ORIGINAL ARTICLE

Overexpression of CD45RA isoforms in carriers of the C77G mutation leads to hyporeactivity of CD4$^+$CD25^{high}Foxp3$^+$ regulatory T cells

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Disorders in regulatory T-cell (T$_{reg}$) function can result in the breakdown of immunological self-tolerance. Thus, the identification of mechanisms controlling the activity of T$_{reg}$ is of great relevance. We used T$_{reg}$ from individuals carrying the C77G polymorphism as models to study the role of CD45 molecules in humans. C77G prevents splicing of CD45 exon A thereby leading to an aberrant expression pattern of CD45 isoforms in affected individuals. Resting and *in vitro* expanded/activated CD4$^+$CD25^{high}Foxp3$^+$ T$_{reg}$ from carriers of C77G strongly expressed CD45RA isoforms whereas these isoforms were almost absent in cells from individuals with wild-type CD45. C77G T$_{reg}$ showed diminished upregulation of activation markers, lower phosphorylation of p56^{lck}(Y505) and a reduced proliferative potential when stimulated with anti-TcR or anti-TcR plus CD28 mAb suggesting decreased responsiveness to activating stimuli. In addition, the capacity to suppress proliferation of conventional CD4$^+$ T cells was impaired in C77G T$_{reg.}$ Furthermore, microarray studies revealed distinct gene expression patterns in T$_{reg}$ from C77G carriers. These data suggest that the changes in CD45 isoform combination resulting from the C77G mutation alter the responsiveness of T$_{reg}$ to TcR-mediated signaling. Targeting CD45 isoform expression might be a useful approach to modulate T$_{reg}$ function.

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INTRODUCTION

Regulatory T cells (T$_{reg}$) belong to the CD4$^+$ T-cell pool[1,2] and can be identified by high-level expression of CD25, expression of Foxp3 (ref. 3) and low-level expression of CD127.[4] T$_{reg}$ prevent autoimmune responses, as well as overreaction of the immune system, thus having an essential role in the control of immune responses.[5] Functional alterations of their regulatory activity can break natural self tolerance, leading to the development of autoimmune diseases.[6]

Cellular therapy using T$_{reg}$ is currently the focus of intense research. On the one hand, the immunosuppressive capacity of T$_{reg}$ is desirable to treat autoimmune diseases or to inhibit immune responses to allografts and the occurrence of graft-versus-host disease after bone marrow transplantation. In these scenarios, treatment is performed by transferring *in vitro* expanded T$_{reg}$ to recipients. On the other hand, since T$_{reg}$ seem to impede immune responses to tumors,[7] downregulation of their suppressive activity is favored in individuals suffering from cancer. The availability of strategies for effective T$_{reg}$ *in vitro* expansion and the identification of target molecules, which allow the modulation of T$_{reg}$ function will be important for further development of T$_{reg}$-mediated therapies. Recent data show that engagement of the glucocorticoid-induced tumor necrosis factor receptor can enhance the activity of T$_{reg}$.[8] Targeting of CTLA4 has also been suggested as a means for altering T$_{reg}$ responses.[9] Evidence is accumulating to show that human T$_{reg}$ must be activated through the TcR to become suppressive,[10] suggesting that the strength of TcR-mediated signaling could also have a role in the control of T$_{reg}$ activity.

An important regulator of the threshold of TcR-mediated signaling is the cell-surface phosphatase CD45,[11] rendering CD45 molecules possible targets to modify T$_{reg}$ activity. Indeed, it has been shown that the suppressive activity of mouse T$_{reg}$ can be abrogated by cross-linking CD45 molecules.[12] CD45 is encoded by a single gene (*PTPRC*, protein tyrosine phosphatase, receptor-type C) containing three alternatively spliced exons A, B and C.[13,14] Depending upon cell type and state of activation, alternative splicing of CD45 results in the expression of different isoform patterns on the cell surface.[15,16] In humans, naive/unprimed T cells express CD45RA isoforms (CD45RA$^+$ cells). During activation, CD45RA molecules are downregulated and CD45R0 isoforms are expressed in which exons A, B and C are spliced out. The CD45R0$^+$ T-cell subset is regarded to contain activated/memory cells.

Various single-nucleotide polymorphisms in CD45 have been described in humans, which lead to aberrant splicing of the variable exons. Some of these variants may be associated with certain autoimmune or infectious diseases.[17–19] The C77G point mutation in exon A[20] disrupts an exon-splicing silencer that normally represses the use of the 5' splice site of exon A.[21] Individuals carrying C77G cannot splice out exon A and thus coexpress CD45RA and CD45R0 on their activated/memory cells instead of CD45R0 only.[22] We have shown previously that the aberrant CD45 isoform pattern on CD4$^+$ activated/memory T cells enhances sensitivity of TcR- and cytokine receptor-mediated signal transduction.[23,24] Here, we investigated the effects of C77G in CD4$^+$CD25^{high}Foxp3$^+$ T$_{reg}$. We found, in contrast to the hyperreactivity of activated/memory cells, hyporeactivity of C77G T$_{reg}$ to TcR-mediated stimulation.

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RESULTS

Diminished responsiveness of T$_{reg}$ from C77G carriers to T cell receptor-mediated stimulation

Among the CD4$^+$ T-cell subset from C77G carriers and controls, T$_{reg}$ were identified as cells expressing highest levels of CD25, low levels of CD127, and FoxP3 (Figure 1a). The C77G polymorphism in exon A of the CD45 gene prevents splicing of this exon and thereby the downregulation of CD45RA isoforms. Thus, as expected, all CD4$^+$CD25^{high} T$_{reg}$ from individuals carrying C77G strongly expressed CD45RA molecules whereas only a small proportion of cells from individuals with wild-type CD45 was CD45RA$^+$ (Figure 1b). CD45 isoforms detected by CD45RB, CD45RC and CD45R0 mAb did not differ between T$_{reg}$ from C77G carriers and controls. Correlated analysis of CD45RA and CD45R0 expression in gated CD4$^+$CD25^{high} cells revealed that the small subset of CD45RA$^+$ cells T$_{reg}$ did not express CD45R0 isoforms in wild-type individuals (Figure 1c). In C77G carriers, CD45R0 isoforms were mainly found on cells expressing slightly reduced levels of CD45RA isoforms. Despite overexpression of CD45RA isoforms in T$_{reg}$ from C77G carriers, the overall amount of CD45 molecules was not enhanced as suggested by similar staining intensity found with CD45$_{common}$ mAb in C77G and wild-type T$_{reg}$ (mean fluorescence intensity; wild type: 4048 ± 317, $n = 3$; C77G: 3522 ± 93, $n = 3$) (Figure 1b). We also compared the expression patterns of molecules that are regarded to be directly involved in T$_{reg}$ function (Foxp3, CD127, CTLA-4, CD28, PD-1 and PD-L1) but did not find significant differences between C77G and control cells (data not shown). Furthermore, there was no significant difference in the frequency of CD4$^+$CD25^{high}Foxp3$^+$ cells between C77G carriers and wild-type controls (C77G: $1.5 \pm 0.14\%$, $n = 6$; wild-type: $1.4 \pm 0.12\%$, $n = 6$; $P = 0.26$; data are expressed as percentage of cells among the CD4$^+$ subset).

