Immunity to self co-generates regulatory T cells

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Summary: Novel regulatory T cells co-generated in response to autoantigen are deficient in
individuals at risk for type 1 diabetes.
ABSTRACT

Immune responses to self are kept in check by tolerance mechanisms, including suppression by regulatory T cells (Tregs). The defective generation of Tregs specific for self-antigens may lead to autoimmune disease. We identified a novel population of human CD4+ Tregs, characterized by high surface expression of CD52, which is co-generated in response to autoantigen. Blood CD4⁺CD52hi T cells were generated preferentially in response to low-dose autoantigen and suppressed proliferation and interferon-γ production by other T cells. Depletion of resting CD4⁺CD52hi T cells enhanced the T-cell response to autoantigen. CD4⁺CD52hi Tregs were neither derived from nor distinguished by markers of conventional resting CD4⁺CD25⁺ Tregs. In response to the pancreatic islet autoantigens glutamic acid decarboxylase, the generation of CD4⁺CD52hi Tregs was impaired in individuals with and at-risk for type 1 diabetes, compared to healthy controls and individuals with type 2 diabetes. CD4⁺CD52hi Tregs co-generated to self-antigen may therefore contribute to immune homeostasis and protect against autoimmune disease.
Regulatory T cells (Tregs) are sub-populations of T cells that help maintain immune homeostasis and self-tolerance, and thus protect against autoimmune disease \cite{1,2}. In the mouse, natural Tregs develop in the thymus and adaptive Tregs are induced post-natally by antigenic stimulation in the periphery \cite{2}. Natural CD4\(^+\) Tregs are identified by high expression of CD25, the \(\alpha\) chain of the IL-2 receptor, and by expression of the forkhead family transcription factor, Foxp3 \cite{3-5}. In human blood, CD4\(^-\)CD25\(^-\)Foxp3\(^+\) Tregs have been further characterized by reduced expression of CD127, the \(\alpha\) chain of the IL-7 receptor \cite{6,7}. However, these markers characterize resting CD4\(^+\) Tregs and not Tregs generated in response to specific antigen. The ability to identify Tregs generated to self-antigens (autoantigens) would elucidate the role of Tregs in self-tolerance and autoimmune disease.

Autoimmune disease is the outcome of pathogenic immunity to autoantigens. Studies of Tregs with undefined antigen specificity suggest that development of autoimmune disease is influenced by Treg frequency and/or function. Thus, in contrast to healthy controls, CD4\(^+\)CD25\(^+\) Tregs from humans with type 1 diabetes (T1D) \cite{8,9} or multiple sclerosis \cite{10} fail to suppress non-antigen specific proliferation of autologous CD4\(^-\)CD25\(^-\) T cells. Similarly, non-antigen specific induction of IL-10 secreting Tregs by stimulation through CD3 and CD46 is impaired in humans with multiple sclerosis \cite{11}, and cells with a similar phenotype may be deficient in T1D \cite{12}. In T1D, T-cell tolerance is impaired to antigens in insulin-producing \(\beta\) cells of the pancreatic islets, including glutamic acid decarboxylase-65 (GAD) and proinsulin \cite{13}. In the non-obese diabetic (NOD) mouse model of T1D, the transfer of CD4\(^+\)CD25\(^+\) Tregs directed at a putative islet antigen \cite{14} or the induction of Tregs to administered islet antigens (reviewed in \cite{15}) retards diabetes development. In a therapeutic context, autoantigen-specific Tregs have an

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advantage over polyclonal, non-antigen specific Tregs in being unlikely to compromise immunity against infectious agents or tumours. We set out to test the hypothesis that Tregs are generated in response to autoantigen, reasoning that markers of such cells could be identified by a comparative analysis of autologous, autoantigen-specific regulatory and non-regulatory CD4$^+$ T-cell clones. This ‘function-to-phenotype’ strategy (Fig. 1A) allowed us to identify a novel population of human CD4$^+$ Tregs generated in response to autoantigen and deficient in autoimmune disease.
RESULTS

