

Supporting methods:**1-Donor samples**

Healthy blood donors were recruited with informed written consent. HLA class II typing of donors was performed by PCR-SSO (Dynal Biotech, UK), and HLA-DRB3 high resolution typing was performed by standard PCR-SSP (Dynal Biotech, UK).

2-Real time mRNA quantification:

Plasmid standard curves were generated performing 5 serial, ten-fold dilutions (10^6 to 10^2). The copy number of DRB1-52, DRB3 and GAPDH in each sample was calculated based on the respective plasmid or amplified product standard curves. Samples were run on triplicate for each gene, and the quantification of DRB1-52 accepting variations of less than a 30% of SD. DRB1-52 and DRB3 quantifications were normalized to GAPDH.

Real time PCR protocols:

Real time amplifications were conducted in a LightCycler 480 instrument, using SybrGreen 1 chemistry. The reactions were performed as follows: A total reaction volume of 10 μ l contained 2 μ l of cDNA, 0.5 μ M of each primer and 5 μ l of LightCycler 480 SybrGreen I Master (Roche, Mannheim, Germany). The DRB1-52 and DRB3 PCR program was: 10 min denaturation step, followed by 50 amplification cycles (95 °C-10 s, 60 °C-5 s and 72 °C-20 s) with fluorescence acquisition in the extension step. Melting curve was generated at 95 °C-0 s, 75 °C-15 s and 99 °C-0 s with 10 acquisitions per °C/s in continuous mode of fluorescence monitoring. The GAPDH PCR program was: 10 min denaturation step, followed by 40 amplification cycles (95 °C-10 s, 55 °C-5 s and 72 °C-15 s) with fluorescence acquisition in the extension step. Melting curves were generated as above. The melting curve was generated at 95 °C for 0 s, 75 °C for 15 s and 99 °C for 0 s with 10 acquisitions per °C/s in continuous mode of fluorescence monitoring.

3- Flow cytometry staining with anti-DRB3 and anti-DR11 antibodies

For each individual tested, PBMCs were obtained from 5 ml of heparinized blood; 2×10^5 cells were used for each condition. 7.3.19.1 (Abcam plc, Cambridge, UK) was used at 1/100 dilution, and 4i131 (Abcam plc) was used at 1/90. Cells were incubated 30 min at room temperature (rt) with the corresponding primary antibody, then washed and resuspended with 50 μ l of the secondary antibody Goat Anti-mouse IgG (H+L)-RPE (Southern Biotech, Birmingham, USA) diluted 1/150 in normal mouse IgG (Caltag Laboratories, UK), used as a blocking agent. Then, cells were washed, and labeled with Anti-CD19-FITC (Becton-Dickinson, San Jose, CA) and anti CD14-APC (BD Biosciences, USA).

As a reference for the staining of all HLA-DR molecules, 2×10^5 PBMCs of each donor were incubated with anti-CD3/DR (Becton-Dickinson, San Jose, CA) and anti-CD14-APC (BD Biosciences, USA) during 30 min at 4°C, then washed and immediately analyzed by flow cytometry.

4- Mapping of CD4 T cell epitopes using class II tetramers

Peptide stimulation of CD4+ T cells or PBMC

When stimulating total PBMCs, cells were seeded at 2×10^6 cell/ml in 48 well plates in T cell medium [RPMI-1640 (GIBCO BRL, Rockville, Maryland, USA), supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 mg/ml penicillin/streptomycin and 10% vol/vol pooled human serum].

When stimulating purified CD4 cells, the positively selected fraction was used as a source of APCs as follows: 1×10^6 cells were seeded in 48 well plates in T cell medium and incubated for 1 h at 37°C, 5% CO₂ to allow adhesion of monocytes to the plates. Wells were washed twice with T cell media to remove the non-adhered cells, and then 2×10^6 CD4+ T cells were added to each well.

