Stabilizing mutations increase secretion of functional soluble TCR-Ig fusion proteins

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Abstract

Background: Whereas T cell receptors (TCRs) detect peptide/major histocompatibility complexes (pMHCs) with exquisite specificity, there are challenges regarding their expression and use as soluble detection molecules due to molecular instability. We have investigated strategies for the production of TCR-immunoglobulin (Ig) fusion proteins. Two different TCRs that are characteristic of a mouse model for idiotype (Id) dependent immune regulation were engineered. They are structurally unrelated with different variable (V), diversity (D) and joining (J) segments, but each share one V gene segment, either Va or Vb, with the well characterized murine TCR, 2C.

Results: Several TCR-Ig formats were assessed. In one, the TCR V domains were fused to Ig constant (C) regions. In others, the complete extracellular part of the TCR was fused either to a complete Ig or an Ig Fc region. All molecules were initially poorly secreted from eukaryotic cells, but replacement of unfavourable amino acids in the V regions improved secretion, as did the introduction of a disulfide bridge between the TCR C domains and the removal of an unpaired cysteine. A screening strategy for selection of mutations that stabilize the actual fusion molecules was developed and used successfully. Molecules that included the complete heterodimeric TCR, with a stabilizing disulfide bridge, were correctly folded as they bound TCR-specific antibodies (Abs) and detected pMHC on cells after specific peptide loading.

Conclusions: We show that fully functional TCR-Ig fusion proteins can be made in good yields following stabilizing engineering of TCR V and C region genes. This is important since TCR-Ig fusions will be important probes for the presence of specific pMHCs in vitro and in vivo. In the absence of further affinity maturation, the reagents will be very useful for the detection of kinetic stability of complexes of peptide and MHC.

Background

Whereas the use of recombinant soluble peptide-MHC (pMHC) molecules for identification of specific T cells has increased dramatically over the last years [1-3], the reciprocal approach of using recombinant soluble TCRs (that is, lacking the transmembrane and intracellular part) for specific detection of peptide presentation and targeting to specific pMHC on cells has proven far more difficult.

A few pMHC specific antibodies have been described, but are often cross-reactive [4-10]. The limitation may be overcome by the use of combinatorial antibody technology as demonstrated for pMHC class I [11]. However, neither antibody libraries, nor the full range of specific, recombinant pMHCs required for panning in the selection step, are readily available.

TCRs have evolved to recognize pMHC. They are detection molecules with exquisite specificity, and exhibit, like antibodies, an enormous diversity. Soluble TCRs also offer unique opportunities for novel, highly specific therapeutic molecules. Different approaches have therefore been taken for production and testing of soluble TCRs, most of which have been derived from established T cell clones of known specificity [12].

Soluble TCRs have been produced as heterodimers of α/β chains [13-15], or as two variable (V) domains joined in single chain TCRs (scTCRs) of various formats [16-20]. In general however, the development of such molecules is hampered by difficulties associated with low stability causing low expression yields, aggregation of purified proteins and misfolding [21].

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In order to increase stability, the TCR V regions have been optimized by amino acid replacements. Such replacements have been described that increase the surface hydrophilicity of a scTCR derived from the human RFL 3.8 TCR [22], or yeast surface display [23] as well as resistance to thermal denaturation [24] of a scTCR derived from the murine 2C TCR.

In some cases, heterodimeric α/β TCRs have been stabilized by a non-native disulfide bridge between the constant (C) domains [25,26].

The intrinsic affinity of a TCR for its pMHC is in the lower micromolar range [27]. While all TCRs on the surface of a T cell are identical, only a few copies of a particular pMHC are displayed on the surface of an antigen presenting cell. Multimerization to increase avidity has therefore been obtained by either indirect capture on beads [28], direct biotinylation and binding to streptavidin [17] or by expressing TCRs on larger particles such as phage [29], viral capsids [19], or cells [30-32].

TCRs have been fused to other soluble polypeptides, amongst Igs, which have a number of advantages as fusion partner since they are naturally secreted, stable molecules. TCR-Ig molecules should be secreted and acquire increased stability and binding avidity upon dimerization, and detection of binding to target cells should be facilitated utilizing the vast repertoire of available methods developed for detection of Ig. In that way, one might tap into the successful strategies developed for monoclonal antibodies, including widely used purification methodology. In addition, the Fc region of TCR-Ig fusion proteins may well provide the targeting TCRs with effector functions in vivo, such as prolonged half life and other FcRn mediated effects, as well as the ability to kill target cells by complement activation and binding to Fc receptors.

However, early attempts to fuse TCRs to Ig domains failed to produce secreted TCRs [33-35]. Later, soluble TCR fusion proteins in which both TCR α- and β-chains were fused to simple Cα domains [36,37] were found to be secreted and recognized by anti-TCR Abs. More recently, a scTCR consisting of Vα-linker-Vβ-Cβ was fused to the human IgG1 constant region [18].