CD45 molecules have an important role in the regulation of T cell receptor (TcR) associated signaling pathways. To study whether the different CD45 patterns in C77G and wild-type T$_{reg}$ may influence signaling, peripheral blood mononuclear cells (PBMC) were stimulated with increasing concentrations of plate-bound anti-TcR antibodies and upregulation of the early activation marker CD69 was studied in gated CD4$^+$CD25^{high} T$_{reg}$. These experiments revealed that CD69 upregulation in response to TcR triggering was more pronounced in T$_{reg}$ from individuals with wild-type CD45 than in cells from C77G carriers (Figure 2a).

The diminished upregulation of CD69 in C77G T$_{reg}$ suggested reduced intensity of TcR-mediated signaling. To follow this assumption, we decided to study an early event in the TcR signaling cascade. It has recently been shown that optimal TcR stimulation induces phosphorylation of both the Y392 and Y505 residue of p56^{lck}. Under such conditions, when Y392 was phosphorylated, simultaneous phosphorylation at Y505 does not inhibit T-cell activation.[25] Since Y505 of p56^{lck} is a well-defined target of CD45 phosphatase activity, we monitored phosphorylation of p56^{lck} in response to TcR triggering. In resting T$_{reg}$ from C77G individuals and wild-type controls, there was weak if any binding of the anti-p56^{lck}/Y505 antibody suggesting mainly a non-phosphorylated status of tyrosine 505 (Figure 2b). The residue became quickly phosphorylated in C77G and control cells after antibody-mediated triggering of the TcR, as shown by a clear-cut increase in p56^{lck}/Y505 binding. However, the intensity of phosphorylation was diminished by about 25% in T$_{reg}$ from C77G carriers (Figures 2b and c). To compare a signaling event downstream of p56^{lck}, we studied phosphorylation of ZAP70 at Y319 in response to TcR triggering (Figure 2d). There was a very small shift in fluorescence intensity suggesting some phosphorylation of ZAP70. However, we did not observe differences between T$_{reg}$ from CD45 wild-type individuals and C77G carriers.

We next compared the proliferative potential of C77G T$_{reg}$ and cells from individuals expressing wild-type CD45. T$_{reg}$ as defined by the marker profile described above (Figure 1a) were purified by cell sorting and stimulated with CD3/CD28-coated beads in the presence of high doses of IL-2. In all, 5×10^4 cells were seeded and cell numbers were determined on day 9 and day 15 after starting the culture. In T$_{reg}$ cultures from individuals with wild-type CD45, we observed a 7.6-fold expansion on day 9 and a 21.5-fold increase in cell number on day 15 (Figure 3). T$_{reg}$ from C77G carriers also proliferated in response to CD3/CD28 plus IL-2 stimulation. However, the intensity of expansion was significantly reduced with a 3.3-fold expansion on day 9 and an 8.5-fold increase in cell number on day 15. To exclude enhanced cell death of C77G T$_{reg}$ as reason for reduced *in vitro* expansion, we analyzed the proportion of viable cells at the end of the expansion period by flow cytometry (forward/side scatter characteristics to distinguish between viable and dead cells). These studies revealed great variability between individual experiments (22–39% of viable cells). However, we did not find in general reduced levels of viable cells in T$_{reg}$ cultures from C77G carriers.

Impaired suppressive capacity of C77G T$_{reg}$

In vitro expanded T$_{reg}$ populations were used for a more detailed analysis of cells from C77G carriers and controls. Despite the significant differences in the proliferative capacity of C77G and control T$_{reg}$ (Figure 3) both expressed a similar T$_{reg}$ marker profile (CD4$^+$CD25^{high} Foxp3$^+$) after *in vitro* expansion (Figure 4a). However, as in resting T$_{reg}$, there was clear overexpression of CD45RA isoforms in T$_{reg}$ from C77G carriers (Figure 4b). CD45RA mAb detects exon A-encoded epitopes. Thus, reactivity of CD45RA mAb could mean that the cells express CD45(ABC) or CD45(AB) isoforms ((CD45(AC) and CD45(A) do not exist in humans)). To distinguish between these possibilities, we performed RT-PCR studies allowing detection of mRNA coding for individual CD45 isoforms. Gel electrophoresis revealed three mRNA species in wild-type T$_{reg}$ corresponding to the isoforms CD45(BC), CD45(B) and CD45(0). The same isoforms were detected in C77G T$_{reg}$, and in addition, an mRNA corresponding to CD45(AB) (Figure 4c).

To study whether aberrant expression of CD45(AB) may influence T$_{reg}$ function, we compared the suppressive capacity of T$_{reg}$ from C77G carriers and controls in co-cultivation experiments. To prevent a possible influence of alloreactivity on the response patterns, we decided to perform the experiments in an autologous setting. CD4$^+$ T cells (T$_{conv}$) from C77G and wild-type individuals responded with strong proliferation to CD3/CD28-mediated activation whereas the corresponding T$_{reg}$ did not proliferate (Figure 5). As expected, when wild-type T$_{conv}$ were stimulated in the presence of T$_{reg}$ from the same donor, proliferation was markedly reduced. Compared with proliferation of T$_{conv}$ alone, we observed reduced proliferation also in T$_{conv}$/T$_{reg}$ co-cultures from C77G carriers. However, whereas T$_{reg}$ from wild-type individuals suppressed proliferation of T$_{conv}$ on average by 85%, C77G T$_{reg}$ suppressed proliferation of T$_{conv}$ only by about 50%.