Positive peptide pool definition

For the definition of the epitopes, positive pools were defined by staining with tetramers loaded simultaneously with all peptides from the pool. Subsequently, the peptides from within the pool which contained the epitope were defined by staining with tetramers loaded individually with single peptides from the pool. In all cases tetramer staining was considered positive based on the following criteria: tetramer staining over the 1%, grouped events constituting a defined population, and an intensity of staining more than 10^2 with tetramer-PE. For the definition epitopes, positive staining was always observed using tetramers loaded with the entire pool and using tetramers loaded with an individual peptide (as previously described [1]).

5- Evaluating the natural processing and presentation of DRB3 tetanus epitopes.

Peptide stimulation of CD4+ T cells with whole toxin or tetanus vaccine

1 μ g/ml of whole tetanus toxin (Sigma-Aldrich Co, Spain) or 0.4 UI of the DITANRIX (Glaxo-Smith-Kline, Spain) tetanus-diphtheria vaccine was used to stimulate 2×10^6 purified CD4+ T cells mixed with 1×10^6 autologous APCs. The peptide stimulation (included as an experimental control) was carried out as described above. At day 7, 20 UI of IL-2 were added to the culture; tetramer staining was done at day 14.

Generation of peptide specific T CD4+ cell lines and clones.

For the generation of the peptide specific TT102 and TT36 CD4+ cell lines, CD4 cells were stimulated with the specific peptide using autologous or irradiated HLA-matched PBMCs every 14 days. For the generation of the TT64 specific clones, tetramer positive cells were purified with a FACSVANTAGE cell sorter with a purity of 99.0%. Stimulation of the single cells was performed with 2 μ g/ml of PHA (Roche, Germany) using irradiated PBMCs. Specificity of the growing clones was tested by tetramer staining.

6- Dose dependent proliferation of CD4+ cell lines stimulated with Tetanus toxin or individual peptides.

Dose dependent proliferation assays of TT102 or TT36 specific CD4+ T cell lines were carried out using 2×10^4 cells/well and autologous irradiated PBMCs (2×10^5 cells/well) as APC. The cells were plated in quadruplicate wells of 96 well plates and stimulated with each of the following antigens: 0.25 $\mu\text{g/ml}$ of the Tetanus Toxin (Sigma-Aldrich-Co, Spain), 1 $\mu\text{g/ml}$ of the specific peptide or 20 UI/ml IL-2. Basal growth rate was determined by quadruplicate wells containing only T CD4+ cell lines and APCs. After 6 days of proliferation, cells were pulsed for 16 h with ^3H -thymidine (1 μCi per well) and collected with a Titertek cell harvester. Incorporation of ^3H -thymidine was measured by liquid scintillation.

7- Direct ex-vivo staining of PBMCs with DRB3 tetramers.