_GAD- and proinsulin-specific T-cell clones display Treg and non-Treg function_

CD4⁺ T-cell clones specific for the islet autoantigens GAD and proinsulin were initially isolated from the blood of three healthy, islet autoantibody-negative individuals and one healthy individual at risk for T1D with autoantibodies to islet antigens, as previously described⁰. Treg clones were identified initially by the ability to suppress proliferation of autologous T-cell lines specific for tetanus toxoid (TT), in an autoantigen-dependent manner (Fig S1). Clones that consistently suppressed proliferation of the TT line by at least 30% in response to autoantigen, e.g. clones 4.3, 4.4 and 4.6 in Fig.1B, were defined as Treg. Clones that did not suppress the TT line and proliferated in response to autoantigen, e.g. clones 4.1, 4.18 and 4.19 in Fig. 1B, were defined as non-Treg. Of 361 clones generated from four individuals, 81 (22%) were classifiable as GAD-specific Treg.

GAD-specific Treg and non-Treg clones expanded in vitro were re-tested for suppressor function and proliferative capacity. In this case, suppressor function was measured by co-culturing autologous GAD-specific Treg and non-Treg clones at different ratios in the absence or presence of GAD. In response to GAD, proliferation of non-Treg clones was suppressed by increasing numbers of Treg clones. In the example shown (Fig. 1C), suppression by the Treg clone of the non-Treg clone was observed at a Treg:non-Treg ratio as low as 0.1:1 and exceeded 80% at a ratio of 2:1. No suppression was observed when two non-Treg clones were co-cultured under identical conditions (Fig. 1C). When the response
of individual Treg and non-Treg clones to GAD was measured, only the non-Treg clones proliferated (Fig. 1D). However, Treg clones proliferated robustly if cultured in the presence of IL-2 at 20U/ml (data not shown). This most likely reflected conditions in bulk PBMCs from which the T cells were cloned because clonal anergy was reversed without loss of suppressor function when Treg clones were stimulated by autoantigen in the context of stimulated bulk PBMCs (Fig. S2).

*Increased surface expression of CD52 distinguishes autoantigen-activated CD4+ Treg and non-Treg clones*

Markers reported to characterize prototypic CD4+CD25+ Tregs were expressed similarly by autoantigen-specific Treg and non-Treg clones, both resting and following stimulation with 5μg/ml plate-bound anti-CD3 antibody. Thus, Treg clones were not distinguished by increased intracellular expression of Foxp3 or CTLA-4, increased surface expression of glucocorticoid-induced TNF receptor (GITR) or CD103 or decreased surface expression of CD127 (data not shown). We therefore compared cell surface expression of other CD antigens between pairs of autologous Treg and non-Treg clones. Clones were analysed by DotScan CD antibody array, resting and following stimulation for 24 hours with 5μg/ml of plate-bound anti-CD3 antibody. In the example shown (Fig. 2A), pairs of Treg and non-Treg clones from two healthy subjects expressed similar levels of the activation marker CD44 but, following stimulation, expression of CD52 was consistently higher (up to 5-fold) on Treg clones, as confirmed by flow cytometry (Fig. 2B).
To determine the possible significance of CD52 on CD4+ T cells in bulk PBMCs, we measured Treg and non-Treg clones isolated from divided CD4+ T cells expressing different levels of CD52, following incubation of PBMCs with GAD. The majority of GAD-specific Treg clones were derived from CD4+ T cells that were CD52hi, corresponding to the upper 10% of undivided CD4+CD52+ PBMCs (Table S1). In further studies, we used the upper 5% as a stricter cut-off to define CD4+CD52hi T cells. Of 151 clones isolated from the CD52hi fractions of two healthy donors, 21 suppressed proliferation of an autologous TT-specific T-cell line in a GAD-dependent manner, whereas only 3/101 of clones isolated from the respective CD52lo fractions suppressed (Fig. 2C). As predicted by our earlier finding, the proliferative response of the clones to GAD was inversely correlated with their suppressor function (Fig. 2D). Treg clones isolated from CD4+CD52hi cells of a subject at-risk of T1D and a subject with T1D had the same phenotype and function as those from the healthy individuals.

