺3G11⁺ T cells

To determine whether naturally occurring 3G11⁺ T cells are suppressive, we isolated 3G11⁺CD4⁺ T cells from splenic CD4⁺ T cells of naïve MOG TCR transgenic mice (Bettelli E et al., 2003). These cells were co-cultured with splenocytes of the transgenic mice at a ratio of 1:5 (3G11⁺CD4⁺ T cells : entire splenocytes) in the presence of MOG₃₅₋₅₅ (10 µg/ml). 3G11⁺CD4⁺ T cells modestly but significantly suppressed MOG₃₅₋₅₅-induced T cell proliferation (Fig. 3A). In parallel, we co-cultured 3G11⁺CD4⁺ T cells of MOG TCR transgenic mice with anti-CD3 stimulated CD4⁺ T cells of naïve C57BL/6 mice as effector cells, at a ratio of 1:1. A modest degree of suppression was also observed, at a level similar to MOG-induced T cell response (Fig. 3B). A significantly higher level of IL-10 was found in the co-culture of anti-CD3 stimulated CD4⁺ T cells with 3G11⁺ T cells than in those with 3G11⁺ T cells or CD4⁺ T cells alone (Fig. 3C; $p < 0.01$). Similar results were also observed at a ratio of 1:2, but not at 1:5 (purified 3G11⁺CD4⁺ T cells : effector T cells) (data not shown).

3.4. CD4⁺3G11⁺ T cells express markers indicating both activation/memory and naïve phenotype

Correlation between 3G11 expression and T cell activation status, as indicated by expression of CD44, CD45RB, CD62L, CD103 and CD154 is shown in Fig. 4A. Data were then further analyzed by gating separately the 3G11⁺ and 3G11⁺ subpopulations, and finding the percentages of 3G11⁺ vs. 3G11⁺ T cells expressing those T cell activation/memory markers (Fig. 4B). A large portion (61.7-68.9%) of 3G11⁺CD4⁺ T cells expressed low levels of CD45RB and CD62L and a high level of CD44, indicating prior activation or memory phenotype (Fig. 4B). In contrast, only 5.1-7.5% of 3G11⁺CD4⁺ T cells expressed these activation/memory markers. While among both 3G11⁺CD4⁺ and 3G11⁺CD4⁺ T cells only small fractions expressed CD154 (0.6-0.7%), a significantly higher percentage of 3G11⁺CD4⁺ T cells expressed CD103^{hi}, a marker for memory T cells, than did 3G11⁺CD4⁺ T cells ($12.1 \pm 2.2\%$ vs. $1.7 \pm 0.4\%$). Yet, up to 30-40% of 3G11⁺CD4⁺ T cells also retained a naïve phenotype, indicating that this cell population can be found in various states of activation.

3.5. The lack of 3G11 expression largely correlates with CD25 and Foxp3 expression

Given that 3G11⁺ T cells exhibit an immunoregulatory effect, we investigated the correlation between 3G11 and CD25 expression on spleen CD4⁺ T cells of naïve mice. Interestingly, we found that the lack of expression of 3G11 overlaps to a great extent with expression of CD25 (CD25⁺3G11⁺) (Fig. 5A). As shown in Fig. 5B, only 9.5% of CD25⁺ T cells were 3G11⁺, while in CD25⁺ T cells the percentage of 3G11⁺ was 54.4. Similarly, only 3.5% of 3G11⁺ T cells were CD25⁺ but 29.4% of 3G11⁺ T cells were CD25⁺ (Fig. 5C; all $P < 0.01$).

We also examined Foxp3 expression in the four CD25- and 3G11-related subpopulations. The highest percentage of Foxp3-expressing cells was found in CD25⁺3G11⁺ T cells ($82.2 \pm 4.3\%$). Intermediate percentage of Foxp3⁺ cells was observed in CD25⁺3G11⁺ ($72.2 \pm 1.8\%$) and CD25⁺3G11⁺ cells ($24.1 \pm 3.5\%$), both of which exhibited lower immunoregulatory capacity than CD25⁺3G11⁺ T cells. CD25⁺3G11⁺ cells contained the lowest percentage of Foxp3⁺ cells ($1.8 \pm 0.3\%$), consistent with our expectation that this population contains T effector cells (Fig. 5D, all $p < 0.01$). While significantly larger fractions of both CD25⁺ T cells and 3G11⁺ T cells expressed Foxp3 than total CD4⁺ T cells

(Fig. 5D, both $p < 0.01$), a better correlation of Foxp3 expression with CD25⁺ than with 3G11⁺ was observed, indicating that CD25⁺ is a more accurate marker for Tregs than 3G11.