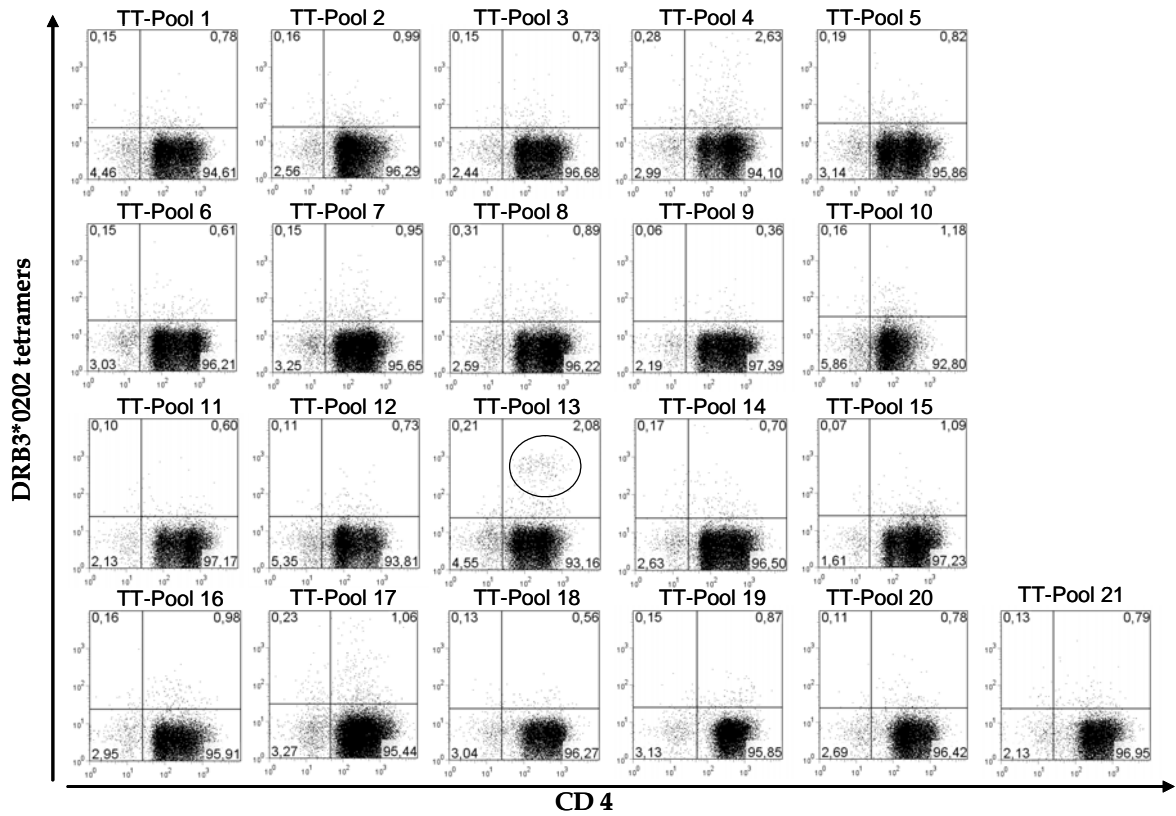
PBMCs were isolated from heparinized blood of immunized healthy donors by density centrifugation (Lymphoprep; Nycomed Pharma AS Diagnostics, Oslo, Norway). PBMCs were resuspended in PBS (supplemented using 1% FCS) at a concentration of 10×10^7 /ml and immediately stained using 25 $\mu\text{g/ml}$ of tetramer. 30×10^6 PBMCs were stained with irrelevant tetramers (DRB3*0101-MP42-PE and DRB3*0101-MP42-APC) and 50×10^6 PBMCs were stained with relevant tetramers (DRB3*0101-TT36-PE and DRB3*0101-TT102-APC). Cells were subsequently stained with anti-CD4-FITC at 4°C for 30 min, washed twice in PBS and analyzed using a FACSCalibur (BD biosciences, San Jose, CA). Statistically significant differences in the number of tetramer positive cells from irrelevant and relevant staining were evaluated using a Mann Withney test. A level of significance of 5% was used in all the statistical evaluations, and a one tailed P value assignation.

- 1 Roti, M., Yang, J., Berger, D., Huston, L., James, E. A. and Kwok, W. W., Healthy human subjects have CD4+ T cells directed against H5N1 influenza virus. *J Immunol* 2008. 180: 1758-1768.

Supporting Table I. Samples used for DRB3/DRB1(52) mRNA quantification and cell surface staining.