**CD4+CD52hi Tregs are co-generated in response to T-cell activation by autoantigen**

A minor proportion (approximately 15%) of CD4+ cells that divided in response to autoantigen were CD52hi (Fig. 3A). Sorted CD4+CD52hi and CD4+CD52lo T cells were tested for their ability to suppress the proliferation of sorted TT-specific, autologous CD4+ T cells. CD4+CD52hi cells that divided in response to proinsulin (Fig. 3B) or GAD (data not shown) suppressed proliferation of TT-specific T cells in an autoantigen-dependant manner, whereas suppression was not observed with CD52lo cells. CD8+ T cells also divided in response to autoantigen, but CD8+CD52hi cells did not exhibit suppressor
function (data not shown). ELISpot assays consistently demonstrated that the number of cells producing IFN-γ (Fig. 3C) or IL-17 (Fig. S3) in sorted CD4⁺CD52^{hi} cells re-stimulated with autoantigen was significantly less than in the CD4⁺CD52^{lo} population. Furthermore, recombined at a 1:1 ratio, CD52^{hi} cells almost completely suppressed cytokine production by CD52^{lo} cells (Fig. 3C). These results predicted that CD52^{hi} cells dynamically suppress pro-inflammatory CD52^{lo} cells within the bulk population of dividing CD4⁺ T cells. To investigate this, we first showed that CD4⁺CD52^{hi} T cells co-generated to autoantigen are derived entirely from CD4⁺CD52^{hi} cells in resting PBMCs that, in the absence of activation, have no suppressor function (data not shown). We then showed that when CD4⁺CD52^{hi} cells were depleted from resting PBMCs, the proliferative response of the remaining cells to autoantigen was significantly increased (Fig. 3D); in contrast, depletion of CD4⁺CD52^{lo} cells reduced the response (Fig. 3D).

Selection for CD52^{hi} expression therefore enriched for autoantigen-activated CD4⁺ T cells with suppressor function, hereafter termed CD4⁺CD52^{hi} Tregs. These cells were routinely analysed 7 days after culturing PBMCs with autoantigen, but could be detected as early as 3-4 days when it was first possible to identify divided cells by CFSE dilution. Similar to Treg clones, CD4⁺CD52^{hi} Tregs generated from PBMCs in response to GAD could not be distinguished from CD4⁺CD52^{lo} T cells by markers of conventional CD4⁺CD25⁺ Tregs (data not shown). Moreover, depletion of CD4⁺CD25⁺ cells from PBMCs (5.4-7.2% of CD4⁺ cells) had no effect on the ability to generate CD4⁺CD52^{hi} Tregs to autoantigen (data not shown).
**CD4⁺CD52hi cells are generated preferentially in response to low-dose autoantigen**

Generation of Tregs preferentially to autoantigen would be an evident mechanism of self-tolerance. We therefore compared the generation of CD4⁺CD52hi T cells in response to autoantigen compared to non-self antigen. In relation to total divided cells, defined as the cell division index, the ratio of CD4⁺CD52hi to CD4⁺CD52lo cells was maximal at the lowest concentration of GAD at which an accurate measurement of cell division was possible (Fig. 4A). In contrast, although CD4⁺CD52hi cells with suppressor function were also generated in response to the non-self antigen TT, the ratio of CD4⁺CD52hi to CD4⁺CD52lo cells was 5-8 fold lower than for autoantigen and did not demonstrate a strong inverse relationship with antigen dose (Fig. 4B).

**CD4⁺CD52hi T cells generated in response to autoantigen are deficient in type 1 diabetes**

To investigate their potential in protecting against autoimmune disease, we measured CD4⁺CD52hi T cells in response to GAD in individuals with pre-clinical and established T1D, compared to HLA-matched healthy controls and individuals with type 2 diabetes (T2D) (Table S2). The proportion of CD4⁺CD52hi cells generated in response to GAD in individuals with pre-clinical and established T1D was significantly lower than in healthy controls or individuals with T2D (Fig. 5A). This difference was not observed with the control antigen TT (Fig. 5B). There was no association between HLA phenotype and the proportion of CD4⁺CD52hi cells.
DISCUSSION

We describe for the first time a sub-population of human CD4+ T cells with suppressor function generated in response to activation by antigen. We identified a marker, CD52, which allows these cells to be monitored during immune responses in human blood. When T cells are activated by antigen, the CD52^hi sub-population of divided cells is functionally distinct and suppresses proliferation and pro-inflammatory cytokine production by other T cells within the population. We propose that this is a physiological mechanism of immune homeostasis operating proximally to regulate responsive CD4+ T cells. Our findings suggest that this mechanism is adapted for chronic, low level expression of self-antigen. The relative deficiency of CD4^+CD52^hi Tregs activated by the islet autoantigen GAD in individuals at-risk of T1D implies that impaired generation of these cells contributes to the pathogenesis of this autoimmune disease.

