NATURALLY ACQUIRED TOLERANCE AND SENSITIZATION TO MINOR HISTOCOMPATIBILITY ANTIGENS IN HEALTHY FAMILY MEMBERS.

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ABSTRACT

Bidirectional cell transfer during pregnancy frequently leads to post-partum persistence of allogeneic cells and allo immune responses in both the mother and in her offspring. The life-long consequences of naturally acquired allo immune reactivity are likely of importance for the outcome of allogeneic Stem Cell Transplantation (SCT). We investigated the presence of CD8<sup>pos</sup> minor Histocompatibility (H) antigen-specific cytotoxic T lymphocytes (T<sub>CTL</sub>) and CD8<sup>pos</sup> minor H antigen-specific T regulator cells (T<sub>REG</sub>) in peripheral blood cells obtained from 17 minor H antigen disparate mother-offspring pairs. Absence of minor H antigen-specific T<sub>REG</sub>, as marked by the feasibility to expand T<sub>CTL</sub> from isolated tetramer<sup>pos</sup> populations, was observed in six mothers and in one son. Presence of minor H alloantigen-specific T<sub>REG</sub> was observed in four mothers and in five sons. These T<sub>REG</sub> were detected within isolated tetramer<sup>dim</sup> staining fractions and functioned in a CTLA-4 dependent fashion. Our study indicates that both T<sub>CTL</sub> and T<sub>REG</sub> mediated allo immunity against minor H antigens may be present in healthy female and male hematopoietic stem cell donors, potentially influencing Graft-versus-Host reactivity in different ways.
INTRODUCTION

Mismatches between the Human Leukocyte Antigen (HLA) haploidentical mother and child may lead to mutual priming of allo immune cells. Although pregnancy frequently results in activation of maternal B cells\(^1\) and T\(_{\text{CTL}}\) directed against fetal Inherited Paternal Alloantigen (IPA), such as HLA\(^2\) and minor H antigens\(^3\), not all parous women develop cytolytic activity against the latter alloantigens\(^3\). Importantly, long-lasting tolerance may also be induced in offspring exposed to Non-Inherited Maternal Alloantigen (NIMA) such as Rhesus D\(^4\) or HLA\(^5\). The latter is illustrated by a failure to generate allo antibodies after re exposure to the relevant alloantigens through pregnancy\(^4\) or through multiple blood transfusions\(^5\).

The immunological mechanism(s) involved in these apparent states of naturally acquired allo tolerance is still poorly understood. The presence of fetal or maternal microchimeric cells may play a role in the induction and/or maintenance of a tolerant status\(^6\). There is ample evidence for a mutual exchange of mature blood and progenitor cells between the mother and her fetus. While mature blood cells have a limited lifespan, hematopoietic stem cells\(^7\) and HLA\(^{\text{dim}}\) mesenchymal stem cells\(^8\) may engraft in the bone marrow, where they remain throughout life. Cells obviously derived from fetal hematopoietic progenitor cells can be detected in the maternal circulation up to several decades after the delivery\(^9\). Likewise, hematopoietic\(^6;10\) and non-hematopoietic\(^11\) cells from maternal origin may persist into adulthood. The tolerogenic potential of chimerism, established either through bone marrow transplantation (macrochimerism)\(^12\) or through pregnancy (microchimerism)\(^7\), has been documented in both rodent\(^13;14\) and in human transplant settings\(^15;16\).

We earlier described the presence of minor H antigen HA-1 specific T\(_{\text{CTL}}\), minor H antigen HA-1 specific T\(_{\text{REG}}\) and HA-1\(^{\text{pos}}\) circulating microchimeric cells in the setting of kidney transplantation\(^17\). The latter cell populations were observed in a long term tolerant
HA-1neg patient transplanted with a renal allograft from her HLA identical HA-1pos sister. These TCTL and TREG could be physically separated based on differences in their capacity to bind HLA-A2/minor H peptide tetramers. While HA-1 tetramerbright staining T cells were found to mediate delayed type hypersensitivity reactions and produced IFNγ in response to HA-1 allo peptide, their function was suppressed in the presence of TGFβ and IL-10 producing HA-1 tetramerdim staining T cells. Thus, differences in tetramer staining intensity may indicate the presence of functionally different types of T cells17;18.

The dominant presence of minor H antigen-specific alloimmune TREG or TCTL in a hematopoietic stem cell (SC) graft may have differential impacts on the outcome of HLA identical minor H antigen non-identical SCT. As a first step towards understanding how the minor H antigen allo immunization status of SC donors may affect SCT outcome, we analyzed whether ‘natural’ exposure to fetal or maternal minor H allo antigens induces functionally different T cells in healthy parous female and male blood donors respectively. Regarding the latter donors, only first born sons were selected thereby avoiding any confounding effects of transmaternal transfer of earlier born sibling cells19. Peripheral Blood Mononuclear Cells (PBMC) from selected minor H antigen mismatched mother-child pairs were collected and a detailed analysis on the presence of minor H antigen-specific TCTL and TREG was performed.
MATERIALS AND METHODS

Study participants

Familial allo-immunization to the HLA-A2 restricted minor H antigens HA-1, HA-2, HA-8 and HY20 was studied (Table 1). Donors with a history of blood transfusion were excluded from the analysis21. After receiving written informed consent, in accordance with the Declaration of Helsinki, blood samples were obtained either by leukapheresis or by extraction of the buffy-coat from whole blood donations. Approval for this study (P06.008) was obtained from the Institutional Review Boards of the Leiden University Medical Center and of Sanquin Blood Bank South West.

HLA class I/minor H antigen peptide tetrameric complexes and monoclonal antibodies.