T$_{reg}$ from carriers of C77G and individuals with wild-type CD45 show distinct gene expression patterns

To further characterize the differences between C77G T$_{reg}$ and wild-type controls, we carried out global gene expression profiling. Pooled RNA from *in vitro* expanded and re-stimulated CD4$^+$CD25^{high} T$_{reg}$ (three C77G blood donors and three wild-type controls, respectively) was used. Expression data from all arrays analyzed in this study are provided as supporting information stored in the EMBO-EBI ArrayExpress database (URL: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31209) under accession no. GSE31209. Comparison of C77G and control T$_{reg}$ revealed 3650 genes that were differentially expressed (unpaired t-test, P-value $\leqslant 0.05$). Seventy-nine of these genes were associated with the term 'immune response' in accordance with gene ontology
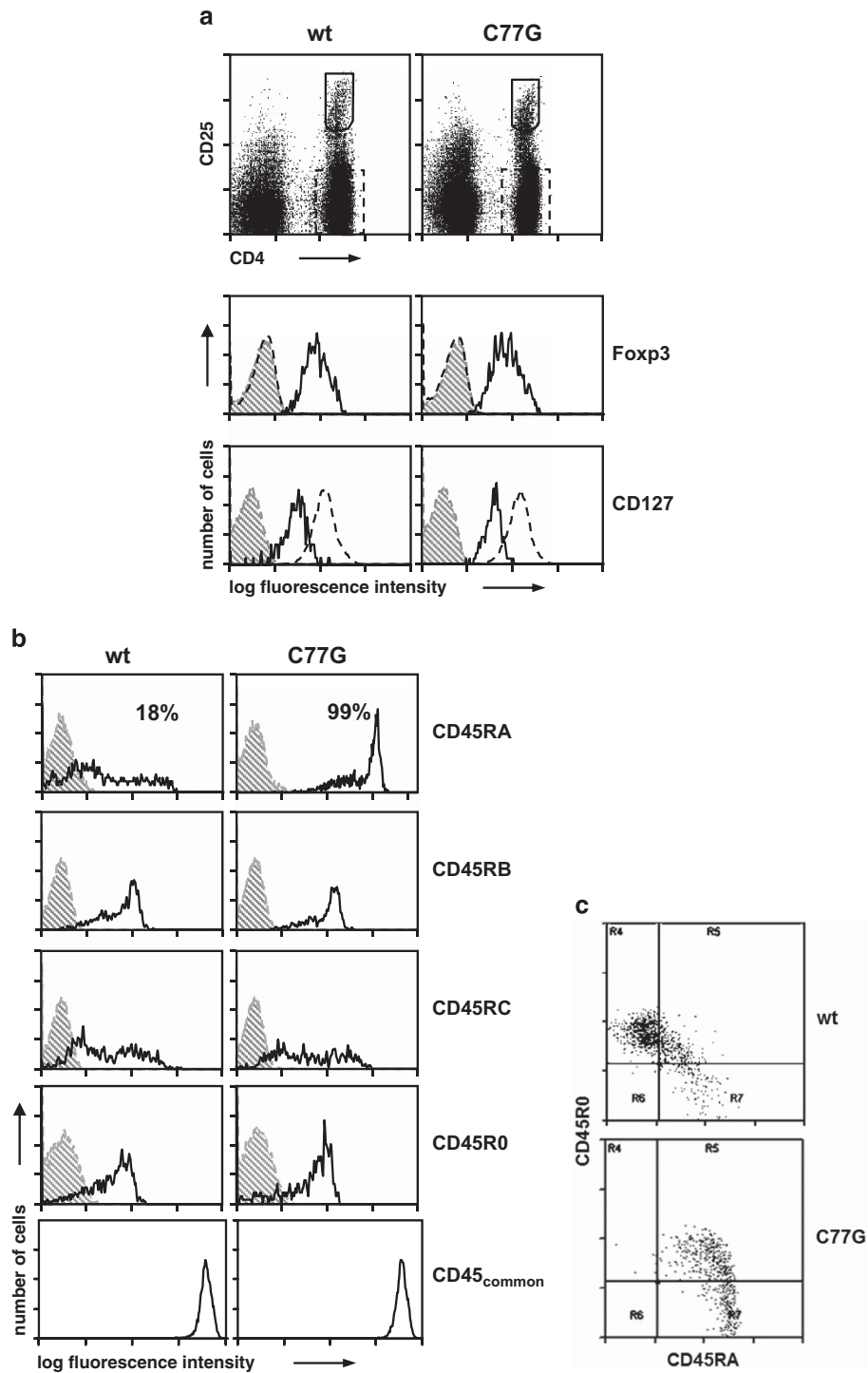
Figure 1. Phenotypic characterization of T$_{reg}$. Peripheral blood mononuclear cells (PBMC) were isolated from a donor with wild-type CD45 (wt) and from a carrier of C77G. Analysis was performed in gated lymphocytes as identified by forward- and side-scatter characteristics. (**a**) Expression patterns of CD4, CD25, Foxp3 and CD127. Representative dot plots of CD4/CD25 coexpression are shown. The CD4$^+$CD25^{high} population is indicated by the solid-line box. The broken-line box contains CD4$^+$CD25$^-$ cells. Foxp3 histograms (solid line) represent expression in gated CD4$^+$CD25^{high} cells, CD127 expression is shown for both, CD4$^+$CD25^{high} cells (solid line) and CD4$^+$CD25$^-$ cells (broken line). Fluorescence obtained with control mAb is shown by filled histograms. (**b**) Aberrant expression of CD45RA in CD4$^+$CD25^{high} T$_{reg}$ from C77G individuals. The cells were stained with mAb to CD4 and CD25 in combination with mAb to CD45RA, CD45RB, CD45RC, or CD45R0. Histograms represent binding of CD45 mAb (unfilled histograms) on gated CD4$^+$CD25^{high} cells and fluorescence obtained without mAb (filled histograms). Lowest panels show binding of a CD45$_{common}$ mAb detecting epitopes present on all isoforms. Similar expression patterns were observed in at least two further experiments. (**c**) CD45RA/CD45R0 coexpression patterns in T$_{reg}$. Representative dot plots of gated CD4$^+$CD25^{high} cells are shown.
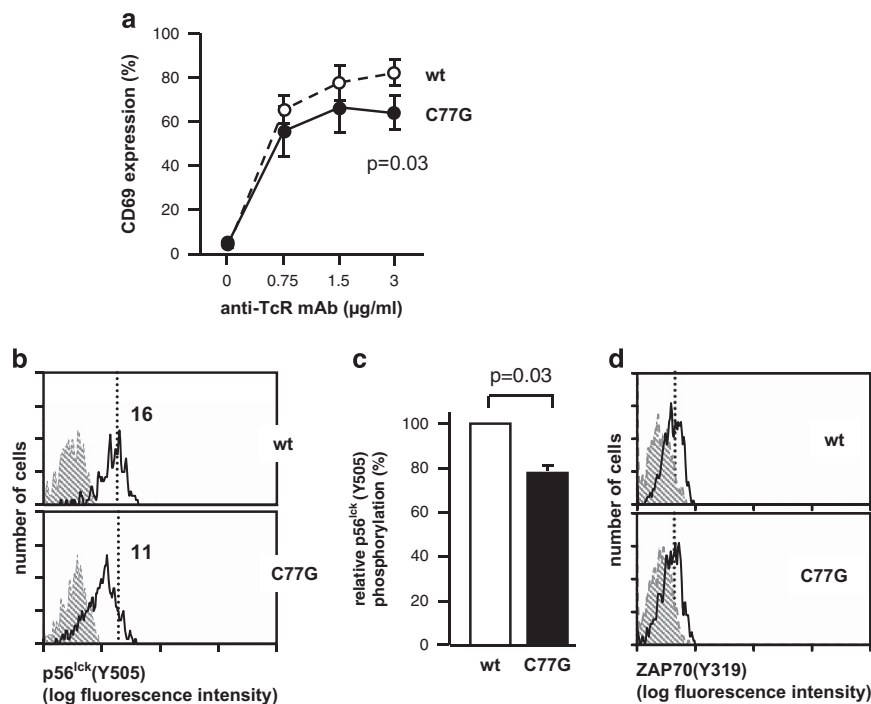
Figure 2. Differences in TcR-mediated signaling between T_{reg} from C77G carriers and individuals with wild-type CD45. (**a**) Impaired CD69 upregulation in anti-TcR stimulated C77G T_{reg}. PBMC from blood donors with wild-type CD45 (broken line) and C77G carriers (solid line) were cultured in microtiter plates precoated with increasing concentrations of the anti-TcR mAb BMA 031. The cells were harvested after 3 h, stained with the antibody combination anti-CD4/-CD25/-CD69 and analyzed by flow cytometry. CD69 expression was determined on gated CD4$^+$CD25^{high} cells. Results are expressed as percentages of CD4$^+$CD25^{high} cells coexpressing CD69 (mean $\pm$ s.e.m. of three independent experiments). Differences between C77G and wild-type T_{reg} were statistically significant ($P = 0.03$, paired t-test) when high anti-TcR antibody concentrations (3 µg ml^{-1}) were used for stimulation. (**b**) Diminished phosphorylation of p56^{lck}(Y505) in anti-TcR stimulated C77G T_{reg}. Unstimulated PBMC (filled histograms) and cells that had been stimulated for 1 min with crosslinked anti-CD3 mAb OKT3 (unfilled histograms) were stained with the antibody combination anti-CD4/-CD25/-p56^{lck}(505) and analyzed by flow cytometry. p56^{lck}(Y505) phosphorylation was determined in gated CD4$^+$CD25^{high} cells. The numbers represent mean fluorescence intensity obtained with anti-p56^{lck}(Y505) in stimulated cells. (**c**) Quantitative comparison of p56^{lck} phosphorylation in cells from four carriers of C77G and four individuals with wild-type CD45. Fluorescence intensity obtained after binding of anti-p56^{lck}(Y505) in anti-TcR stimulated gated CD4$^+$CD25^{high} T_{reg} from individuals with wild-type CD45 was set 100% and compared with fluorescence intensity obtained with cells from C77G carriers (mean $\pm$ s.e.m). Phosphorylation was reduced by about 25% in C77G T_{reg} ($P = 0.03$, paired t-test). (**d**) Phosphorylation of ZAP70 (Y319) in anti-TcR stimulated T_{reg}. Unstimulated PBMC (filled histograms) and cells that had been stimulated for 1 min with crosslinked anti-CD3 mAb OKT3 (unfilled histograms) were stained with the antibody combination anti-CD4/-CD25/-ZAP70 (319) and analyzed by flow cytometry. ZAP70 (Y319) phosphorylation was determined in gated CD4$^+$CD25^{high} cells. Similar response patterns were observed in a second experiment.
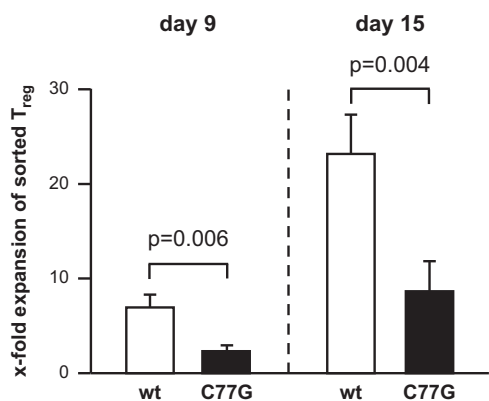