To our knowledge, only two other reports document human autoantigen-specific CD4^+ Treg clones, to the skin autoantigen, desmoglein 3, in pemphigus vulgaris\textsuperscript{19}, and to a red cell autoantigen in haemolytic anemia\textsuperscript{20}. In both cases, Tregs were selected based on expression of IL-10. Our approach was unbiased, based only on autoantigen specificity and T-cell function. Clones might not necessarily be representative of fresh polyclonal T cells but they allow comparison of pure autologous, antigen-specific T cells, which is otherwise not possible. Screening for Treg clones by antigen-specific suppression of a proliferating TT line may not have been optimal to distinguish the function of all clones. Although
stringent for Treg clones, it may have been relatively insensitive for non-Treg clones that proliferated weakly to autoantigen compared to proliferation of the TT line to TT. Despite these potential limitations, multiple Treg clones from different individuals were distinguished by high expression of CD52, which was then shown to be a marker of autoantigen-activated CD4+ Tregs in PBMCs. A role for CD4+CD52hi Tregs in immune homeostasis was evidenced by their ability to suppress proliferation and pro-inflammatory cytokine production by CD4+CD52lo T cells, and by the increased proliferation in response to autoantigen when PBMCs were first depleted of CD4+CD52hi T cells. CD4+CD52hi Tregs do not arise from resting CD4+CD25+ T cells, or natural Tregs, but are a product of division of T cells within the resting population of CD4+CD52hi T cells.

Human CD52 is a small glycopeptide tethered to the surface of lymphocytes by a glycosylphosphatidylinositol anchor, well known as the target of the therapeutic complement-dependent, lymphocyte-depleting monoclonal antibody, Campath-1. The function of CD52 is unknown, but it is reported to be a co-stimulation molecule for T cells, including CD4+ Tregs. CD52 has not been shown previously to distinguish antigen-activated Tregs. In some experiments CD52hi cells underwent fewer rounds of division than CD52lo cells, suggesting they were relatively anergic in the bulk PBMC population and might rely on bystander ‘help’, eg via IL-2 from the CD52lo cells, to proliferate. CD52 is downregulated as lymphocytes undergo division and induction of quiescence in Jurkat T-cell lines is associated with markedly higher CD52 expression. Retention of CD52 expression in response to antigen stimulation could therefore be due to relative anergy. However, suppression by CD4+CD52hi Tregs was not just a reflection of
anergy, because Tregs could not be enriched by selecting for cells that had undergone fewer divisions, unless they were CD52\(^{hi}\). Furthermore, Treg clones were not enriched in the CD52\(^{lo}\) fraction even though this fraction included a significant proportion of more slowly dividing cells. It is highly unlikely that Tregs were induced by the very low concentration of antibody used to detect CD52 because the majority of cells dividing in response to antigen expressed CD52 yet only the CD52\(^{hi}\) population was enriched for autoantigen-activated Tregs and Treg clones. The mechanistic relationship between CD52 and the suppressor function of CD4\(^+\)CD52\(^{hi}\) cells awaits exploration.

The existence of autoantigen-activated T cells in healthy individuals \(^{29,30}\) underscores the importance of immune tolerance mechanisms necessary to maintain homeostasis and to prevent autoimmune disease. Our findings provide potential new insights into this process. The preferential generation of CD4\(^+\)CD52\(^{hi}\) Tregs at low concentrations of autoantigen (GAD), in higher numbers compared to an infectious antigen/immunogen (TT), suggests that CD4\(^+\)CD52\(^{hi}\) Tregs could be continuously generated in response to chronic, low level expression of ‘self’, representing a default mechanism of self-tolerance. Two concepts of immunology derived from early experimental mouse studies are that persistence of antigen is required to maintain tolerance \(^{31}\) and that T-cell tolerance is induced in the ‘low zone’ of antigen concentration \(^{32}\). Our results are consistent with these concepts. The response to autoantigen that we observed may be based on one or more features of self antigens or their presentation, e.g. concentration, avidity for the T-cell receptor, chronicity of presentation to the T cell or the nature of co-stimulation signals expressed by self-antigen presenting cells. Efficacious generation of Tregs at low concentrations of autoantigen also raises the
possibility that differences in autoantigen-activated T-cell responses between healthy individuals and those with autoimmune disease might be more obvious at concentrations of autoantigen at the lower end of the dose-response range.