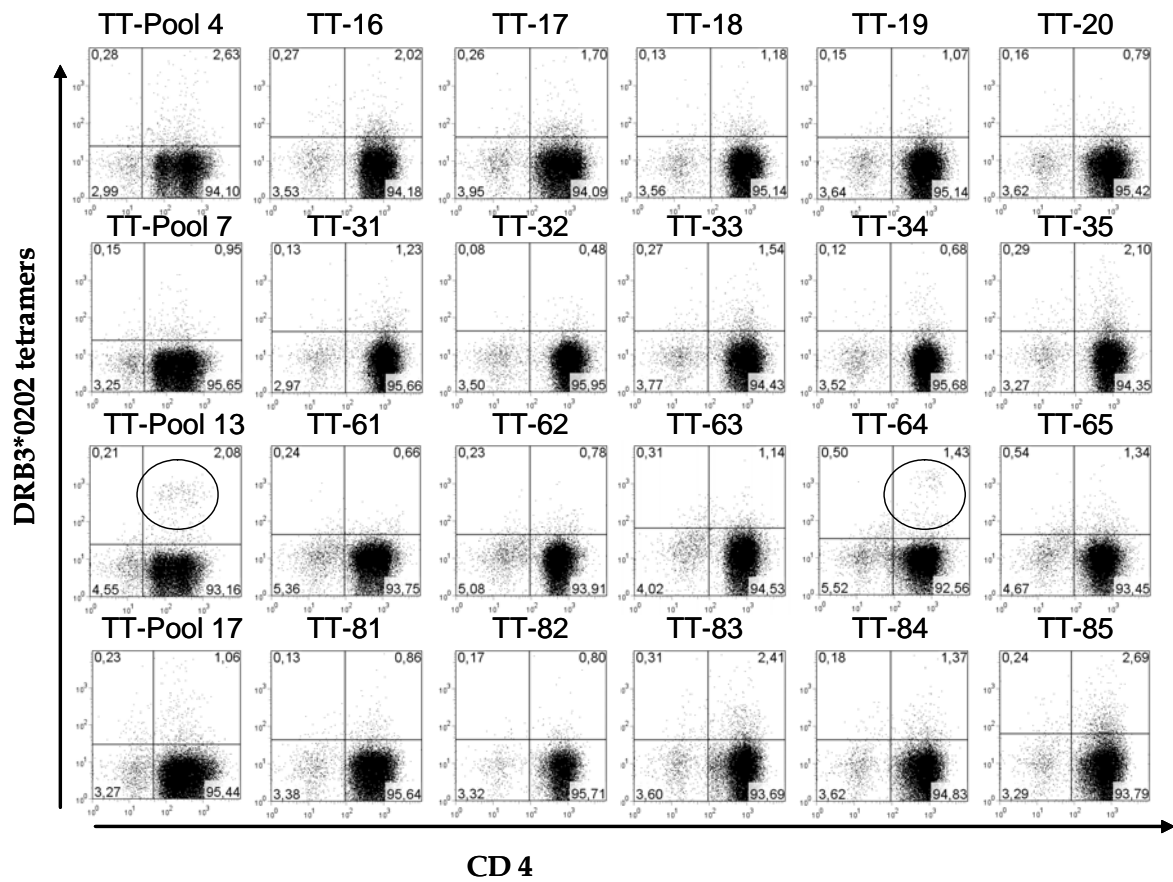
<i>Donor number</i>	<i>DRB1 typing</i>	<i>DRB3 typing</i>	<i>Enrichment of^a</i>	<i>CD3 %</i>	<i>CD19 %</i>	<i>CD14 %</i>
1	07/11	0202	CD19+	9	82	0.95
			CD14+	6.95	12	72
2	01/11	0202	CD19+	9.29	86	2.03
			CD14+	3.70	<0.1	88
3	04/13	0101	CD19+	<0.1	100	<0.1
			CD14+	7.92	<0.1	91
4	07/13	0101	CD19+	3	94	1
			CD14+	1	<0.1	97
5	03/07	0101	CD19+	9	85	1
			CD14+	9	1	88
6	03/04	0101	CD19+	30	67	2.42
			CD14+	9.57	<0.1	86
7	03/13	0101/0101	CD19+	26.10	69	0.68
			CD14+	13	3.36	82

^a The table contains also the purity of the CD14+ and CD19+ cell fractions used for the mRNA quantification. Bold numbers showed the % of the specific fraction enrichment (the other values refer to contaminating cell populations in the sample).