Figure 3. Reduced *in vitro* expansion of C77G T_{reg}. CD4$^+$CD25^{high} cells from wild-type and C77G individuals were isolated by cell sorting. Defined cell numbers (5×10^4) were seeded to microtiter plates and cultured with CD3/CD28-coated beads plus IL-2. After a cultivation period of 9 and 15 days, the cells were counted again. The cell count was divided by the cell count of the starting population to calculate the rate of expansion. Results are expressed as x-fold expansion $\pm$ s.e.m. obtained with cells from 16 individuals with wild-type CD45 and 7 carriers of C77G. P-values were determined using an unpaired t-test with Welch's correction.

analysis and are depicted in the cluster map presented in Figure 6. Further analysis of these 79 genes using pathway databases (Kyoto Encyclopedia of Genes and Genomes, KEGG and Gene Map Annotator and Pathway Profiler, GenMAPP) revealed four pathway modules: TcR signaling, regulation of proliferation, inflammatory response and apoptosis (Table 1). Components of TcR-associated signaling pathways seemed to be less affected. Nevertheless, in the remaining three modules several genes coding for molecules with regulatory potential were differentially expressed. There was no homogeneous pattern of upregulation or downregulation in genes from C77G carriers. However, it was a striking finding that all CC and CXC chemokines that were identified as differentially expressed showed decreased levels in C77G cells. Furthermore, granzyme A and tumor necrosis factors showed lower expression levels in C77G cells. These observations, together with the weak cellular responses of C77G T_{reg}, are in line with the assumption that aberrant expression of CD45 isoforms is associated with diminished T_{reg} reactivity.

DISCUSSION

It is well established that various signaling pathways are modulated by the tyrosine phosphatase activity of CD45.[26,27] The role of individual CD45 isoforms in these processes, however,

is still poorly understood. In mouse models, such aspects can elegantly be addressed by studying genetically modified mice expressing particular CD45 isoform patterns.[28] In humans, CD45 polymorphisms that alter the composition of CD45 isoforms are interesting models. We have previously shown that CD4$^+$ activated/memory T cells from carriers of the C77G polymorphism overexpress CD45RA isoforms resulting in enhanced sensitivity of

TcR- and cytokine receptor-mediated signal transduction.[23,24] Here we show that aberrant expression of CD45RA also changes the response patterns of CD4$^+$CD25^{high}Foxp3$^+$ T$_{reg}$. Interestingly, however, in contrast to the hyperreactivity of C77G activated/ memory cells, C77G T$_{reg}$ showed diminished responses to TcR stimulation.