Two TCRs have previously been fused to complete IgG1 and used to stain pMHC on cell surfaces as well as intracellularly after exogenous specific peptide loading [38,39]. We found that such molecules were secreted at very low levels, and therefore explored how select mutations might increase expression. We focused on stabilizing mutations, as no reports exist as to how stabilizing mutations in the TCR V or C domains may affect TCR-Ig fusion molecule production and specificity. Here, we describe the generation of a panel of TCR-Ig fusion proteins based on two different TCRs (4B2A1 and 7A10B2) that are specific for an Ig light chain CDR3 Id peptide presented on an MHC class II molecule in a mouse model for autoimmunity and tumour immunology [40-42]. Three different fusion formats were investigated.

**Results**

**Design of TCR-Ig fusion proteins**

Two different TCRs were fused to Ig, namely 4B2A1 and 7A10B2. Both recognize amino acid 91-101 from the λ2315 light chain of myeloma protein M315 presented on I-EK MHC class II molecules [43]. The TCR-Ig proteins were made in three different fusion formats (Figure 1). In a first format, the TCR V regions were fused to IgG C regions (TCRV-IgC). In two other formats, both V and C TCR domains were included. Either, the extracellular domains of the TCRs were fused to a complete IgG (cTCR-clg) or to the IgG hinge and Fc region (cTCR-IgFc). Thus, the term “cTCR” refers to the complete extracellular part of the TCR, including V and C regions, whereas “clg” refers to complete Ig.

**The fusion proteins are secreted from HEK 293E cells**

Genes encoding either TCRV-IgC or cTCR-clg were assembled on vectors designed for Ig production in mammalian cells as described in Materials and Methods. For each TCR, both α and β chains were tested for fusion to both heavy and light Ig chains, a total of eight constructs. To investigate fusion protein secretion, HEK 293E cells were transiently transfected with the genes, and cell supernatants examined in ELISA specific for the Ig portion of the fusions after two days. Low

![Figure 1 TCR-Ig fusion protein formats](http://www.biomedcentral.com/1472-6750/10/61)
secretion levels were observed in all cases (Figure 2). Fusion proteins based on the 4B2A1 receptor were secreted at higher levels than those based on 7A10B2. The fusion protein secreted at highest levels (~100 ng/ml) was the 4B2A1-based TCRV-IgC construct with the complete TCR α chain fused to the heavy chain C region and the TCR Vβ domain on the light chain C region (VαH + VβL) or vice versa in the TCRV-IgC format. The complete TCR α chain was fused to the complete heavy chain and the TCR β chain fused to the light chain (H+VβL) or vice versa in the cTCR-clg format. TCR domains are filled, Ig domains are open circles. Cell supernatant was tested in triplicates. Error bars: Standard deviation (SD). One representative experiment out of three is shown.

**Stability engineering of V regions improves secretion**

To stabilize the TCR V domains and thus increase secretion levels, amino acids were replaced by site directed in vitro mutagenesis. A large number of mutants were generated and those that increased secretion levels in the actual TCRV-IgC format selected. Targeted positions have been described [22-24], and the substitutions tested were: S82R in 4B2A1 Vα3.1; G17E, H47Y, and L80S in 4B2A1 Vβ3.1; L43P and W82R in 7A10B2 Vα3.1 with 2C. Thus, when the V(D)J encoded complete V domain sequences are considered, 7B2A1 exhibits high homology to 2C Vα (86.8% identity and 88.6% similarity), while 4B2A1 exhibits high homology to 2C Vβ (89.5% identity and 92.1% similarity).

The mutagenesis reactions were performed such that each gene acquired one or several alterations. All VαH variants were tested in combination with all VβL variants, and the best pair regarding secretion levels from transiently transfected cells identified for each receptor as described in Materials and methods. Analyses were done on the constructs that were secreted at the highest level in the previous section, namely TCRV-IgC with VαH+VβL.

For 4B2A1, the highest protein production was obtained when the wt α-chain was co-expressed with a β-chain that had acquired three mutations, namely G17E, H47Y, and L80S. As shown in Figure 3A, the mutant fusion protein, denoted 4Vmut, was secreted thirteen times better than the wt. For 7A10B2, the selected combination was L43P and W82R in Vα together with wt Vβ. The protein is denoted 7Vmut, and production was increased seventeen fold (Figure 3A). Thus, amino acid replacements selected to improve the thermodynamic properties of 2C, also improved secretion of 4B2A1 as well as 7A10B2.