3.6. CD25⁺3G11⁺CD4⁺ T cells exhibit the most potent suppressive effect in vitro

We then defined the biological function of CD25⁺3G11⁺, CD25⁺3G11⁺, CD25⁺3G11⁺ and CD25⁺3G11⁺ T cells. These 4 populations from spleen of naïve C57BL/6 mice were purified by FACS sorting and a purity of >90% was obtained for all 4 populations. Cells were activated with anti-CD3 antibody and analyzed 72 h later. The 3G11⁺CD25⁺ population had the lowest proliferation rate (3-fold less than CD25⁺3G11⁺). The CD25⁺3G11⁺ and CD25⁺3G11⁺ population had a relatively low proliferation rate, whereas CD25⁺ 3G11⁺ cells proliferated most vigorously (Fig. 6A).

We then co-cultured these 4 subpopulations with anti-CD3 antibody stimulated CD25⁺CD4⁺ effector T cells of naïve C57BL/6 mice at a 1:1 ratio to examine their potential regulatory function. As shown in Fig. 6B, CD25⁺3G11⁺ T cells suppressed effector T cell proliferation most potently, and intermediate levels of suppression were mediated by CD25⁺3G11⁺ cells and CD25⁺3G11⁺ cells. In contrast, CD25⁺3G11⁺ cells did not exhibit any immunosuppressive capacity in the co-culture. Similar results were obtained in co-cultures employing either transwells, indicating that the suppressive effect of CD25⁺3G11⁺ T cells is not contact dependent.

To determine if IL-10 plays a role in suppression by 3G11⁺ cells, T cell subpopulations were purified from IL-10^{-/-} mice and co-cultured with anti-CD3 mAb stimulated CD25⁺CD4⁺ effector T cells as described above. Again, the most potent suppression was observed by 3G11⁺CD25⁺ T cells, and the suppressive potency of 3G11⁺ T cell subpopulations from IL-10^{-/-} mice was significantly reduced compared with wild type T cells (Fig. 6C).

Consistent with these findings, we also found that CD25⁺3G11⁺ T cells secreted large amounts of IL-2, IL-6 and TNF- α but a low level of IL-10; however, 3G11⁺CD25⁺ T cells secreted a large amount of IL-10 but not IL-2. CD25⁺3G11⁺ and CD25⁺3G11⁺ T cells secreted more IL-10 than CD25⁺3G11⁺ T cells, but not IL-2, IL-6, INF- γ or TNF- α (Fig. 7).

3.7. In vivo suppressive efficacy of different 3G11- and CD25-related T cell subpopulations on EAE

To test the suppressive function of 3G11⁺ and CD25⁺ related subpopulations *in vivo*, we separated CD25⁺3G11⁺, CD25⁺3G11⁺, CD25⁺3G11⁺, and CD25⁺3G11⁺ CD4⁺ T cells from MOG TCR transgenic mice and injected them into immunized mice at days 0 and 7 p.i. As shown in Fig. 8, EAE was suppressed in all three groups of mice that received 3G11⁺ and CD25⁺ related T cell subpopulations. Among these groups, mice that received CD25⁺3G11⁺ T cells exhibited significantly delayed EAE onset (until day 19 p.i.) and, thereafter, developed a mild EAE compared to PBS-treated control mice. EAE onset in mice that received CD25⁺3G11⁺ or CD25⁺3G11⁺ T cells was similar to that of control mice; however, the disease severity in these groups was significantly lower than in control mice. No suppression was observed in mice that received CD25⁺3G11⁺ T cells compared to control mice (Fig. 8).

4. DISCUSSION

Our previous studies suggested that loss of the surface antigen 3G11 on CD4⁺ T cells may represent a unique characteristic of Treg cells (Zhang GX et al., 2006). In this study, we further characterized the 3G11⁺CD4⁺ population; we also explored the relationship between 3G11⁺ T cells and the well-defined CD25⁺ Treg cells. Our results provide *in vitro* and *in*

vivo evidence that CD25⁺3G11⁻ T cells represent the most potent Treg population among different CD4⁺ T cell subpopulations.