Conclusions that can be drawn from experiments with primary human cells are often somewhat limited because of the heterogeneity of blood donors. Thus, one might argue that the observed differences between C77G T$_{reg}$ and cells from wild-type controls are not direct effects of altered CD45 isoforms in C77G cells. This is unlikely because of two lines of arguments. First, we tested cells from a large group of C77G individuals and found similar response patterns in all of them. For example, a reduced capacity of C77G T$_{reg}$ to expand *in vitro* was found in a series of experiments using cells from seven different C77G carriers (Figure 3). Second, only cell types with prominent differences in CD45RA expression exhibited differential functions. CD4$^+$CD25^{high} T$_{reg}$, for example, strongly expressed CD45RA isoforms in C77G individuals but weakly in wild-type controls (Figure 1) and responded differently to TcR triggering (Figure 2). Naïve/resting
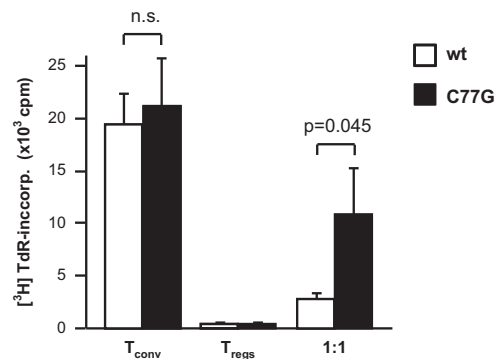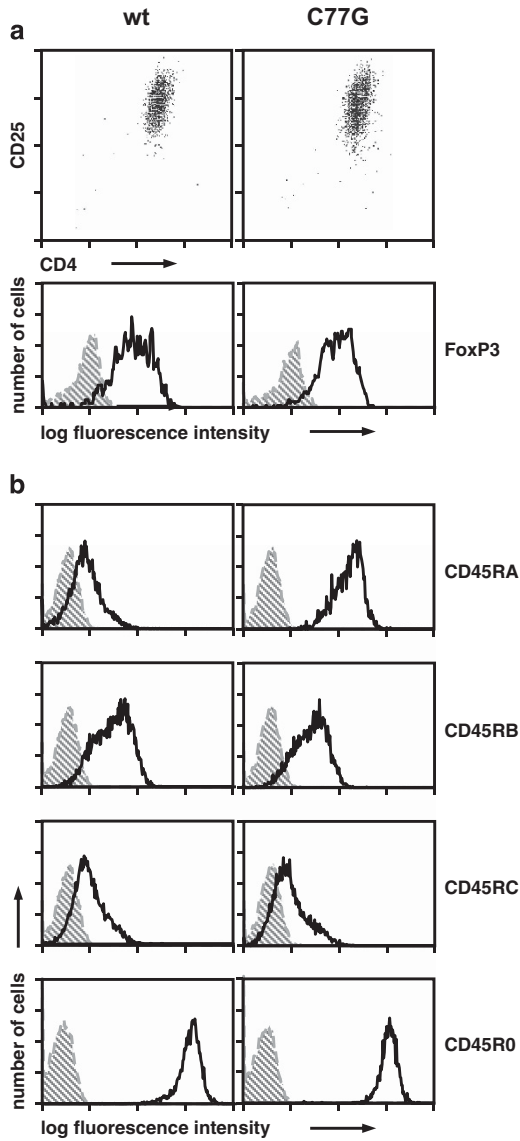

Figure 4. Characterization of *in vitro* expanded/activated T$_{reg}$ from wild-type individuals and C77G carriers. CD4$^+$CD25^{high} cells were isolated by cell sorting and cultured with CD3/CD8 beads plus IL-2. Analysis was performed on day 13 after starting the culture. Data shown were obtained with cells from one C77G carrier and one wild-type control. Similar results were achieved using cells from two additional C77G carriers and two blood donors with wild-type CD45. (**a**) Flow-cytometry analysis of CD4, CD25 and Foxp3 expression. Representative dot plots of CD4/CD25 co-stainings are shown. Histograms were obtained after staining of permeabilized cells with anti-Foxp3 antibody (unfilled histograms) or an isotype-matched control Ab (filled histograms). (**b**) Assessment of CD45 isoform patterns by flow cytometry. The cells were stained with CD45RA, CD45RB, CD45RC or CD45R0 mAb (open histograms) or without mAb (filled histograms). (**c**) Detection of CD45 mRNA species by RT-PCR analysis. Total RNA was isolated and RT-PCR was performed using primers located in exons 2 and 7 of CD45, ensuring amplification of all alternatively spliced isoforms. PCR products were electrophoresed, stained with ethidium bromide and visualized under UV light.

Figure 5. Impaired suppressive capacity of C77G T$_{reg}$. CD4$^+$ T cells (T$_{conv}$) and *in vitro* expanded CD4$^+$CD25^{high}Foxp3$^+$ T$_{reg}$ (1x10^4 per well) were stimulated with CD3/CD28 beads separately or in cocultures containing 1×10^4 T$_{conv}$ and 1×10^4 T$_{regs}$ (1:1). Cells from C77G carriers (filled bars) were compared with cells from individuals with wild-type CD45 (open bars). Proliferation was determined after 5 days by [^{3}H]-TdR incorporation. Results are expressed as mean cpm $\pm$ s.e.m. obtained with cells from 12 individuals with wild-type CD45 and 6 carriers of C77G. *P*-values were determined using an unpaired *t*-test with Welch's correction.