Supplementary figure 1**Supplementary figure 1. Identification of DRB3*0202-restricted TT specific epitopes with tetramer pools.**

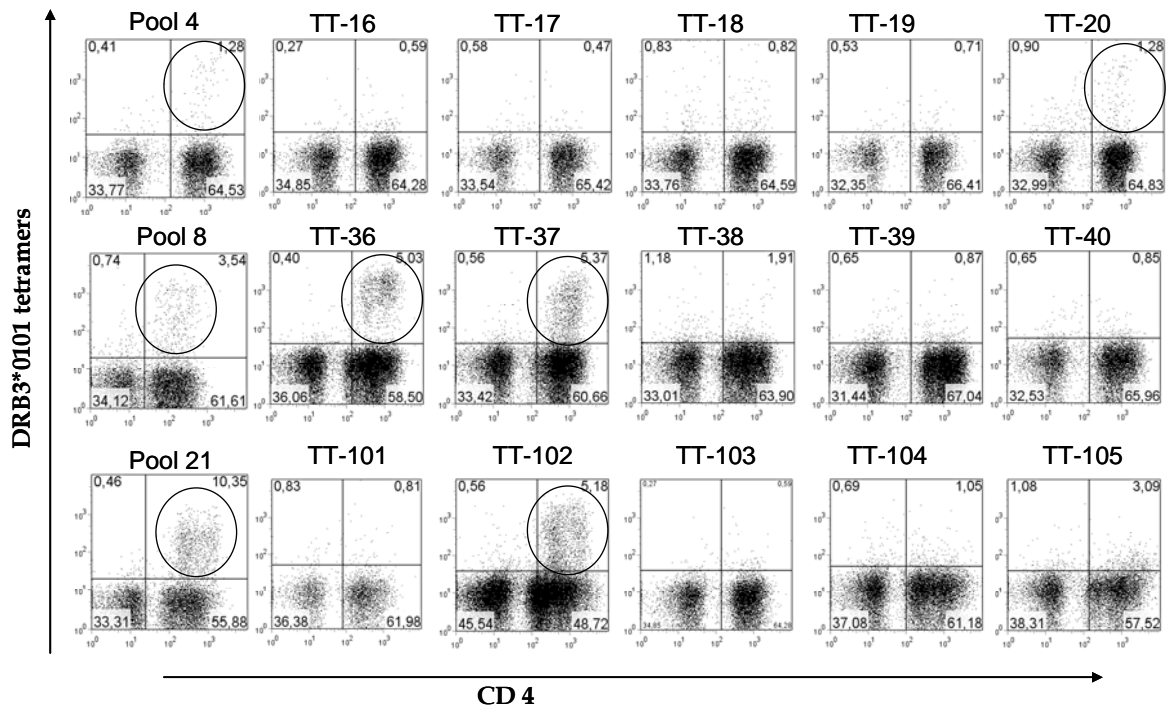
Representative staining profiles with DRB3*0202-TT tetramer pools. CD4⁺ T cells from a DRB3*0202 subject were stimulated with 21 pools of TT peptides, and stained with DRB3*0202 pooled peptide tetramers 14 days after stimulation. Circled events indicate the populations that were tetramer positive.