We have found in the current study that, in a naïve state, a large portion (up to two-thirds) of 3G11⁻ T cells express markers CD62L^{lo}CD44^{hi}CD45RB^{lo}, indicating activated/memory T cell phenotype, while the remainder exhibit a naïve phenotype (CD62L^{hi}CD44^{lo}CD45RB^{hi}). These results are consistent with previous studies showing that anergic/Treg cells can be found in various states of activation (Ring S et al., 2006 and Fisson S et al., 2003). Indeed, it has been found that naturally occurring Tregs in normal, non-manipulated mice are composed of two subsets that have distinct phenotypes in homeostasis. Some Tregs remain quiescent and have a long lifespan (months), while others express multiple activation markers such as CD62L^{lo}, CD44^{hi}, CD45RB^{lo}, CD69^{hi}, and CD103^{hi}. This subpopulation with activated phenotype may be composed of autoreactive Tregs that are continuously activated by tissue self-antigens (Banham AH et al., 2006). Interestingly, a higher percentage of 3G11⁻CD4⁺ T cells expressed CD103^{hi}, a marker for memory T cells, than did 3G11⁺CD4⁺ T cells. Given that CD103⁺ T cells are considered to be a population of inducible Tregs (Zhu J et al., 2009 and Zhao D et al., 2009), increased CD103 expression on 3G11⁻CD4⁺ T cells supports our theory that this cell subpopulation represents a Treg subset. The correlation among CD103, CD25, Foxp3 and 3G11 expression on CD4⁺ T cells and their significance as definitive markers for Tregs require further investigation.

Although it is well established that CD25⁺CD4⁺ T cells are naturally occurring Treg cells (Shevach EM, 2002, Sakaguchi S et al., 2008, Liang S. et al., 2005, Lohr J et al., 2006 and Vieira PL et al., 2004), it is not known whether there are other naturally occurring Treg subpopulations that cannot be identified by CD25 expression. We have identified a fraction of CD4⁺ T cells from tolerized mice, that is, 3G11⁻ (Zhang GX et al., 2006), but it is not known whether naturally occurring 3G11⁻CD4⁺ T cells possess Treg properties. In the present study we isolated these cells and characterized their immunological properties. As the expression of Foxp3 is essential for the generation of Treg cells and serves as a reliable marker for both naturally occurring and peripherally induced Treg cells (Reddy J et al., 2004, Selvaraj RK et al., 2007, Campbell DJ et al., 2007 and Banham AH et al., 2006), we analyzed the expression of Foxp3 in 3G11⁻CD4⁺ T cells. We found that these T cells more frequently expressed Foxp3 than 3G11⁺CD4⁺ T cells, strongly suggesting the potential regulatory properties of this population. Compared to 3G11⁺CD4⁺ T cells, 3G11⁻CD4⁺ T cells upon TCR stimulation exhibited significantly lower proliferative responses and produced less IL-2 and TNF- α , but higher levels of IL-10. These properties are similar to those of 3G11⁻CD4⁺ T cells isolated from i.v. tolerized mice, which have been shown to be regulatory (Zhang GX et al., 2006). Further, these data indicate that 3G11⁻CD4⁺ T cells from naïve mice possess properties similar to CD25⁺CD4⁺ T cells and are potentially also a subpopulation of naturally occurring Treg cells. Low IL-2 and high IL-10 secretion in 3G11⁻ T cells suggested that this population might exert suppressive effect through soluble mediators (e.g. IL-10). This notion was supported by our finding that 3G11⁻CD4⁺ T cell-induced suppression was largely blocked in IL-10^{-/-} mice. The observation that 3G11⁻ T cells from MOG TCR transgenic mice suppressed both MOG- and anti-CD3 induced T cell responses indicates an antigen-non-specific immunosuppressive function of naturally occurring 3G11⁻ T cells, similar to CD25⁺CD4⁺ Treg cells (Zhou G et al., 2007, Golshayan D et al., 2007 and Belkaid Y et al., 2006).

Given that both CD25⁺CD4⁺ and 3G11⁻CD4⁺ T cells of naïve mice exhibit Treg function, we asked whether there is a relationship between these two populations. We hypothesized that CD4⁺ T cells exhibiting 3G11⁻CD25⁺ phenotype possess the strongest Treg capacity among all CD25- and 3G11-related T cell populations. To test this hypothesis, we characterized CD4⁺ T cells for their 3G11, CD25 and Foxp3 expression, and purified