Phycoerythrin (PE)-conjugated HLA-A2/minor H peptide HY (HYA2), HA-1 (HA-1A2) and HA-2 (HA-2A2) tetramers were generated and validated as previously described22. The following cell surface markers and isotype control antibodies (all from BD Biosciences, Amsterdam, the Netherlands) were used for multicolor flow cytometric (FACS) analysis: allophycocyanin (APC)-conjugated CD45RO, peridinin chlorophyll protein (PerCp)-conjugated CD8 and fluorescein isothiocyanate (FITC)-conjugated CD25 and CTLA-4. Intracellular staining for FoxP3 and the corresponding isotype control were performed with FITC labeled antibodies according to the supplier’s manual (eBioscience, via Bioconnect, Huissen, the Netherlands).
Detection, isolation and expansion of minor H antigen-specific CD8<sup>pos</sup> T cells from peripheral blood samples

Detection and isolation of circulating minor H antigen-specific T cells from CD8 enriched PBMC was performed according to a previously described protocol<sup>3</sup>. In short, PBMC were isolated by Ficoll-Isopaque density gradient centrifugation and depleted for various cell subsets using CD4, CD14, CD16 and CD19 MACS beads according to manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). The depleted fraction was subsequently stained with the relevant tetramers and CD8 antibodies. Enrichment of CD8<sup>pos</sup> tetramer<sup>pos</sup> T cells was performed on a FACS-ARIA cell sorter (Becton Dickinson) using the non-stringent “enrich mode”. The enriched population was immediately resorted using the more stringent “normal-R” mode. Double FACS-sorted populations were expanded in the presence of 10<sup>5</sup> irradiated autologous minor H antigen<sup>neg</sup> PBMC in Iscove’s Modified Dulbecco’s Medium containing 10% pooled human serum, 1% phytohemagglutinin and 150 IU/ml recombinant IL-2.

Functional assays

The cytolytic capacity was tested in a standard chromium release assay. In brief, 2500 <sup>51</sup>Cr labeled target cells were incubated with serial dilutions of T cells as indicated in the figures. Supernatants were harvested after 4 hour of incubation for gamma counting. Percentage specific lysis was calculated as follows: (experimental release - spontaneous release)/(maximal release – spontaneous release) x 100%. The data are shown as the mean of duplicate samples.

T<sub>REG</sub> function was tested in the <i>trans-vivo</i> Delayed Type Hypersensitivity (tvDTH) assay. This assay identifies the presence of a dominant population of T<sub>REG</sub>, exploiting bystander immune suppression as their main mode of action<sup>17,23</sup>. Briefly, 7-9x10<sup>6</sup> cryopreserved
PBMC were injected into the footpads of CB17.SCID mice in the presence of either PBS (negative control), 0.25 LF recall antigens Tetanus Toxoid/Diphtheria Toxoid (TT/D, positive control), 1 μg minor H allo peptide (HY or HA-1\(^{1\text{st}}\)), or TT/D plus 1 μg minor H allo peptide. Control swelling responses to 1 μg minor H self peptide (HA-1\(^{R}\), HA-2\(^{V}\) or HA-2\(^{M}\) +/- TT/D were tested in parallel. Footpad swelling responses were measured 24 hours later by using a dial thickness gauge. Values of the response to PBS were subtracted from test values to determine the net swelling response (NSR), reported in units of 10\(^{-4}\) inches. The percent of inhibition is calculated as follows: % suppression = 1 – (NSR to co injection of recall antigens + minor H allo peptide/ NSR to recall antigens alone) x 100%. The following classification criteria for DTH phenotypes were used: a DTH regulator phenotype is defined by a low minor H allo peptide-induced swelling response and ≥50% inhibition of TT/D recall response in the presence of the same minor H allo peptide. A DTH non-regulator phenotype is also defined by a low minor H allo peptide-induced swelling response together with <50% inhibition of TT/D recall response in the presence of minor H allo peptide. Where appropriate, the minor H allo peptide-induced bystander suppression was reversed by addition of 1 μg of neutralizing CTLA-4 or control IgG antibody (Antibody Solutions, Palo Alto, CA).

Detection of circulating Y-chromosome\(^{\text{pos}}\) and HA-1\(^{\text{H}}\) microchimeric cells

PBMC were isolated from the interphase after Ficoll-Isopaque density gradient centrifugation of whole blood samples. Granulocytes were obtained from the remaining cell pellet after lysing red blood cells with NH\(_4\)CL and KHCO\(_3\) containing lysis buffer. PBMC were subsequently labeled with FITC, PE, PerCp or APC conjugated antibodies (all from BD Biosciences) for isolation of CD3\(^{\text{pos}}\) T cells, CD20\(^{\text{pos}}\) B cells, CD14\(^{\text{pos}}\) monocytes and CD3\(^{\text{neg}}\)CD20\(^{\text{neg}}\)CD14\(^{\text{neg}}\)CD11c\(^{\text{pos}}\) myeloid Dendritic Cells (DC) by FACS.
sorting. The QIAamp DNA blood minikit (Qiagen via Westburg b.v., Leusden, the Netherlands) was used for genomic DNA extraction from all fractions. For the detection of Y-chromosome specific microchimerism, we adapted the real-time PCR protocol kindly provided by Prof. Dr. D.W. Bianchi (Tufts-New England Medical Center, Boston, MA). To standardize the data, a second PCR reaction detecting the human Hematopoietic Cell Kinase gene (HCK) was carried out in parallel\textsuperscript{24}. Table 2 lists the corresponding primers and probes. For the detection of HA-1 positive (HA-1\textsuperscript{H}) microchimeric cells, we adapted an earlier established nested PCR protocol\textsuperscript{25}. The different primer sets for the first and second PCR reactions performed in this study are listed in Table 2. The sensitivity of each PCR reaction was routinely determined by incorporating a titration series of a minor H antigen\textsuperscript{pos} Epstein-Barr Virus transformed Lymphoblastoid Cell Line (EBV-LCL) diluted in minor H antigen\textsuperscript{neg} EBV-LCL\textsuperscript{25}. The differences in sensitivity between both methods are due to significant differences in copy numbers of both genes; while the lower threshold of sensitivity in the HA-1\textsuperscript{H} specific PCR is 1 HA-1\textsuperscript{pos} cell in 10\textsuperscript{4} HA-1\textsuperscript{neg} cells, the Y chromosome-specific PCR reliably detects 1 male cell in 10\textsuperscript{5} female cells.
RESULTS

Isolation, expansion and functional testing of total tetramer-binding fractions obtained from female and male blood donors.