The weight of evidence favours the view that people at risk for autoimmune disease have a deficiency in the number and/or function of Tregs for disease-specific autoantigens, but this hypothesis was not able to be tested previously because no marker was known that distinguished Tregs from non-Tregs in response to autoantigen. We found that individuals with T1D and at risk for T1D had a lower proportion of CD4⁺CD52⁺ Tregs in response to GAD but not TT, compared to HLA-matched healthy individuals and those with T2D. These findings are not explained by age, clinical diabetes or insulin therapy and suggest that a defect in the mechanism that generates CD4⁺CD52⁺ Tregs in response to islet autoantigens may contribute to the development of T1D. Studies in the NOD mouse show that Tregs induced by administration of autoantigen prevent diabetes and trials of autoantigen-based immunotherapy are currently underway in humans at risk of T1D (http://www2.diabetestrialnet.org/, http://www.stopdiabetes.com.au/). A causal relationship between deficiency of islet autoantigen-activated CD4⁺CD52⁺ Tregs and development of T1D would be strengthened if the deficiency could be corrected by autoantigen-based immunotherapy and shown to prevent diabetes. The discovery of Tregs co-generated in response to autoantigen should provide new insights into immune tolerance in health and disease.
METHODS

Blood samples

Venous blood was obtained with informed consent and the approval of Melbourne Health Human Ethics Committee from 11 subjects with islet autoantibodies at risk for T1D (Pre-T1D), 12 subjects with established T1D, nine healthy control subjects and six subjects with T2D (Table S2). Preclinical T1D subjects were relatives of someone with T1D, with autoantibodies to at least two islet autoantigens, including GAD, and a 5-year risk of developing T1D of 26-50% \(^{33}\). T1D subjects were a median of 21.4 months since diagnosis and all except three had autoantibodies to GAD. Preclinical T1D, T1D and control subjects did not differ in frequency of shared HLA DR 3 or 4 risk alleles, but the median age of the preclinical subjects (9 years) was significantly lower than that of the T1D (29 years) or control (43 years) subjects. Diabetes was diagnosed by American Diabetes Association criteria \(^{34}\). PBMCs were isolated over Ficoll/Hypaque (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and washed twice in PBS.

Antigens

Tetanus toxoid (TT) was supplied by CSL (Parkville, Victoria, Australia). Recombinant GAD65, produced in Baculovirus and purified as described \(^{35}\), was obtained from Dr Peter van Endert, Hôpital Necker, Paris. The endotoxin concentration of the GAD stock solution, measured by Limulus lysate assay (BioWhittaker, Walkerville, MD, USA), was 1.2 EU/mg/ml. Recombinant human proinsulin was produced in-house according to Cowley and Mackin \(^{36}\). After refolding and reversed phase high performance liquid chromatography
(RP-HPLC) purification, the protein resolved as a single species of expected molecular mass by matrix-assisted laser desorption/ionisation-time of flight mass spectrometry. The endotoxin concentration of proinsulin stock solution was 0.51 EU/mg/ml.