different subpopulations for further functional studies. Indeed, a substantial overlap (>50%) between 3G11[−] and CD25⁺ populations was observed compared to 3G11⁺ and CD25⁺ populations (10% overlap), while most 3G11⁺ T cells were CD25[−]. Among CD4⁺ T cells, the CD25⁺3G11[−] subpopulation had the greatest number of cells expressing Foxp3, whereas the CD25[−]3G11⁺ subpopulation had the smallest Foxp3-expressing fraction (Fig. 5D). Intermediate percentages of Foxp3⁺ cells were observed in CD25⁺3G11⁺ and CD25[−]3G11[−] populations. Among these four populations, CD25⁺3G11[−] T cells exhibited the most potent immunosuppressive capacity, while CD25[−]3G11⁺ T cells were not immunosuppressive. More importantly, these findings were further confirmed by in vivo experiments, in which the CD25⁺3G11[−] subpopulation most effectively suppressed EAE (Fig. 8). Although it has been suggested that naturally occurring Treg cells exert their suppressive effect via cell-cell contact (Valmori D et al., 2005), a contact-independent mechanism has also been observed (Golshayan D et al., 2007). Our data derived from transwell cultures suggest a mechanism of suppression by CD25⁺3G11[−] T cells that is independent of cell-cell contact. Taken together, these results indicate that CD25⁺3G11[−] T cells are the most potent naturally occurring Treg cells among the four CD25- and 3G11-related T cell subpopulations.

In summary, our study provides evidence that the lack of surface molecule 3G11 characterizes a distinct population of Treg cells and that, among nTregs, CD25⁺3G11[−] T cells represent a more effective Treg subpopulation than CD25⁺3G11⁺ T cells.

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Fig. 1. Foxp3 expression in 3G11⁺ and 3G11⁻ CD4⁺ T cells

To determine the relationship between Foxp3 and 3G11 expression, splenocytes were harvested from naïve C57BL/6 mice and stained with anti-3G11 and anti-CD4 mAbs. Cells were then intracellularly stained with anti-Foxp3 mAb after being fixed and permeabilized. Cells that were intracellularly stained with isotype antibody served as control. **(A)** CD4⁺ T cells were gated and the percentage of 3G11 expression was determined. **(B)** Percentages of Foxp3⁺ cells in gated 3G11⁻CD4⁺ and 3G11⁺CD4⁺ T cells, which are indicated by arrows. Columns refer to the mean values and bars to SD (n= 5 each group). *** p<0.001. Data are representative of three experiments.

Fig. 2. Proliferation and cytokine secretion of 3G11⁺ and 3G11⁻ CD4⁺ T cells
 Total CD4⁺, 3G11⁺CD4⁺ and 3G11⁻CD4⁺ T cells were purified from naïve C57BL/6 mice by FACS sorting following the gating strategy in Fig 1A. Cells were labeled with CFSE and cultured at 1×10^6 /ml with 1.0 μ g/ml anti-CD3 mAb. **(A)** Fluorescence intensity of CFSE after 72 h of culture. Numbers represent the average percentage of CFSE-low cells. **(B)** Levels of IL-2, IL-10, and TNF- α in supernatants of 72-hr cultures were measured in triplicate by CBA-Flex (BD Bioscience) assay. Columns refer to mean values and bars to SD. ** $p < 0.01$. **(C)** IL-10 production of 3G11⁺ and 3G11⁻ CD4⁺ T cells after 3 days of culture with 1.0 μ g/ml anti-CD3 mAb was determined by intracellular staining. All data are representative of three experiments.

Fig. 3. Immunosuppressive effects of 3G11⁻ T cells in co-culture assay

3G11⁺CD4⁺, 3G11⁻CD4⁺, and total CD4⁺ T cells of naïve MOG TCR transgenic mice were purified (purity >90%) using MACS microbeads. **(A)** Unseparated splenocytes of MOG-transgenic mice were co-cultured in triplicate (1×10^6 /ml) with purified T cell subpopulations at a ratio of 5:1 (unseparated splenocytes : purified T cells), in the presence of MOG₃₅₋₅₅ (20 μ g/ml). After 60 h of incubation, proliferative response was determined by [³H]-methylthymidine incorporation. **(B)** The culture plates were pre-coated with anti-CD3 mAb. Purified CD4⁺ T cells (1×10^6 /ml) from naïve C57BL/6 mice were co-cultured with 3G11⁻ T cells of MOG-transgenic mice (B6 background) at a 1:1 ratio. Proliferative response was determined by [³H]-methylthymidine incorporation. **(C)** Three days after culture, supernatants from co-culture described under (B) were harvested and IL-10 levels were measured by CBA-Flex assay. Columns refer to mean values and bars to SD. ** $p < 0.01$. All data are representative of three experiments.

Fig. 4. Correlation of T cell activation markers with 3G11 expression
Splenocytes of naïve C57BL/6 mice were harvested, stained with antibodies to CD4, 3G11, and several T cell activation/memory markers, and analyzed by flow cytometry. **(A)** CD4⁺ T cells were gated and expression of T cell activation/memory markers on 3G11⁺ vs. 3G11⁻ T cells is shown. **(B)** 3G11⁺ or 3G11⁻ T cells shown in (A) were gated and the percentages of each T cell activation/memory marker on total 3G11⁺ or 3G11⁻ T cells were calculated. Values represent averages ± SD of three experiments.