We earlier described the usefulness of HLA-A2/minor H peptide tetramers to visualize\textsuperscript{22} and isolate minor H antigen-specific CD8\textsuperscript{pos} T\textsubscript{CTL} from peripheral blood cells for subsequent functional analyses after polyclonal expansion\textsuperscript{3,26}. The latter protocol was used to study the presence of T\textsubscript{CTL} within total tetramer-binding fractions isolated from 10 female donors with minor H antigen HY or HA-1 mismatched offspring and from 6 HA-1\textsuperscript{pos} male donors, i.e. first born sons, all delivered by an HA-1\textsuperscript{pos} mother. The minor H genotypes of these mother/offspring combinations are listed in Table 1 and the various routes of donor alloantigen exposure are exemplified in Figure 1.

Female donor analyses: HY\textsuperscript{A2} (♀1-7 and ♀9) or HA-1\textsuperscript{A2} (♀10-11) tetramer\textsuperscript{pos} populations were isolated, as exemplified in Figure 2A, and cultured in the absence of the relevant minor H alloantigen. While outgrowth of cytolytic T cells failed in five out of ten cases (♀1-5), T\textsubscript{CTL} were obtained from the five other females (Table 3). Figure 2B shows the cytolytic capacity and corresponding tetramer staining plots of two representative T\textsubscript{CTL} populations. T\textsubscript{CTL} that lysed target cells expressing the natural ligand were obtained from female donor ♀6 (Figure 2B, bottom panel) and ♀11 (data not shown). Peptide-specific T\textsubscript{CTL} were present in T\textsubscript{CTL} populations expanded from female donor ♀9 (Figure 2B, top panel), ♀7 and ♀10 (both data not shown).

Male donor analyses: HA-1\textsuperscript{A2} (♂1-5) or HA-2\textsuperscript{A2} (♂6) tetramer\textsuperscript{pos} populations were isolated, as exemplified in Figure 2C, and cultured as described for the female donors. T\textsubscript{CTL} outgrowth occurred in 3 out of 6 cases (Table 3). Functional testing revealed the presence of natural ligand-specific T\textsubscript{CTL} in CD8\textsuperscript{pos} populations obtained from donor ♂1 (Figure 2D, bottom plot). Peptide-specific T\textsubscript{CTL} were present in CD8\textsuperscript{pos} populations generated from donor ♂3 (Figure 2D, upper plot) and ♂5 (data not shown).
Collectively, these experiments show that minor H antigen-specific T_{CTL} can be identified in approximately half of the analyzed female and male donors.

*Minor H antigen-specific T cells with a suppressive function identified in mothers and in first born sons.*

We next questioned whether failure to expand T_{CTL} from total tetramer^{pos} fractions, as observed in the other half of female and male donors tested, could be due to the co-presence of minor H antigen-specific T_{REG}. The tvDTH assay was applied to analyze the putative presence of minor H antigen-specific T_{REG} within total PBMC^{17}. These PBMC were derived from the same blood sample as used for the identification of minor H antigen-specific T_{CTL}. Sixteen blood donors, 9 females and 7 males, were selected for these experiments (Table 3). Figure 3 shows the data of two mothers with male offspring, representing either the tvDTH regulator phenotype (♀4, left graph), indicating the presence of functional minor H antigen-specific T_{REG}, or the tvDTH non-regulator phenotype (♀7, right graph), indicating the absence of functional minor H antigen-specific T_{REG}. Four out of nine mothers (44%) were classified as a tvDTH regulator; the remaining five mothers (55%) as tvDTH non-regulators. The latter group included the two females with HA-1 disparate offspring (♀10 and 11). Thus, natural exposure of mothers to fetal inherited minor H alloantigens seems to lead to either sensitization or tolerization to minor H alloantigens.

Likewise, five HA-1^{neg} sons, all delivered by a HA-1^{pos} mother, were tested in the tvDTH assay (Table 3). Only one donor (♂1) could be assigned as a tvDTH non-regulator. Unlike the other male donors, this donor even displayed significant swelling responses (>25x10^{-4} inches) when his PBMC were injected with HA-1 allo peptide alone (data not shown), indicating the presence of high numbers of minor H antigen-specific T_{CTL} mediating delayed type hypersensitivity reactions^{17}. The remaining four males were all
classified as tvDTH regulators. Two additional males (♂ 6 and ♂ 7), with an HA-2 and
HA-8 mismatched mother respectively, were also tested for regulatory capacity. Both
donors displayed profound suppression of recall responses in the presence of HA-2\(^{V}\)
(60%) and HA-8\(^{R}\) (75%) allo peptide respectively. Thus, six out of seven male donors
tested could be classified as a tvDTH regulator, indicating that tolerization to non-inherited maternal minor H antigens may be more prevalent than sensitization in male donors.

*Minor H antigen-specific T cell populations contain tetramer\(^{\text{bright}}\) and tetramer\(^{\text{dim}}\) staining cells.*

In a previous study, we have identified the presence of minor H antigen HA-1 specific
T\(_{\text{REG}}\) in a renal allograft tolerant patient\(^{17}\). Dissection of the total tetramer-binding CD8\(^{\text{pos}}\) T cell population into tetramer\(^{\text{bright}}\) and tetramer\(^{\text{dim}}\) staining T cells proved to be a crucial
step in the identification of these T\(_{\text{REG}}\) in the latter population. Likewise, we dissected the
total tetramer-binding populations observed in our healthy subjects. Nine out of eleven
female donors and six out of seven male donors were used for these analyses. Figures
4A and C exemplify how the distinction between brightly and dimly tetramer staining T
cells was made. Two different staining patterns were observed, i.e. a dual tetramer
staining pattern\(^{17}\) and a pattern dominated by tetramer\(^{\text{dim}}\) staining T cells. Figure 4B, left
panel shows a representative example of coexisting HYA2 tetramer\(^{\text{bright}}\) and tetramer\(^{\text{dim}}\) staining T cells that were observed in donor ♀ 9; the corresponding ratio bright:dim
staining T cells is 1:6. This dual staining pattern was observed in 4 out of 7 females who
gave birth to sons (Table 4). Predominant tetramer\(^{\text{dim}}\) staining T cells, i.e. a ratio
bright:dim staining T cells greater than 1:10, were observed in the three other mothers
with sons. Female 6 illustrates the latter phenotype; the ratio of bright:dim staining T
cells in this donor is 1:32 (Figure 4B, right panel). Both dual and predominantly
tetramer\textsuperscript{dim} staining profiles were also found in the two mothers with HA-1 mismatched offspring respectively (Table 4). Collectively, dual tetramer\textsuperscript{bright} and tetramer\textsuperscript{dim} staining T cells are observed in five out of a total of nine women analyzed in this study. Predominantly tetramer\textsuperscript{dim} staining T were observed in the four remaining women.