CD4^{+} T cells and B cells, however, expressed similar amounts of CD45RA in wild-type and C77G individuals and we did not find significant functional differences between cells from control and C77G individuals[23] (and unpublished). If reasons other than the aberrant CD45 isoform pattern were responsible for the functional
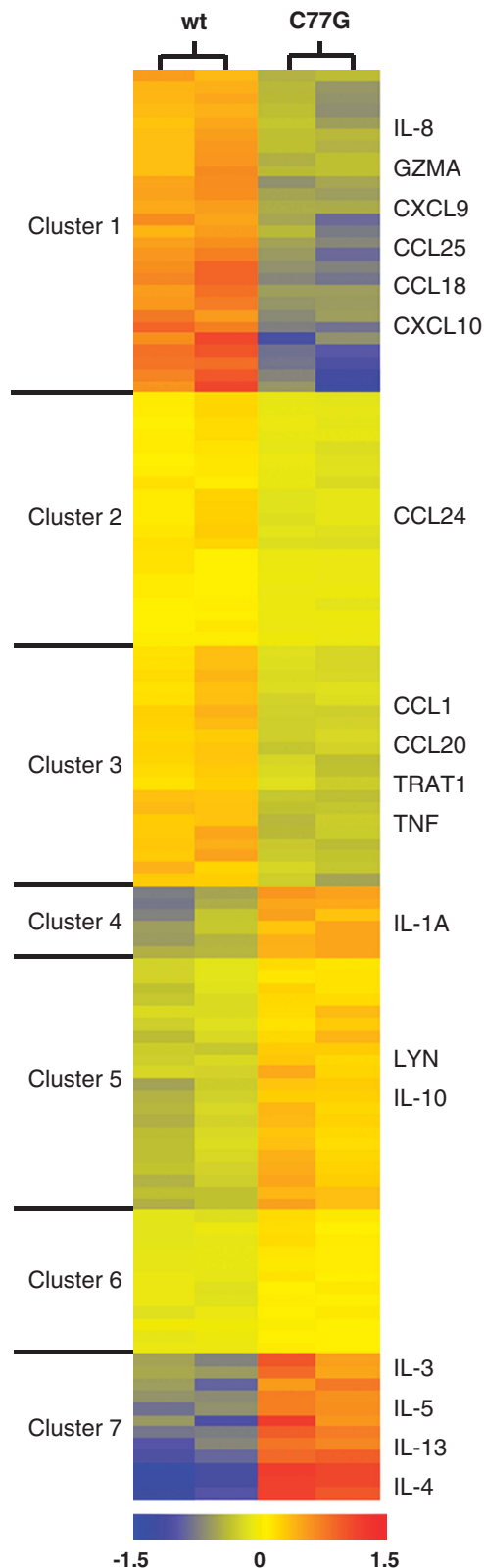
peculiarities of C77G cells one would not expect the functional differences to be limited to subsets of cells showing the most pronounced differences in CD45 isoform expression between C77G carriers and controls expressing wild-type CD45. Despite these arguments, it cannot fully be excluded that altered CD45 isoform expression in C77G carriers may cause other differences in the milieu that contribute to abnormal development or responsiveness of T$_{reg}$. For example, the abnormalities in the T-cell compartment of motheaten mice, which are deficient in Src homology 2 domain-containing phosphatase 1, are due to an aberrant cytokine milieu resulting from deficiencies in myeloid cells.[29–31]

Assessment of CD45 isoform patterns in freshly isolated CD4^{+}CD25^{high}Foxp3^{+} T$_{reg}$ revealed only a small subset of cells expressing CD45RA isoforms in individuals with wild-type CD45 whereas all T$_{reg}$ from C77G carriers expressed high levels of CD45RA (Figure 1). Since there were no significant differences in the expression levels of other molecules having a role in the regulation of T$_{reg}$ activity (for example, Foxp3, CTLA-4), we surmise that overexpression of CD45RA is crucial for hyporeactivity of C77G T$_{reg}$. Recent studies analyzing the function of different T$_{reg}$ subsets as defined by expression of CD45 isoforms support this assumption. Thus, in patients with systemic lupus erythematosus CD45RA expressing T$_{reg}$ have been described that exhibit impaired suppressive activity.[32] Furthermore, the CD45RA^{+}DR^{−} T$_{reg}$ subset is characterized by weak suppressive activity in comparison with CD45RA-negative subsets.[33] In addition, the CD45RA^{+} T$_{reg}$ subset has been reported to be composed of cells with low proliferative potential.[34] Together, these observations suggest that CD45RA expression on T$_{reg}$ is associated with hyporeactivity and also implies that CD45 isoforms are not only markers for functionally distinct subsets but may also be directly involved in the regulation of T$_{reg}$ activity. The observation that the suppressive activity of mouse T$_{reg}$ can be abrogated by crosslinking of CD45 molecules[12] is in line with this assumption. Furthermore, CD45 ligation can expand T$_{reg}$.[35]

Figure 6. Transcriptional profiling of CD4^{+}CD25^{high} T$_{reg}$ from C77G carriers and individuals with wild-type CD45. A total of 3650 genes were differentially expressed. The 79 depicted genes were associated with the term 'immune response' by gene ontology (GO) analysis. Expression data are presented as a heat map with relative values to averaged gene expression (mean centralized): blue coloring shows gene expression below mean value, red colorings show gene expression above mean value. Genes associated with pathway modules (Table 1) are shown on the right side. The microarray analysis using pooled mRNA samples from wild-type and C77G T$_{reg}$ was performed in duplicate.

Table 1. Pathway modules affected by differential gene expression in T$_{reg}$ from carriers of C77G and individuals with wild-type CD45[a]

Module	Differentially expressed genes
TcR signaling pathway	LYN, *TRAT1*[b]
Inflammatory response	*CCL1, CCL18, CCL20, CCL24, CCL25, CXCL9, CXCL10,* IL-13, IL-4
Regulation of proliferation	*CXCL10,* IL-3, IL-5, *IL-1 A, IL-8,* IL-10
Apoptosis	GZMA, TNF

[a]Analysis was performed using Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Map Annotator and Pathway Profiler (GenMAPP) software. [b]Plain type indicates increased gene expression, italic type indicates decreased expression in C77G T$_{reg}$ compared with wild-type control cells.

Due to the limited number of T$_{reg}$ we could not perform detailed biochemical studies to compare TcR-associated signaling pathways in T$_{reg}$ from individuals with wild-type CD45 and C77G carriers. Nevertheless, the observation that TcR-induced phosphorylation of the CD45 substrate Y505-p56^{lck} was diminished in C77G T$_{reg}$ (Figures 2b and c) suggests differential signaling. Since dephosphorylation at Y505 is regarded to enhance p56^{lck} kinase activity, cells with lower levels of Y505 phosphorylation would contain a more active pool of p56^{lck}. This, however, is difficult to reconcile with a less sensitive TcR in C77G T$_{reg}$ as suggested by decreased CD69 upregulation (Figure 2a), reduced proliferative capacity (Figure 3) and impaired suppressive potential (Figure 5). Studies to identify T$_{reg}$-specific signaling pathways are still in the early stages but first data suggest that biochemical events of TcR-mediated signaling differ between T$_{reg}$ and conventional T cells.[36,37] Although no direct experimental evidence is available so far, one might speculate that—in contrast to conventional CD4$^+$ T cells—enhanced CD45 phosphatase activity in T$_{reg}$ is associated with decreased sensitivity of TcR signaling.

Depending on the signaling pathways, the phosphatase activity of CD45 can positively or negatively influence immune responses. Whereas TcR and BcR-associated pathways are mainly positively influenced by CD45, cytokine-mediated signaling via Jak/STAT pathways is regarded to be negatively regulated.[38,39] Opposed effects of CD45 in different signaling pathways suggest a high degree of functional plasticity of the phosphatase. The observation that aberrant expression of CD45 isoforms enhanced TcR signaling in activated/memory T cells[23] but decreased responses in T$_{reg}$ indicates another level of plasticity. Thus, the same signaling pathway (TcR) seems to be differently affected by CD45 in different T-cell subsets.