Fig. 5. Correlation of 3G11 and CD25 expression on CD4⁺ T cells

Splenocytes of naïve C57BL/6 mice were harvested and stained with anti-3G11, anti-CD25 and anti-CD4 mAbs. CD4⁺ T cells were gated and expression of CD25 and 3G11 of these cells is shown (A). The percentages of 3G11⁺ cells in gated CD25⁺CD4⁺ vs. CD25⁻CD4⁺ T cells (as indicated by arrows) were calculated in (B). The percentages of CD25⁺ cells in 3G11⁻CD4⁺ vs. 3G11⁺CD4⁺ T cells were calculated in (C). (D) These cells were then intracellularly stained with anti-Foxp3 mAb and its expression in four different subpopulations was determined. Columns refer to the percentage of Foxp3⁺ cells in different T cell populations and bars to SD. ** $p < 0.01$, comparisons between total CD4⁺ T cells and other populations. ## $p < 0.01$, comparisons between entire CD25⁺ T cells and 3G11⁻ T cells. All data are representative of three experiments.

Fig. 6. Proliferation and trans-well co-culture assays of CD25⁻ and 3G11⁺-expressing T cell subpopulations

Splenocytes of naïve C57BL/6 mice were stained with anti-3G11 and anti-CD25 mAbs. CD25⁺3G11⁻, CD25⁺3G11⁺, CD25⁻3G11⁻, CD25⁻3G11⁺ cells were sorted by BD FACS Aria sorter (purity >96%). (A) Separated cells were cultured (1×10^6 /ml) for 72 h in the presence or absence of 1.0 μ g/ml anti-CD3. After 60 h of incubation, the cells were pulsed for 12 h with 1 μ Ci of [³H]-methylthymidine. (B) To determine the immunosuppressive effect of these T cell subpopulations, they were co-cultured at a 1:1 ratio with anti-CD3 stimulated CD25⁻CD4⁺ effector T cells of naïve C57BL/6 mice (both at 1×10^6 /ml), either as a mixed suspension or separated in transwell plates. Proliferative responses were determined in co-cultures with or without transwells. The proliferative responses are those of the CD25⁻CD4⁺ effector cells in the lower wells of a transwell. (C) Different CD25- and 3G11-related T cell subpopulations from IL-10^{-/-} mice and their wild type control were purified by FACS sorting and were co-cultured with anti-CD3 stimulated CD25⁻CD4⁺ effector T cells (both at 1×10^6 /ml). Columns refer to mean values and bars to SD. * represents the comparison of CD25⁻CD4⁺ effector T cells only with other groups; @ represents the comparison of CD25⁻3G11⁺ T cells with other groups; and # represents the comparison of IL-10^{-/-} mice with wild type groups. *, p<0.05; **, @, and #, p<0.01; *** and @@@, p<0.001. All data are representative of two experiments.

Fig. 7. Cytokine production of CD25⁻ and 3G11⁻ related T cell subpopulations

CD25⁺3G11⁻, CD25⁺3G11⁺, CD25⁻3G11⁻, and CD25⁻3G11⁺ cells were purified as in Fig. 6. Separated cells were cultured (1×10^6 /ml) for 72 h in the presence or absence of 1.0 μ g/ml anti-CD3 mAb. Supernatants were collected and cytokines levels were determined in triplicate by BD CBA-Flex assays. All data are representative of three experiments. * represents the comparison of CD25⁺3G11⁻ T cells with other groups; # represents the comparison of CD25⁺3G11⁺ T cells with other groups; and & represents the comparison of CD25⁻3G11⁻ with other groups. *, p<0.05; **, ## and &&: p<0.01; ***, ### and &&&: p<0.001.

Fig. 8. Effect of different 3G11⁺ and CD25⁺ related T cell subpopulations on EAE
 CD25⁺3G11⁺, CD25⁺3G11⁻, CD25⁻3G11⁺, and CD25⁻3G11⁻ cells were purified as in Fig. 6. EAE was induced by MOG₃₅₋₅₅ + CFA immunization of female C57BL/6 mice, 8-10 weeks of age. The same number of cells (4×10^6 /mouse) was i.v. injected into these immunized mice at days 0 and 7 p.i. Data were expressed as the mean clinical score on each day. * represents the comparison of PBS i.v. groups with other groups; @ represents the comparison of CD25⁺3G11⁺ T cells i.v. group with other groups; * and @: $p < 0.05$; ** and @@: $p < 0.01$. All data are representative of two repeated experiments.