Likewise, PBMC from six male donors were analyzed for the presence of HA-1\textsuperscript{A2} or HA-2\textsuperscript{A2} tetramer bright and/or dim staining T cells (Table 4). Four out of six males predominantly displayed tetramer\textsuperscript{dim} staining T cells. A representative example is depicted in Figure 4D, right panel illustrating the ratio of 1:16 bright:dim staining T cells as observed in male donor 2. Donors ♂1 (Figure 4D, left panel) and ♂4 displayed dual tetramer\textsuperscript{bright} and tetramer\textsuperscript{dim} staining T cells (Table 4). The corresponding ratios of bright:dim staining T cells in these males are 1:7 (♂1) and 1:5 (♂4). Thus, similar tetramer staining profiles as observed in parous women were detected in the male donors, although the predominant tetramer\textsuperscript{dim} phenotype seems more frequent.

Minor H antigen-specific tetramer\textsuperscript{dim} staining T cells express phenotypic markers typically associated with regulatory function.

A predominant tetramer\textsuperscript{dim} staining profile was observed in five out of eight donors who were classified as strong tvDTH regulators (♀1, ♀3, ♂3, ♂5, ♂6). To further characterize putatively present minor H antigen-specific T\textsubscript{REG}, we determined the percentages of respectively CD25, CTLA-4 and FoxP3 expressing CD8\textsuperscript{pos} T cells within the isolated tetramer\textsuperscript{dim} staining fractions obtained from six donors: ♂1 (tvDTH regulator), ♀6 (tvDTH non-regulator), ♂4 (tvDTH regulator), ♂5 (tvDTH regulator), ♀8 and ♀9. The latter two donors were not tested for immune regulation in the tvDTH assay due to insufficient numbers of PBMC. A large variation in CD25 and CTLA-4 expression was observed within these tetramer\textsuperscript{dim} staining populations. The percentages of HY\textsuperscript{A2} tetramer\textsuperscript{dim} staining T cells expressing these markers varied from 1 to 58%; the highest percentage
of these cells were detected in ♀1 (Figure 5A), followed by ♀8 (12% and 13% respectively), ♀6 (Figure 5A) and ♀9 (both 1%). HA-1A2 tetramer<sub>dim</sub> staining T cells expressing CD25 and CTLA-4 were also detected in the two male donors analyzed. Whereas donor ♂4 only displayed very low numbers of CD25 and CTLA-4 positive cells (both 1%), higher numbers of this phenotype were detected in donor ♂5 (Figure 5A).

Neither CTLA-4 nor CD25 was expressed by control CD8<sup>pos</sup> tetramer<sup>neg</sup> cells analyzed in parallel (data not shown). Furthermore, none of the CD8<sup>pos</sup> tetramer<sub>dim</sub> or CD8<sup>pos</sup> tetramer<sup>neg</sup> populations expressed FoxP3 (data not shown). Thus, minor H antigen-specific tetramer<sub>dim</sub> staining CD8<sup>pos</sup> T cell populations contain variable numbers of T cells expressing CTLA-4, but not FoxP3.

Minor H antigen-mediated regulation involves CTLA-4.

We next questioned whether CTLA-4 expressed by minor H antigen-specific tetramer<sub>dim</sub> staining T cells would be functionally involved in the suppression of recall antigen-specific T cells as measured in the tvDTH assay. To address this question, mother ♀1 and her son ♂5 were selected, displaying a distinctive population of CTLA-4<sup>pos</sup> cells within respectively the HY and HA-1 tetramer<sub>dim</sub> staining fractions (Figure 5A). Addition of CTLA-4 neutralizing antibodies was tested for its expected uncovering of recall antigen-induced footpad swelling responses<sup>17</sup>. Both donor ♀1 and her son ♂5 showed partly (50% and 75% respectively) restored footpad swelling responses to recall antigens when the CTLA-4 antibody was added (Figure 5B). Donor ♀6, classified as a tvDTH non-regulator and displaying only 2% of CTLA-4<sup>pos</sup> cells (Figure 5A), did not show such an effect upon addition of CTLA-4 blocking antibodies. Likewise, addition of an isotype control antibody could not reverse minor H allo peptide induced suppression of footpad swelling. These first observations suggest that the regulatory effect of the tetramer<sub>dim</sub> population, provided the presence of sufficient numbers of T<sub>REG</sub> cells, is partially
depending on functional CTLA-4 signaling.
DISCUSSION