*Isolation of antigen-specific CD4⁺ T-cell clones/lines*

PBMCs were stained with CFSE dye as previously described 30. Stained cells (2 x 10⁵ in 150μl) were cultured in 96-well round-bottom plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) either with medium alone, GAD (5μg/ml), proinsulin (20μg/ml) or TT (10 Lyons flocculating units (LFU)/ml). After 7 days in culture, cells with each antigen were pooled, washed in PBS and stained on ice with anti-human CD4-PE (BD Pharmingen, San Diego, CA, USA). A single, viable (propidium-iodide negative) CFSEdim CD4⁺ cell that had undergone division in response to antigen was sorted with a FACS Aria (Becton Dickinson) into each well of a 96-well plate, as previously described 16. Each well contained 2x10⁵ irradiated allogeneic PBMC, 2x10⁴ irradiated JY EBV cells and 30ng/ml anti-CD3 (OKT3) in medium supplemented with 20U/ml IL-2 (NCIBRB Preclinical Repository, Fisher Biosciences, Rockville, MD) and 5ng/ml IL-4 (Peprotech, Rocky Hill, NJ, USA). Fungizone (Amphotericin B, Bristol-Myers Squibb, Princeton, NJ, USA) was added to cultures at a final concentration of 2μg/ml. Cultures were supplemented on days 7 and 14 with fresh cytokines in 50μl of medium, to the final concentrations indicated. Growing clones were identified by visual inspection, expanded into 48-well plates after ~2 weeks and then tested for antigen-specific suppressor function (see below). TT-specific T-cell lines were generated by culturing 1x10⁷ PBMC with 10LFU/ml TT; IL-2 (20U/ml) was
added on day 3 and the cells were used between day 7 and 10. Autoantigen-specific clones were expanded by re-stimulating with 2.5μg/ml PHA (Sigma, St. Louis, MO, USA) in the presence of 20U/ml IL-2 and 5ng/ml IL-4.

**Suppressor T-cell assay**

Clones were screened for autoantigen-specific suppressor function by measuring their ability to inhibit proliferation of autologous T-cell lines/clones. For screening, 200μl of each clone was taken from culture, washed once with PBS and once with medium. Clones were added to 4 replicate wells each containing 5x10^4 irradiated autologous PBMC as APCs, 1x10^4 autologous TT-specific T-cell line and 10LFU/ml TT. GAD or proinsulin at the indicated concentration was added to half of the wells. Following 48 hours of culture, 0.5μCi ³H-thymidine was added to each well. Plates were harvested 16 hours later and proliferation was determined by ³H-thymidine incorporation. Clones were operationally defined as Treg if, in the presence of autoantigen, they inhibited proliferation of the TT line by >30%. This threshold was chosen to exceed twice the inter-assay coefficient of variation of the response of the TT line to TT.

**Two-colour suppressor T-cell assay**

Treg or non-Treg clones were labelled with the fluorescent dye, PKH26 (Sigma, St Louis, MO, USA), at a final concentration of 2μM and co-cultured at a 1:20 ratio with autologous PBMCs labelled with 0.5μM CFSE. Following 5-6 days in culture, cells were analysed on a
FACSAria flow cytometer and the proportion of PBMC or clone responding to antigen was determined.

**Immunophenotyping**

For surface phenotyping, cells were collected and stained on ice with the appropriate concentrations of PE-labelled antibodies to GITR (eBioscience, San Diego, CA, USA), HLA-DR and CD103 (BD Pharmingen, Franklin Lakes, NJ, USA). Foxp3 expression was detected with a flow cytometry kit (Biolegend, San Diego, CA, USA). In some experiments, PE-labelled antibody to CTLA-4 (BD Pharmingen) was added at the same time as antibody to Foxp3 to detect intracellular expression of CTLA-4. The expression of CD antigens on the surface of Treg and non-Treg clones was measured by cell capture with an array of membrane-bound CD antibodies and captured cells quantified by optical densitometry with a DotReader (MEDSAIC Pty Ltd, Sydney, Australia). Clones (1x10⁶) were taken directly from culture on day 10 and analysed resting or after stimulation for 24 hours with 5μg/ml of plate-bound anti-CD3.

**Analysis and cloning of autoantigen-activated CD4⁺CD52 hi and CD4⁺CD52 lo T cells**

PBMC were labelled with CFSE or PKH26 and cultured with and without autoantigen, as described above. Five to seven days later, usually the latter, cells were collected and incubated on ice with unconjugated anti-CD52 antibody (clone H24-930, BD Pharmingen) followed by APC-labelled anti-mouse IgG (Caltag). After washing in FACS buffer (PBS, 0.1% pooled human serum), cells were stained with PE-Cy7-labelled anti-CD4 antibody.
(BD Pharmingen) before a final wash in FACS buffer. In some experiments, anti-CD52 antibody directly conjugated with PE (clone CF1D12, Caltag) was used. Cells that had divided in response to GAD or proinsulin were identified as CD52<sup>hi</sup> and CD52<sup>lo</sup> and sorted in the FACS Aria, washed in PBS and counted. CD4<sup>+</sup> T cells that divided separately in response to TT were also sorted, washed and counted. Cells in the CD4<sup>+</sup>CD52<sup>+</sup> population were depleted by sorting on the FACS Aria; as a control, undepleted PBMCs were also passed through the FACS Aria.