Supplementary figure 2

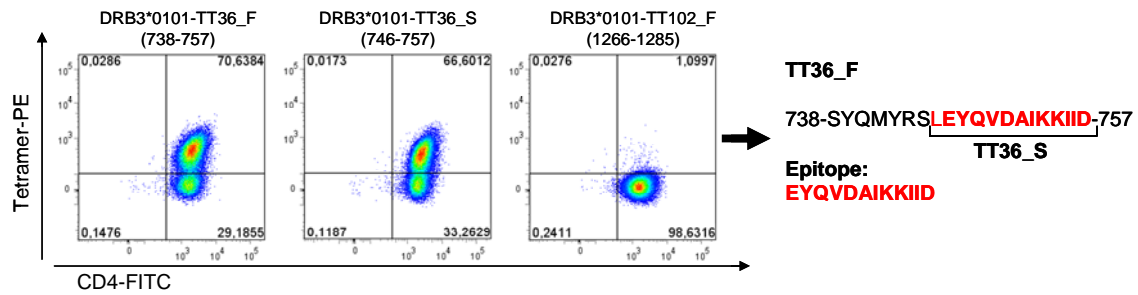


Supplementary figure 2. Fine mapping of DRB3*0202-restricted TT specific epitopes.

Representative staining profiles with DRB3*0202 tetramers loaded with individual peptides from TT pools 4, 7, 13 and 17. Circled events indicate the populations that are tetramer positive: TT pool 13 and TT peptide 14.

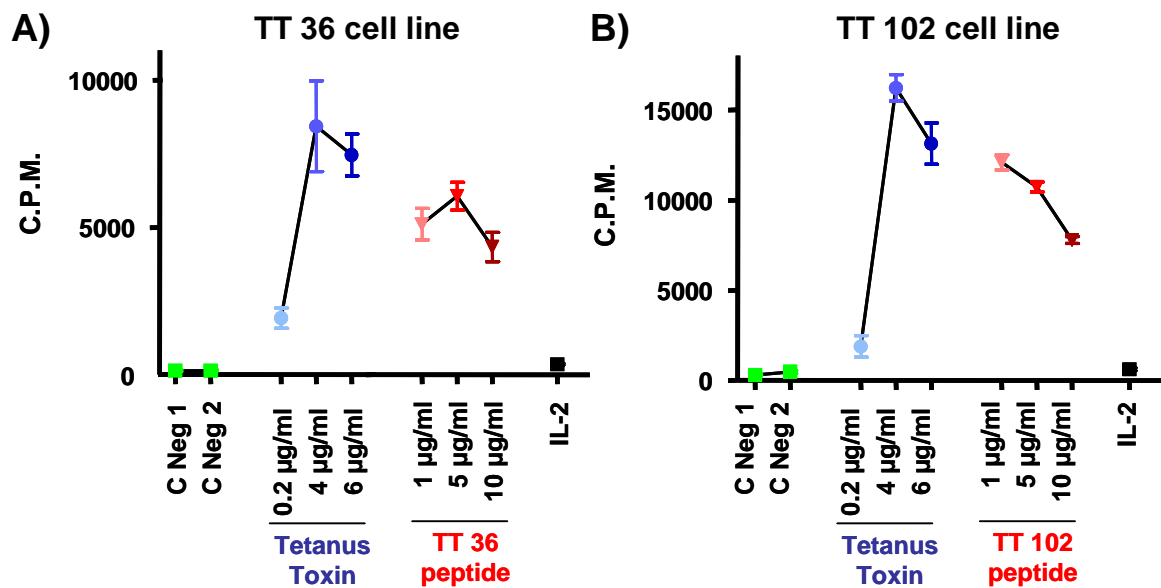
Supplementary figure 3**Supplementary figure 3. Identification of DRB3*0101-restricted TT specific epitopes.**

Representative staining profiles with DRB3*0101 tetramers loaded with pooled peptides from TT pools 4, 8 and 21. Next to each pool is shown the fine mapping with individual loaded TT tetramers from these pools. Circled events indicate the populations that are tetramer positive.

Supplementary figure 4**Supplementary figure 4. Identification of DRB3*0101 minimal epitopes.**

Staining profiles of the T cell lines with tetramers loaded with minimal epitopes. A) TT 36 cell line stained either with the full length peptide DRB3*0101-TT36_F(738-757), with the minimal epitope peptide DRB3*0101-TT36_S (746-757) and with a negative control.