Bilateral cell trafficking during pregnancy and delivery induces allo immunization in mutual direction as illustrated by the presence of minor H antigen-specific T<sub>CTL</sub> populations in the mother and the newborn child<sup>3,26,27</sup>. In this study, we questioned whether beside T<sub>CTL</sub> also minor H antigen-specific CD8<sup>pos</sup> T<sub>REG</sub> are generated and if so, whether both populations can be demonstrated in adult donors. To this end, we collected PBMC from 17 minor H antigen mismatched familial combinations and analyzed the presence of functionally different types of CD8<sup>pos</sup> minor H antigen-specific T cells. We show for the first time the presence of minor H antigen-specific T<sub>REG</sub> in healthy adult women and men. These T<sub>REG</sub> seem to coexist with variable numbers of minor H antigen-specific T<sub>CTL</sub>. Both T cells subsets arise across HY and autosomal minor H antigen barriers; the latter occurs in mutual direction. The minor H antigen-specific CD8<sup>pos</sup> T<sub>REG</sub> co-express the cell surface CD25 and CTLA-4, but no FoxP3. Although adaptive CD8<sup>pos</sup> CD25<sup>pos</sup> CTLA-4<sup>pos</sup> FoxP3<sup>pos</sup> T<sub>REG</sub> have been identified in transplant patients or in patients with autoimmune or infectious disease<sup>28</sup>, not all thus far identified CD8<sup>pos</sup> T<sub>REG</sub> express FoxP3<sup>29</sup>. In fact, FoxP3 is not a very specific marker for human T<sub>REG</sub> given that activated non-regulatory T cells may transiently upregulate this transcription factor. The CD8<sup>pos</sup> T<sub>REG</sub> described in this study were identified within the tetramer dim staining fraction. These results are in agreement with two earlier studies describing reduced HLA/peptide multimer binding by T<sub>REG</sub><sup>17,18</sup>. Low avidity CD8<sup>dim</sup> staining HY-specific T<sub>REG</sub> have been described in a murine allograft tolerance model<sup>30</sup>. These observations collectively point to incomplete T cell receptor signaling as a common feature of T<sub>REG</sub> function. This phenotypic characteristic can however not be used for identification of minor H antigen-specific T<sub>REG</sub>, because some tetramer<sup>dim</sup> staining fractions also contained minor H antigen-specific T<sub>CTL</sub>. Indeed, functional testing (data not shown)
demonstrated the presence of $T_C^\text{CTL}$ in three expanded populations derived from isolated tetramer$^{\text{dim}}$ staining fractions obtained from donor ♀3, ♂5 (both HA-1) and ♀9 (HY).

Recent observations show that human tolerogenic fetal CD4$^{\text{pos}}$ CD25$^{\text{high}}$ FoxP3$^{\text{pos}}$ T$^{\text{REG}}$ specific for undefined maternal alloantigen(s) may be primed \textit{in utero} and persist long after birth$^{31}$. In line with this report, our results show that also minor H antigen-specific CD8$^{\text{pos}}$ $T_C^\text{CTL}$ may be (partly) functionally tolerized in mother and child as a consequence of exposure to minor H alloantigens through pregnancy. The minor H antigen-specific CD8$^{\text{pos}}$ T$^{\text{REG}}$ identified in our study may, like their CD4$^{\text{pos}}$ CD25$^{\text{high}}$ counterparts, persist for decades. Indeed, the majority of adult males and approximately half of the women analyzed in our study clearly displayed minor H allopeptide-specific regulatory function in the tvDTH assay. Whereas primary contact with alloantigens during fetal/neonatal life may be a far more favorable setting for the induction of tolerance, (repetitive) exposure to such antigens in adulthood should, theoretically, result in a sensitized phenotype as earlier proposed$^{32}$. Although further studies are necessary to determine the effect of more than one pregnancy on the maternal allo-immunization status, the in this study observed T cell heterogeneity may provide a first clue why donor parity is still a controversial factor affecting HLA identical SCT outcome. Indeed, unequivocal conclusions regarding the effect of donor parity on the Graft-versus-Host Disease (GvHD) risk still exist as reviewed$^{33}$. Interestingly, in eight out of nine females analyzed, a correlation was found between their tvDTH test result, which was generated \textit{ex vivo} in a blinded fashion, and the outcome of the in parallel performed \textit{in vitro} experiments on $T_C^\text{CTL}$ outgrowth (Table 3). Dissection of tolerized donors from sensitized donors may also be useful in male-to-male SCT settings. Because of the presumed lowest GvHD risk, an HLA identical male donor is considered as the optimal SC donor for male patients$^{33}$. This study indicates the presence of CD8$^{\text{pos}}$ T$^{\text{REG}}$ directed against the hematopoietic system-restricted minor H antigens HA-1 or HA-
2 in male donors and thus potentially also in their hematopoietic SC graft. Such T_{REG} may impair HA-1 or HA-2 T_{CTL}-driven Graft-versus-Leukemia reactions\textsuperscript{34,35}. Evidently, the presence of these T_{REG} must also be considered when HA-1 or HA-2 specific T_{CTL} are generated from unselected PBMC or CD8 enriched populations for the purpose of cellular adoptive immunotherapy\textsuperscript{36}.

Given our current observations that non-functional CD8\textsuperscript{pos} T cells, i.e. failure to lyse or produce IFN\textsubscript{γ} when stimulated with minor H alloantigen, can also be expanded from some of our donors (♀\textsuperscript{2}, ♀\textsuperscript{3} and ♂\textsuperscript{2}, data not shown), we speculate that naturally established tolerance to minor H antigens may be due to either the presence of minor H antigen-specific T_{REG} and/or the induction of T cell anergy. Several experimental transplantation models have demonstrated that the establishment and maintenance of stable allograft tolerance depend on systemically persisting alloantigen-expressing microchimeric cell types such as lymphocytes\textsuperscript{37,38}, dendritric cells (DC)\textsuperscript{39-42} or both\textsuperscript{17}. These cell types continuously present alloantigen to host T cells under non-inflammatory conditions, which may lead to active deletion of host alloreactive T cells\textsuperscript{37} and/or the induction of alloreactive adaptive T_{REG}\textsuperscript{42}. Similar observations were recently made in a murine F1 breeding model, in which detectable levels of maternal CD11b\textsuperscript{pos} monocytes or CD11c expressing mDC correlated with the presence of a dominant population of TGFβ-producing CD4\textsuperscript{pos} T_{REG} in NIMA tolerant offspring (P. Dutta & W.R. Burlingham, \textit{manuscript submitted}). From eight donors (6 females and 2 males) sufficient numbers of PBMC were available to determine the presence of microchimeric cells expressing the relevant minor H alloantigen. Using DNA extracted from highly purified hematopoietic cell fractions and real-time PCR technology, we were able to demonstrate the presence of minimally one microchimeric cell type in seven out of eight donors tested (data not
shown). It remains to be studied whether or not particular microchimeric cell types are associated with either a sensitized or a tolerized minor H antigen immunization status.