T$_{reg}$ can suppress conventional T cells by several mechanisms including release of immunosuppressive cytokines, IL-2 consumption or apoptosis induction.[40] We do not know which effector mechanisms are impaired in C77G T$_{reg}$ leading to diminished suppressive activity (Figure 5). It has been suggested that crosslinking of CD69 induces the production of the inhibitory cytokine TGF-β.[41,42] Since we found reduced levels of CD69 on the cell surface after TcR triggering of C77G T$_{reg}$ (Figure 2), it is tempting to speculate that CD69-mediated induction of TGF-β may be impaired in C77G T$_{reg}$. On the other hand, by gene expression profiling we did not find different levels of TGF-β mRNA in T$_{reg}$ from variant individuals and wild-type controls (Figure 6). It should be noted, however, that the RNA used for these experiments was isolated from cells that had been stimulated with mAb specific for CD3/TcR and CD28 without direct triggering of CD69. The observation that the expression levels of granzyme A and tumor necrosis factor were reduced in C77G T$_{reg}$ (Figure 6 and Table 1) may indicate a differential role of apoptosis inducing mechanisms in T$_{reg}$ from C77G carriers and individuals with wild-type CD45 expression. Although the gene arrays did not allow definite conclusions on the effector mechanisms that are impaired in C77G T$_{reg}$ cells, we found differential expression of various genes, further supporting the hypothesis that aberrant CD45RA expression alters the quality of TcR-mediated signaling.

The question of whether T$_{reg}$ can be used clinically for downregulation of immune responses to allografts or to re-establish tolerance to self-antigens in patients suffering from autoimmunity has been intensively discussed.[43,44] An important prerequisite for clinical application is the availability of protocols for effective *in vitro* expansion of T$_{reg}$ to achieve sufficient cell numbers for patient treatment. It has been suggested that the CD45RA$^+$ T$_{reg}$ subset is the ideal starting population because these cells are stable and remain suppressive during culture and expansion by repeated stimulation with anti-CD3/CD28-coated beads.[45,46] If T$_{reg}$ from a C77G carrier would to be used for *in vitro* activation, then several peculiarities have to be considered: (1) the

fact that all T$_{reg}$ express CD45RA does not mean that all of them functionally resemble the small CD45RA$^+$ subset of wild-type individuals. (2) Downregulation of CD45RA isoforms cannot be used as a marker for the activation of T$_{reg}$ because C77G cells do not downregulate CD45RA (Figure 4b). (3) Expansion of T$_{reg}$ by CD3/CD28-coated beads will be inefficient (Figure 3). Although the frequency of the 77G allele is low in the normal European population (0–3.5%)[17] the knowledge of this polymorphism is important for future clinical application of T$_{reg}$ to avoid misinterpretation of experimental data and/or therapeutic failure when C77G carriers have to be treated.

The experiments presented here were performed using cells from healthy individuals carrying C77G. An enhanced frequency of the 77G allele has been described in certain autoimmune and infectious diseases, suggesting a possible role of C77G as a risk factor.[47–50] The existence of C77G in healthy individuals and the excess of heterozygous carriers in some diseases indicate that C77G may act as a genetic modifier. Thus, C77G alone does not necessarily result in disease but modifies the susceptibility of the individual. If additional genetic or environmental components are also present, which further enhance susceptibility, then manifestation of the disease may occur. Detailed knowledge of the functional consequences of C77G is essential to understand how this polymorphism can become a risk factor. We hypothesized that the particularly sensitive TcR and the resulting hyperactive activated/memory T cells of C77G carriers will lower the signaling threshold thereby increasing the chance of autoaggressive T cells to be activated by autoantigens.[23,24] One can assume that hyperreactive T cells from C77G carriers may also be less controllable by other cell types. Impaired regulation by T$_{reg}$ is considered to be an important cause of autoimmune diseases.[6] Thus, it is conceivable that hyporeactive T$_{reg}$ as found in C77G carriers will contribute to a reduced control level for autoaggressive cells.

MATERIALS AND METHODS

Ethical statement and blood donors

Blood samples were obtained from healthy voluntary blood donors recruited at the Department of Transfusion Medicine (Hannover Medical School). PBMC were isolated from leukotrap filter, which are usually discarded after the collection of whole blood. The filters are anonymized/coded and cannot be allocated to an individual blood donor. Thus, written or verbal consent was not obtained. The local ethics committee of Hannover Medical School approved this procedure. Cells were isolated by Ficoll gradient centrifugation and cryopreserved until usage. Heterozygous individuals carrying C77G were identified by the absence of CD45RA-negative lymphocytes as determined by flow cytometry.[51] In samples showing this phenotype, the C → G exchange at position 77 in CD45 exon A was confirmed by restriction fragment length polymorphism analysis.[20] Among a cohort of 743 tested blood donors 22 individuals were found to carry C77G.

Antibodies and flow cytometry

CD4$^+$CD25^{high} T$_{reg}$ were identified by incubating PBMC with anti-CD4-FITC or -PE (RPA-T4; BD Biosciences; San Jose, CA, USA) in combination with anti-CD25-PE or –APC (M-A251; BD Biosciences). Activation induced upregulation of CD69 in T$_{reg}$ was monitored using anti-CD69-PE (CH/4; Life Technologies, Carlsbad, CA, USA). Phosphorylation at Y505 of p56^{lck} was detected by anti-p56^{lck}/Y505-FITC (4/LCK-Y505), ZAP70 phosphorylation at Y319 by anti-ZAP70/pY319-Alexa Fluor488 (17A/P-ZAP70), both from BD Biosciences. Exon-specific anti-CD45 mAb were used to characterize the expression patterns of CD45 isoforms on CD4$^+$CD25^{high} cells. CD45 isoforms containing exon A were identified by anti-CD45RA (MEM56; provided by V Horejsi, Prague, Czech Republic) or anti-CD45RA-FITC (HI100; BD Biosciences), exon B and C containing isoforms by anti-CD45RB (MEM55; Serotec, Düsseldorf, Germany) and anti-CD45RC (MT2; IQ Products, Groningen, The Netherlands), respectively. Anti-CD45R0-PE (UCHL1, BD Biosciences) was used to detect CD45 isoforms containing no exon A, B or C encoded epitopes. If unlabeled primary antibodies were

applied, then they were detected using FITC-conjugated goat anti-mouse IgG and IgM (Dianova, Hamburg, Germany) or PE-conjugated rat anti-mouse IgG (BD Biosciences). Detection of intracellular Foxp3 was performed using the Alexa Fluor 647 Foxp3 Flow Kit according to the manufacturer's instructions (206D, isotype control: MOPC-21; BioLegend, San Diego, CA, USA). Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and data were processed by using Summit 5.1 software (Beckman Coulter, Krefeld, Germany).