The function of autoantigen-activated CD52<sup>hi</sup> and CD52<sup>lo</sup> cells was analysed in two ways. First, CD52<sup>hi</sup> or CD52<sup>lo</sup> cells were co-cultured with TT-responsive CD4<sup>+</sup> T cells (1,000/well) at a 1:1 ratio in 6 wells of a 96-well plate. Each well also contained 5x10<sup>4</sup> irradiated autologous PBMCs as APCs and 10LFU/ml TT antigen to stimulate proliferation of the autologous TT-responsive CD4<sup>+</sup> T cells. The relevant autoantigen, GAD (5µg/ml) or proinsulin (20µg/ml), was added to 3 of the 6 wells to activate sorted ‘Tregs’ or ‘non-Tregs’. As a control, irradiated PBMCs were also cultured with or without antigen. Second, following culture of CFSE-labelled PBMCs with antigen for up to 7 days, CD52<sup>hi</sup> or CD52<sup>lo</sup> cells (5,000 each) sorted on the FACS Aria were cultured alone or co-cultured at a 1:1 ratio for 24 hr, in 6 wells of a 96-well ELISpot plate pre-bound with anti-IFN-γ antibody (Mabtech, NSW, Australia). Each well also contained 25x10<sup>3</sup> irradiated autologous PBMCs as APCs. The relevant autoantigen, GAD (5µg/ml) or proinsulin (20µg/ml), was added to 3 of the 6 wells to activate the sorted cells. Irradiated PBMCs were also cultured with or without antigen.
Autoantigen-specific CD4+ T cells were cloned from either the CD52hi or CD52lo fractions and expanded as described above. Clones were screened for autoantigen-dependent Treg function in the suppressor assay: 10,000 cells from each clone were added at a 1:1 ratio with autologous TT line cells in the presence of TT alone or TT and autoantigen. Proliferation of the TT line without antigen, with TT or with TT plus autoantigen was also measured.

*Enumeration of CD4+CD52hi cells in blood*

PBMCs were isolated, labelled with CFSE as described above and cultured without antigen or in the presence of GAD or TT at the concentrations above. After one week, the cells were labelled with antibodies to CD4 and CD52 and the ratio of CD52hi to CD52lo cells within the divided CD4+ population was determined. To standardise comparisons between individuals, undivided CD4+ cells were used as an internal reference in each assay. The majority of GAD-specific Treg clones generated were derived from divided CD4+CD52+ cells that corresponded to the top 10% of undivided CD4+CD52+ PBMCs (Table S1). Therefore, to define cells as CD52hi we chose a stringent cut-off of divided CD4+ cells that corresponded to the top 5% of undivided CD4+CD52+ PBMCs. In three consecutive weekly assays on the same two healthy donors the coefficient of variation of the CD52hi to CD52lo ratio in response to GAD was 21%.
*Statistical analysis*

Group differences were analysed by non-parametric Mann-Whitney test (two-tailed). Correlation was determined by Spearman rank-log test. The proportions of Treg clones from CD52\textsuperscript{hi} and CD52\textsuperscript{lo} cells were compared by chi-square test. Analyses were performed with GraphPad Prism version 3.0cx for Macintosh (GraphPad Software Inc., San Diego, California, USA).
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FIGURE LEGENDS

Figure 1. Isolation and expansion of autoantigen-specific Treg and non-Treg clones.  
(A) Schema of the approach used to identify markers of autoantigen-specific Treg clones.  
(B) Treg clones (4.3, 4.4 and 4.6) were identified by their ability to suppress proliferation of an  
autologous tetanus toxoid (TT)-specific T-cell line in the absence (open bars) or presence  
(filled bars) of the cognate autoantigen, GAD. Shown is an example of clones isolated from  
a healthy donor.  
(C) Expanded GAD-specific Treg clones (example clone 4.4) suppressed  
proliferation of autologous GAD-specific non-Treg clones (example clone 4.19) in a dose- 
dependent manner when co-cultured in the presence (filled squares) but not in the absence  
(open squares) of GAD. Suppression was not observed when a non-Treg clone (example  
clone 4.15) was co-cultured with a non-Treg clone (example clone 4.19) in the presence  
(filled circles) or in the absence (open circles) of GAD.  
(D) Treg clones (example clone  
4.4) failed to proliferate in response to GAD, whereas non-Treg clones (example clones  
4.15 and 4.19) proliferated strongly. Results are representative of at least five independent  
experiments. Data are mean ± sem of triplicate wells.