A significant proportion of patients eligible for SCT does not have an HLA-identical family donor nor an HLA-matched unrelated donor and may therefore receive an HLA haplo- or partial identical SC graft. Several clinical studies suggest that mutual allo tolerance, marked by the presence of fetal microchimerism in the maternal SC donor and maternal microchimeric cells in the recipient prior to SCT, is an important prerequisite for successful outcome of haploidentical SCT using mother as SC donor. Although the risk of acute GvHD may be enhanced in the latter transplantation setting, event-free survival seems to be significantly better for patients receiving transplants from their mother when compared to grafts donated by their father. Moreover, HLA haploidentical sibling SC grafts mismatched for NIMA were shown to induce less acute GvHD when compared to NIPA mismatched grafts. Such compelling evidence supporting the powerful effects of naturally acquired NIMA-specific tolerance is also accumulating in the field of solid organ transplantation. These studies include the reported prolonged survival of sibling donated haploidentical NIMA-mismatched kidney transplants, when compared to grafts from NIPA-mismatched sibling donors. Familial alloantigen tolerance induced as a consequence of fetomaternal or transmaternal sibling cell traffic may also account for reduced transplantation related mortality observed in first born patients receiving an HLA-identical SC graft from a younger sibling donor. Not only in the HLA-identical but also in the HLA-haploidentical situation, minor H alloreactivities may be induced. Thus, pre-transplant minor H genotyping combined with functional testing for donor T_{CTL} or T_{REG} activity as well as determination of the presence of microchimeric cell populations may be of clinical value particularly in familial SCT settings.
ACKNOWLEDGEMENTS

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G. de Roo, M. van den Hoorn and J. Stegehuis-Kamp are acknowledged for excellent technical assistance in the FACS sorting experiments. We thank M. Kester for tetramer synthesis, A. Goekoop for collecting blood samples or bucal swabs and the HLA tissue typing lab of the Department of Immunohematology and Blood Transfusion for performing HLA and minor H antigen genotyping. We thank Prof. Dr. F.H. Claas, Prof. Dr. J.J. van Rood, Dr. E. Spierings and Dr. L. Hambach for critical reading of the manuscript.
AUTHOR STATEMENT

Contribution: A.H., W.J.B. and E.G. designed the research; A.H., E.J-G, A.J, E.B and J.P performed experiments and collected data; A.H. analyzed results and prepared figures; A.H., A.B., W.J.B. and E.G. drafted the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interest.
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Table 1. Autosomal minor H antigen genotyping of 17 HLA-A2 sharing mother-offspring pairs included in the study.

<table>
<thead>
<tr>
<th>Donor and relevant family</th>
<th>Age</th>
<th>HA-1 genotype</th>
<th>HA-2 Genotype</th>
<th>HA-8 genotype</th>
</tr>
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<tbody>
<tr>
<td>mother 1</td>
<td>57</td>
<td>HA-1 H/R</td>
<td>HA-2 M/M</td>
<td>HA-8 R/R</td>
</tr>
<tr>
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<td>31</td>
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<td>HA-8 R/R</td>
</tr>
<tr>
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<td>HA-2 M/M</td>
<td>HA-8 R/R</td>
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<tr>
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<td>HA-2 M/M</td>
<td>HA-8 P/R</td>
</tr>
<tr>
<td>mother 3</td>
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<td>HA-2 M/M</td>
<td>HA-8 R/R</td>
</tr>
<tr>
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<td>HA-1 H/R</td>
<td>HA-2 V/M</td>
<td>HA-8 P/R</td>
</tr>
<tr>
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<td>HA-1 R/R</td>
<td>HA-2 M/M</td>
<td>HA-8 P/R</td>
</tr>
<tr>
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<td>HA-1 R/R</td>
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<td>HA-8 R/R</td>
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<tr>
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<tr>
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<tr>
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<td>HA-8 R/P</td>
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<tr>
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<td>HA-2 V/V</td>
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<td>HA-2 V/V</td>
<td>HA-8 P/P</td>
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<td>HA-2 V/V</td>
<td>HA-8 P/P</td>
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<tr>
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<td>HA-2 V/M</td>
<td>HA-8 P/R</td>
</tr>
<tr>
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<td>HA-1 H/R</td>
<td>HA-2 V/M</td>
<td>HA-8 P/R</td>
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<tr>
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<tr>
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<td>HA-2 V/M</td>
<td>HA-8 P/R</td>
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<td>34</td>
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<td>HA-8 R/R</td>
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<tr>
<td>mother 15</td>
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<td>HA-8 P/R</td>
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<tr>
<td>son 4</td>
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<td>HA-2 M/M</td>
<td>HA-8 P/P</td>
</tr>
<tr>
<td>mother 16&lt;sup&gt;4&lt;/sup&gt;</td>
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<td>HA-8 R/R</td>
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<tr>
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<tr>
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<td>HA-8 R/P</td>
</tr>
<tr>
<td>son 7</td>
<td>19</td>
<td>HA-1 H/R</td>
<td>ND</td>
<td>HA-8 P/P</td>
</tr>
</tbody>
</table>
Subjects analyzed in the study are indicated in bold.

Autosomal minor H antigens are encoded by bi-allelic loci comprising an immunogenic allele, e.g. HA-1\(^H\), HA-2\(^V\) and HA-8\(^R\), and a non-immunogenic or a ‘null’ allele, e.g. HA-1\(^R\), HA-2\(^M\) and HA-8\(^P\). Consequently, individuals expressing HA-1\(^H/R\) are referred to as HA-1\(^{pos}\), whereas HA-1\(^R/R\) individuals are designated as HA-1\(^{neg}\); ND not determined.

The same pair.

The autosomal minor H alloantigen under study is indicated in bold. HY is studied in female donors in case no autosomal minor H mismatch exists between the mother and her offspring.