Supplementary figure 5

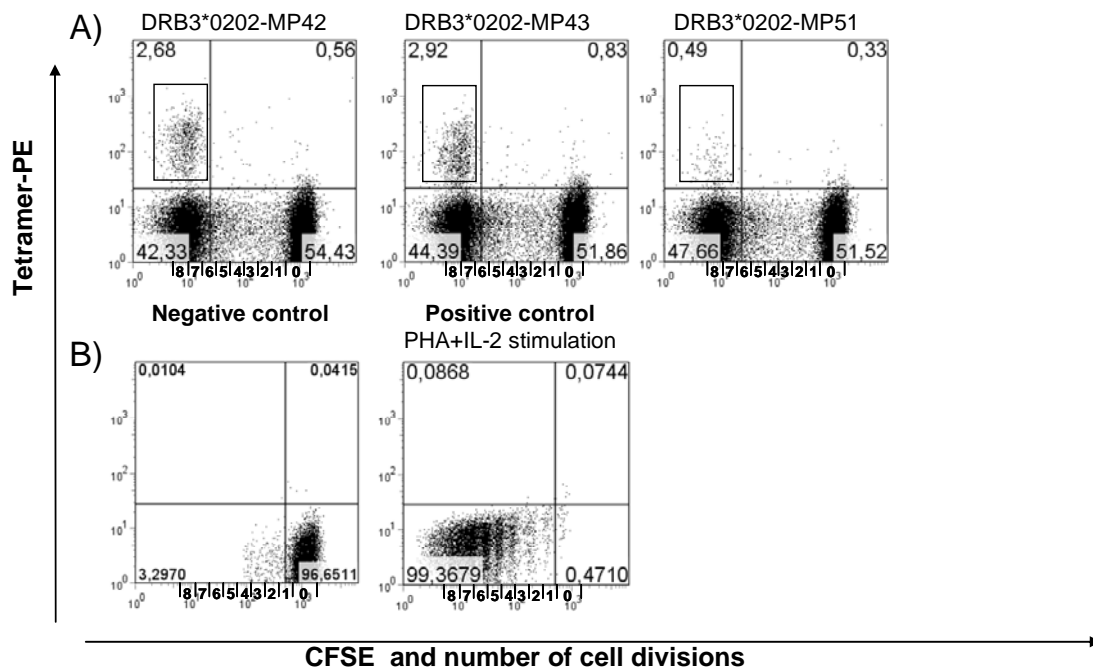


Supplementary figure 5. Dose dependence of the response to the Tetanus Toxin and to the peptides.

A). Level of proliferation of a CD4⁺ T cell line specific for the peptide TT36 stimulated with irradiated autologous PBMCs and different concentrations of the whole tetanus toxin, different concentrations of the specific peptide (TT36) or IL-2.

B). Level of proliferation of a CD4⁺ T cell line specific for the peptide TT102 stimulated with irradiated autologous PBMCs and different concentrations of the whole tetanus toxin, different concentrations of the specific peptide (TT102) or IL-2.