Figure 2. Increased surface expression of CD52 distinguishes autoantigen-activated Treg  
clones.  
(A) CD antigens expressed by GAD-specific Treg (filled bars) and non-Treg (open  
bars) clones after stimulation by anti-CD3 antibody, measured in a CD antibody array.  
Each panel shows pairs of Treg and non-Treg clones (3.3 and 3.73, and 4.4 and 4.19) from  
two healthy subjects.  
(B) CD52 expression on Treg clones 3.3 and 4.4 (open plots) and  
non-Treg clones 3.73 and 4.19 (filled plots) measured by flow cytometry.  
(C) The majority
of CD4+ T-cell clones with suppressor function are generated from CD4+CD52hi not CD4+CD52lo cells, following division of CFSE-labelled PBMCs incubated with GAD for 7 days (P=0.004). Shown are the proportions of Treg and non-Treg clones from two healthy donors (mean + sem). (D) Proliferation in response to GAD is inversely related to GAD-dependent suppressor function of CD4+CD52hi T-cell clones (r = -0.49; P = 0.023).

Figure 3. CD4+CD52hi Tregs are co-generated in response to T-cell activation by autoantigen. (A) A minor proportion of CD4+ T cells dividing in response to proinsulin is CD52hi (healthy donor). (B) Proliferation of CD4+ T cells specific for TT was suppressed by CD52hi cells in a proinsulin-dependent manner. Sorted cells were co-cultured at a 1:1 ratio. Suppression was not observed when CD52lo cells were co-cultured with the TT-specific CD4+ T cells. Data are mean + sem of triplicate wells. (C) IFN-γ production by CD4+CD52hi and CD4+CD52lo T cells, sorted 7 days after incubating CFSE-labelled PBMCs with GAD, measured by ELISpot in the absence (open bars) or presence (filled bars) of GAD. When co-cultured at a 1:1 ratio, CD52hi cells suppressed IFN-γ production by CD52lo cells in response to GAD. Data are mean + sem of triplicate wells and are representative of three independent experiments. (D) Proliferative response of PBMCs to GAD following depletion of either CD4+CD52hi cells (upper 10% of CD4+CD52+ population) or CD4+CD52lo cells (bottom 10% of CD4+CD52+ population). Data are mean + sem of two independent experiments.
Figure 4. CD4⁺CD52⁺ cells are generated preferentially in response to low-dose autoantigen. The ratio (x100) of CD4⁺CD52⁺ to CD4⁺CD52⁻ cells (filled circles) and the cell division index (17) (filled squares) are plotted as a function of GAD (A) and TT (B) concentrations. Data are representative of five independent experiments.

Figure 5. CD4⁺CD52⁺ Tregs generated in response to autoantigen are deficient in type 1 diabetes.

The proportion of CD4⁺CD52⁺ T cells generated from PBMCs in response to GAD (A) and tetanus toxoid (TT) (B) is shown for individuals both at risk for type 1 diabetes (Pre-T1D) and with T1D, compared to healthy individuals or those with type 2 diabetes (T2D) (P = 0.006 for each comparison). Bars are the mean for each group.
FIGURES

Figure 1

A

PBMC + Ag
7 days → Clone CD4+ cells → Define function → Compare surface phenotypes

B

Donor #4

C

D
Figure 2

A

Binding density

CD antigen

CD4  CD25  CD44  CD52

B

Donor #3  Donor #4

Resting

Activated

CD82

C

% of clones with suppressor function

CD52hi  CD52lo

D

Proliferation (SI)

% suppression

r = 0.5153
p < 0.05
Figure 3
Figure 4

A

B

![Graph A](image1)

![Graph B](image2)
Figure 5

A

GAD

CD52hi/lo CD4+ T cells

pre-T1D  T1D  Healthy  T2D

B

Tetanus toxoid

CD52hi/lo CD4+ T cells

pre-T1D  T1D  Healthy  T2D