\(^m\)GM means HLA-A2 positive maternal grandmother.
Table 2. Primer and probe nucleotide sequences used for the detection Y chromosome and HA-1\textsuperscript{H} positive microchimeric cells by real-time PCR.

<table>
<thead>
<tr>
<th>primer</th>
<th>nucleotide sequence</th>
<th>fragment size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forw-DYS1</td>
<td>5'-TCCTGCTTTATCCAAATTCACCATT-3'</td>
<td>86bp\textsuperscript{1}</td>
</tr>
<tr>
<td>Rev-DYS1</td>
<td>5'-ACTTCCCTCTGACATTACCTGATAATTG-3'</td>
<td></td>
</tr>
<tr>
<td>Y probe</td>
<td>5'- (FAM)-AAGTCGCCACTGGATATCAGTTCCCCCTTG-(TAMRA)-3'</td>
<td></td>
</tr>
<tr>
<td>Forw-HCK</td>
<td>5'-TATTAGCACCATCCATAGGAGGCTT-3'</td>
<td>81bp\textsuperscript{1}</td>
</tr>
<tr>
<td>Rev-HCK</td>
<td>5'-GTTAGGGAAAGTGGAGCGGAAG-3'</td>
<td></td>
</tr>
<tr>
<td>HCK Probe</td>
<td>5'- (FAM)-TAACCGCTGCCACCAAGGATGCAG-(TAMRA)-3'</td>
<td></td>
</tr>
<tr>
<td>Forw-I-COM</td>
<td>5'-GACGTCGTCGAGGACATCTCCCCATC-3'</td>
<td>324 bp\textsuperscript{2}</td>
</tr>
<tr>
<td>Rev-I HA-1\textsuperscript{H}</td>
<td>5'-CATCAGATCTTTTAAAAAAAAAGTG-3'</td>
<td></td>
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<tr>
<td>For-II HA-1\textsuperscript{H}</td>
<td>5'-CTTAAGGAGTGTGTGCTGCA-3'</td>
<td>191 bp\textsuperscript{3}</td>
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<tr>
<td>HA-1\textsuperscript{H} Rev-II</td>
<td>5'-ACTCCTACACATCCCTCAGA-3'</td>
<td></td>
</tr>
<tr>
<td>For-I HCK</td>
<td>5'-ACCTCCCCGAAGATTGCAGAC-3'</td>
<td>381 bp\textsuperscript{2}</td>
</tr>
<tr>
<td>Rev-I HCK</td>
<td>5'-TTGGGGGCAAGTGGAGTTGA-3'</td>
<td></td>
</tr>
<tr>
<td>For-II HCK</td>
<td>5'-TATTAGCCACATCCATAGGAGGCTT-3'</td>
<td>81bp\textsuperscript{3}</td>
</tr>
<tr>
<td>Rev-II HCK</td>
<td>5'-GTTAGGGAAAGTGGAGCGGAAG-3'</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{1} Amplification conditions: 95°C for 10 minutes, then 40 cycles at 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds.

\textsuperscript{2} Amplification reactions: 5 minutes at 95°C, then 30 cycles at 95°C for 1 minute, 58°C for 1 minute and 72°C for 1 minute. The cycling was followed by 72°C for 5 minutes.

\textsuperscript{3} Amplification conditions: 4.5 minutes at 95°C, then 5 cycles at 95°C for 15 seconds and 66°C for 45 seconds, followed by 35 cycles at 95°C for 15 seconds, 62°C for 45 seconds and 72°C for 30 seconds.
Table 3. Presence of minor H antigen-specific CD8<sup>pos</sup> T<sub>CTL</sub> or minor H antigen-specific CD8<sup>pos</sup> T<sub>REG</sub> in healthy blood donors.

<table>
<thead>
<tr>
<th>Donor</th>
<th>cytolytic T cells&lt;sup&gt;1&lt;/sup&gt;</th>
<th>tvDTH classification&lt;sup&gt;2&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>♀4</td>
<td>no</td>
<td>regulator (58%)</td>
</tr>
<tr>
<td>♀5</td>
<td>no</td>
<td>regulator (60%)</td>
</tr>
<tr>
<td>♀1</td>
<td>no</td>
<td>regulator (67%)</td>
</tr>
<tr>
<td>♀3</td>
<td>no</td>
<td>regulator (67%)</td>
</tr>
<tr>
<td>♀2</td>
<td>no</td>
<td>non-regulator (0%)</td>
</tr>
<tr>
<td>♀6</td>
<td>yes</td>
<td>non-regulator (0%)</td>
</tr>
<tr>
<td>♀10</td>
<td>yes</td>
<td>non-regulator (0%)</td>
</tr>
<tr>
<td>♀7</td>
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<td>non-regulator (10%)</td>
</tr>
<tr>
<td>♀11</td>
<td>yes</td>
<td>non-regulator (33%)</td>
</tr>
<tr>
<td>♀9</td>
<td>yes</td>
<td>ND</td>
</tr>
<tr>
<td>♂2</td>
<td>no</td>
<td>regulator (50%)</td>
</tr>
<tr>
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<td>regulator (50%)</td>
</tr>
<tr>
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<td>regulator (60%)</td>
</tr>
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<td>regulator (80%)</td>
</tr>
<tr>
<td>♂1</td>
<td>yes</td>
<td>non-regulator (0%)</td>
</tr>
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</table>

<sup>1</sup>Total tetramer-binding T cells were isolated by FACS sorting, polyclonally stimulated and subsequently tested for cytolytic function.

<sup>2</sup>The dominant presence of minor H alloantigen-specific Treg within total PBMC was measured in the tvDTH assay<sup>17</sup>. The percentages indicate that percentage inhibition of recall antigen-induced swelling footpad responses in the presence of the relevant minor H allo peptide; ND not determined.
Table 4. Tetramer staining profiles in female and male blood donors with minor H antigen disparate family members.