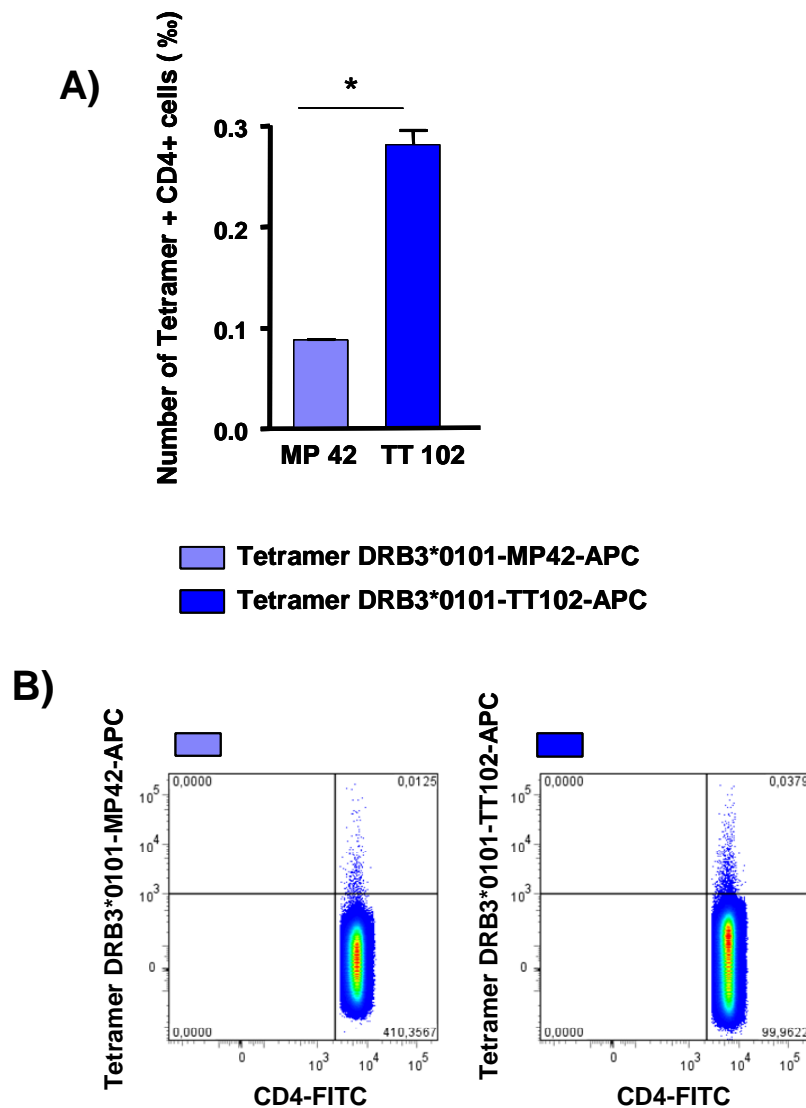
Both cell lines respond to the whole toxin and to the peptide providing evidence of the in-vivo relevance of these epitopes.

Supplementary figure 6**Supplementary figure 6. CFSE staining of DRB3*0202 and DRB1*1101 MP specific epitopes.**

A) Identification of MP42 (MP1-20), MP43 (MP9-28), MP51 (MP73-92) antigen specific cells with DRB3*0202-tetramers. B) Negative control: irrelevant tetramer staining of peptide stimulated cells. Positive control: PHA+IL-2 stimulation to calculate the number of cell divisions. Events in the squares where used to do the calculations of frequency of the T cell precursors.

T CD4 cells isolated using beads where labeled with CFSE, stimulated with autologous adherent cells and peptide, where stained on day 7 with PE-labeled tetramers and analyzed subsequently by flow cytometry. The stimulation was performed with PBMC from a DRB3*0202 individual. In all panels cells are gated on forward and side scatter, the vertical axis shows PE fluorescence of the tetramers indicated in each panel, and the horizontal axis shows CFSE fluorescence over a 4-decade logarithmic scale. In addition, the horizontal axis shows the corresponding number of cell divisions, with “0” depicting the undivided parent population. This scale was calculated from the distinct CFSE fluorescence peaks produced by polyclonal stimulation with PHA and IL-2 as described in Methods. Percentages shown in the margins of each panel represent the percent of total cells present in each quadrant.

Supplementary figure 7



Supplementary figure 7: Tetanus specific DRB3 restricted T CD4+ cells are present at relevant levels in vivo.

A). Number of tetramer positive T CD4+ cells observed in PBMCs from an individual tetanus vaccinated 4 years ago. DRB3*0101-MP42 was used as irrelevant tetramer both with APC and PE. The percentage of staining with DRB3*0101-TT102 was statistically higher ($P=0.0179$) than the observed with the irrelevant tetramer. B). Representative plots of the direct staining with tetramers. Each plot represents the staining of 10,000,000 PBMCs.