Isolation and cultivation of T-cell subsets

$CD4^+CD25^{high}$ T_{reg} were isolated by electronic cell sorting. PBMC from individuals with wild-type CD45 and from C77G carriers were incubated with the antibody combination anti-CD4-FITC (RPA-T4; BD Biosciences)/anti-CD25-PE (M-A251; BD Biosciences). Cell sorting was performed on a FACSAria (Becton Dickinson). Reanalysis of sorted populations typically revealed >97% of cells expressing the $CD4^+CD25^{high}$ phenotype. FACS-sorted cells were expanded with CD3/CD28 T-cell expander beads (Life Technologies) in culture medium containing 300 U ml^{-1} IL-2 (Hoffmann-La Roche, Basel, Switzerland). After 7 days, the cells were counted and restimulated using fresh beads. The beads were removed after an additional 4 days and the cells were further expanded in medium containing IL-2 alone (300 U ml^{-1}). Phenotypic and functional characterization of in vitro activated/expanded $CD4^+CD25^{high}$ cells was then performed after 2 days.

$CD4^+$ T cells were negatively isolated by MACS (magnetic cell sorting) (Miltenyi Biotec, Bergisch Gladbach, Germany). PBMC were incubated with an antibody cocktail consisting of the mouse mAbs anti-CD14 (clone CD14; American Type Culture Collection, ATCC), anti-HLA-DR (ATCC), anti-CD8 (AICD8.1; a gift of B Schraven, University of Magdeburg, Germany), and anti-CD56 (T199; a gift of T Pietsch, University of Bonn, Germany). Labeled cells were washed twice and incubated with magnetic goat anti-mouse IgG Micro Beads. The cells were washed again, resuspended in MACS buffer (phosphate-buffered saline, pH 7.2; supplemented with 0.5% BSA and 2 mM EDTA), applied to separation columns and subjected to a magnetic field according to the manufacturer's instructions. Unbound cells were eluted from the columns and used for further analyses. Purity of the $CD4^+$ T-cell subset was checked by flow cytometry and was typically ⩾95%.

Analysis of CD45 isoform expression by RT-PCR

Total RNA was isolated using the RNeasy Plus Micro Kit (Qiagen, Hilden, Germany). A total of 0.5 μg RNA was reverse-transcribed using the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA). PCR was performed in a final volume of 30 μl containing 2 μl of reverse-transcription transcript, 3 μl of 10-fold PCR buffer (New England BioLabs, Ipswich, MA, USA), 10 mM dNTPs, 0.16 μM of each primer and 1.5U TaqDNA polymerase (New England BioLabs). The following primers were used: CD45-sense, 5′-GGC TGA CTT CCA GAT ATG ACC ATG TAT-3′; CD45-antisense, 5′- GGT TGT TTC AGA GGC ATT AAG GTA GG-3′. Samples were amplified using 33 cycles. PCR products (10 μl) were electrophoresed on ethidium bromide-stained agarose gels (1.5%) and visualized under UV light. Fragment sizes were 627 bp for CD45(ABC), 483 bp for CD45(AB), 429 bp for CD45(BC), 285 bp for CD45(B) and 144 bp for CD45(0).

Functional characterization of $CD4^+CD25^{high}$ T_{reg}

To compare the intensity of TcR-mediated signaling in $CD4^+CD25^{high}$ cells from individuals carrying wild-type CD45 or C77G, PBMC were stimulated for 3 h with increasing concentrations of plate-bound mAb BMA031 (a gift of R Kurrle, Aventis Pharma, Bridgewater, NJ, USA), which detects a monomorphic epitope on the αβ-TcR.[52] Upregulation of the early activation marker CD69 was assessed on gated $CD4^+CD25^{high}$ cells as the readout for TcR signaling. To study the effect of TcR stimulation on $p56^{lck}$ and ZAP70 phosphorylation in $CD4^+CD25^{high}$ cells, PBMC were incubated with the anti-CD3 mAb OKT3 (ATCC). The cells were washed and crosslinking of cell bound OKT3 was performed by adding goat-anti-mouse Ig (Dianova). The stimulus was stopped after 1 min by fixation of the cells using Fix/Perm Buffer (BioLegend). Permeabilization (37 °C, 10 min) was then performed using Perm Buffer (BioLegend). Permeabilized cells were incubated with normal mouse serum to block free binding sites on the goat anti-mouse Ig crosslinker, which could bind the mouse Ig mAb CD4 and CD25 thereby leading to false positive results. The cells were then incubated with the antibody combination CD4-PE/CD25-APC/$p56^{lck}$(Y505)-FITC or /ZAP70(Y319) at room temperature for 30 min. The phosphorylation status of $p56^{lck}$(Y505) or ZAP70(Y319) in T_{reg} was assessed by

establishing fluorescence histograms of gated $CD4^+CD25^{high}$ cells. The potential of $CD4^+CD25^{high}$ cells from wild-type individuals and C77G carriers to expand in cell culture in response to CD3/CD28 stimulation was monitored by cell counting. Co-cultivation experiments were performed to assess the suppressive capacity of $CD4^+CD25^{high}$ cells from controls and C77G individuals. 1×10^4 $CD4^+$ T cells were stimulated with 2×10^3 CD3/CD28 T-cell expander beads (Life Technologies) in the presence or absence of 1×10^4 $CD4^+CD25^{high}$ T_{reg} in a total volume of 200 μl culture medium. Proliferation was determined after 5 days by incorporation of [^{3}H]-TdR added as 1 μCi per well 16 h before harvesting.

Microarray

In vitro expanded $CD4^+CD25^{high}$ T_{reg} were used for gene expression profiling. Cells from individuals with wild-type CD45 and carriers of C77G were stimulated (2 h, CD3/CD28 beads) and total RNA was isolated using the RNeasy Plus Micro Kit (Qiagen). Two pools consisting of three wild-type and three C77G individuals were prepared using equal amounts of RNA from the single donors. The microarray was performed by the array facility of the Helmholtz Centre for Infection Research, Braunschweig, Germany. The two samples were each hybridized twice on an Agilent Custom Gene Expression Microarray 4×44 K. Data were processed using Agilent Feature Extraction 10.5 software (Agilent Technologies, Santa Clara, CA, USA) and analyzed on the basis of Agilent algorithms.

Statistical analysis

Statistical analysis was performed by using GraphPad Prism 6.04 software (San Diego, CA, USA). Center values with error bars are represented as means ± s.e.m. The individual tests applied to estimate levels of significance were always carried out as two-sided tests and are stated in the figure legends. Test results (P-values) ⩽0.05 (*) were considered significant; no adjustments for multiple comparisons were necessary. Exact sample sizes (n) are stated in the figure legends and always represent biological replicates.

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