<table>
<thead>
<tr>
<th></th>
<th>mother anti-child</th>
<th>son anti-mother</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HY mm(^1)</td>
<td>HA-1 mm</td>
</tr>
<tr>
<td>dual staining pattern(^2)</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>predominant tetramer(^{dim}) staining pattern(^3)</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>number of donors analyzed</td>
<td>7</td>
<td>2</td>
</tr>
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</table>

\(^1\)mm mismatch
\(^2\)The ratio tetramer\(^{bright}\) : tetramer\(^{dim}\) is 1 : ≤10.
\(^3\)The ratio tetramer\(^{bright}\) : tetramer\(^{dim}\) is 1 : >10.
FIGURE LEGENDS

Figure 1. Bidirectional routes of familial exposure to minor H alloantigens.
Pathway C illustrates exposure of the offspring to non-inherited maternal minor H alloantigen HA-1^H after transfer of cells during pregnancy (as indicated by arrow).
Maternal exposure to fetal inherited paternal minor H alloantigen HY or HA-1^H is illustrated by pathway A and B respectively.

Figure 2. Tetramer staining patterns and cytolytic function of CD8^{pos} minor H antigen-specific T cells obtained from healthy women and men.
(A and C) Representative tetramer-binding profiles of HY specific T cells (A) and HA-1 specific T cells (C) detected \textit{ex vivo} in CD8 enriched PBMC after non-stringent FACS sorting. The rectangle indicates the total tetramer^{pos} population isolated during a second round of FACS sorting. (B and D) The cytolytic activity, indicated as percentage lysis on the Y-axis, of polyclonally expanded tetramer^{pos} fractions tested against various target cells: (□) HLA-A2^{pos}, HA-1^{neg} female target cells; (■) HLA-A2^{pos}, HA-1^{neg} female target cells pulsed with HY peptide (two top graphs) or HLA-A2^{pos}, HA-1^{neg} female target cells pulsed with HA-1 peptide (two bottom graphs) and (●) HLA-A2^{pos} HA-1^{pos} male target cells. The effector:target cell ratios (as depicted on the X-axis) were calculated according to the corresponding percentages of tetramer-binding T cells as shown in the centre plots.
Figure 3. Presence or absence of minor H antigen-specific regulatory T cells analyzed in the tvDTH assay.

Total PBMC of two mothers with male offspring were tested for minor H antigen HY-driven bystander suppression of recall responses. PBMC were injected together with either a mixture of recall antigens comprising Tetanus Toxoid (TT) and Diphtheria Toxoid (D), minor H allo peptide HY (allo HY) or minor H self peptide HA-2V or HA-2M (self HA-2V, self HA-2M) alone or with a combination of TT/D plus allo or self peptide into the footpads of CB17.SCID mice. Footpad swelling, indicated as net swelling on the Y-axis, was measured 24 hours later. Percentages indicate the percentage of inhibition of the recall response (solid bar) in the presence of allo (hatched bar) or self (open bar) minor H peptide. Mother ♀4 (left graph) displays a tvDTH regulator phenotype, mother ♀7 (right graph) is classified as a tvDTH non-regulator.

Figure 4. Dissection of HLA-A2/minor H peptide tetramer staining profiles.

(A and C) Definition of tetramerbright and tetramerdimgate settings (as indicated by dotted lines) using a tetramerbright staining cytolytic HY (A) or HA-1 (C) specific T cell clone titrated into CD8 enriched PBMC. (B) Analysis of tetramer binding profiles of two female donors (♀9 and ♀6) with male offspring after non-stringent sorting of HYA2 tetramerpos CD8pos cells. (D) Analysis of HA-1A2 tetramer binding profiles of two HA-1R/R male donors (♂1 and ♂2) with a HA-1H/R mother after non-stringent sorting of HA-1A2 tetramerpos CD8pos cells. The solid box indicates the total CD8pos tetramerpos population; tetramerbright staining T cells are plotted in black and tetramerdim staining T cells are plotted in gray. Percentages indicate the distribution of each T cell subset in the total tetramerpos fraction.
Figure 5. Phenotypic and functional analysis of minor H alloantigen-specific TREG.
Results from one male donor (♂5) and two female donors (♀1 and ♀6) with minor H
antigen disparate family members are shown. (A) Cell surface expression of CD25 and
CTLA-4 by CD8\textsuperscript{pos} HA-1\textsuperscript{A2} (top graph) or HY\textsuperscript{A2} (centre and bottom graph) tetramer\textsuperscript{dim}
staining T cells (indicated by circle) isolated by two consecutive rounds of FACS sorting
from CD8 enriched PBMC. (B) Defining the role of CTLA-4 in minor H alloantigen-driven
bystander suppression of recall responses. Total PBMC, obtained from the same blood
sample as shown in A, were injected together with the recall antigens Tetanus Toxoid
(TT) and Diphtheria Toxoid (D), allo peptide (HA-1 for 55; HY for ♀1 and ♀6) alone or a
combination thereof into the footpads of CB17.SCID mice. Footpad swelling indicated as
net swelling is depicted on the Y-axis. Uncovering of recall antigen-induced footpad
swelling was induced by co injection of blocking CTLA-4 antibodies (αCTLA-4) or an
isotype control antibody (iso). The percentages indicate the percentage of inhibition of
the recall response (solid bar) when minor H allo peptide is co-injected with or without
blocking CTLA-4 antibody.
Figure 3
Figure 4

A

HYA2 tetramer

'Bright'

'Dim'

B

♀ 9

15% 85%

♀ 6

3% 97%

C

HA1A2 tetramer

'Bright'

'Dim'

D

♂ 1

13% 87%

♂ 2

6% 94%

CD8
Figure 5

A

▲ 5

CD8

♀ 1

♀ 6

HYA2 tetramer

CD25

CTLA-4

83% 17%

82% 18%

44% 56%

42% 58%

98% 2%

98% 2%

B

TT/D allo HA-1iso-TT/D allo

αCTLA-4

TT/D + allo HA-1 +

net swelling (10^4 inches)

♀ 6

♂ 5

CD25

CTLA-4

67% 33%

0% 0%

0% 0%
Naturally acquired tolerance and sensitization to minor histocompatibility antigens in healthy family members

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