Folding was assessed in ELISA with a panel of anti-TCR mAbs as coat (Table 1). GB113, which is specific for 4B2A1 [44], and F23.2 [45], for which binding is conformation dependent and specific for the Vβ8.2 segment, were used for 4B2A1 [46]. 44-22-1 [47] and RR4-7 [48], both of which recognize the Vβ6 segment, were used for 7A10B2. The two 4B2A1 specific reagents do not recognize 7A10B2 and vice versa. In addition, both receptors were tested for binding to an anti-Cα antibody, H57, which does not recognize this format which lacks Cβ (TCRV-IgC). Importantly, none of the TCRV-IgC molecules were recognized by the anti-TCR Abs (Figure 3B and 3C). Thus, even though the introduced mutations increased secretion, correct folding was not achieved. We therefore turned to other TCR fusion formats.

**Stability engineering of C regions improves folding and secretion**

Comparing the cTCR-clg molecules (Figure 1B) for secretion from HEK293E cells (Figure 2), both 4B2A1 derived molecule were secreted at medium levels. Molecules with complete α chain fused to the light chain and complete β chain fused to the heavy chain (αL + βH) showed the best secretion.

The selected V region mutations were then introduced in molecules of the cTCR-clg format derived from both segments. While 4B2A1 shares the Vβ8.2 segment, 7A10B2 shares Vα3.1 with 2C. Thus, when the V(D)J encoded complete V domain sequences are considered, 7B2A1 exhibits high homology to 2C Vα (86.8% identity and 88.6% similarity), while 4B2A1 exhibits high homology to 2C Vβ (89.5% identity and 92.1% similarity).
4B2A1 and 7A10B2 (denoted 4 mut and 7 mut), the molecules produced in HEK293E cells and secretion levels measured by ELISA as above. For 4 mut (4B2A1 cTCR-cIg), secretion doubled, whereas no change was observed for 7 mut (Figure 4A). Subsequently, the TCR C regions were modified by the introduction of a disulfide bridge between the two C domains (mTRAC T48C and mTRBC S57C) and by replacing an unpaired cysteine in the β-chain with alanine, as previously described [29].

This was done in wt as well as in V region mutated molecules denoted 4wtSS, 4mutSS a.s.o. For 4wtSS, the introduced disulfide bridge did not alter the secretion level. However, when C region mutations were combined with stabilizing V region mutations (4mutSS), protein production was improved five-fold (Figure 4B). For 7wtSS, secretion increased two fold. There was no additive effect of V and C region mutations for the 7A10B2 derived molecule.

Folding was assessed in ELISA using anti-TCR Abs. Both 4 mut and 4 mutSS were well recognized by all relevant antibodies (Figure 4C). For the corresponding 7A10B2 derived receptor (Figure 4D), the V region mutations alone did not improve recognition. Introduction of the disulfide bridge, however, did. The TCR Cβ region specific mAb, H57, bound both 4B2A1 and 7A10B2 derived molecules with the same efficiency as the V region specific mAbs.

Two fusion molecules that were well recognized by TCR-specific antibodies, namely 4 mutSS and 7 wtSS, were secreted at 0.6 and 0.1 μg/ml, respectively (Figure 4B). Using the NS0 myeloma cell line, the production rate was lower, and the supernatants of stably transfected cells regularly contained approximately 1/10 the amount of fusion protein as that of the HEK 293E supernatants (data not shown). In comparison, IgGs can be obtained from HEK293E cells at 10 μg/ml 2 days after transient transfection. This prompted us to investigate whether production of TCR-Igs could be increased in insect cells.

The fusion proteins are secreted from insect cells

Initially, we compared mammalian and insect cell production of the TCRV-IgC format. The complete fusion genes were transferred from pLNOH2 and pLNO/C20 to pAc/C20-Fc, which is designed for Ig production using baculoviral and insect cell infection. The Fc encoding gene originally in the vector was deleted. Both 4B2A1 and 7A10B2 receptors were tested. We found that the secretion levels doubled in Sf9 insect cells compared to HEK 293E cells (Figure 5A). The effect of V region mutations was less prominent, however, and again, the molecules were not recognized by anti-TCR Abs (results not shown).

We then introduced the cTCR encoding genes upstream of the Fc region encoded in the pAc-κ-Fc vector. The 4B2A1 receptor, with or without V region mutations as well as with or without C region mutations, was tested, and a total of four different molecules were included in the experiment. In all, the 4B2A1 α-

Table 1 TCR-specific Abs.

| Ab clone | Specificity | T cell clone | References |
|----------|-------------|--------------|------------|
| F23.2    | Murine TRBV13-2 | 4B2A1       | [45,46]    |
| H57-597  | Murine Gp | 4B2A1 + 7A10B2 | [64]       |
| GB113    | TCR 4B2A1 | 4B2A1       | [44]       |
| 44-22-1  | Murine TRBV19 | 7A10B2     | [47,63]    |
| RR4-7    | Murine TRBV19 | 7A10B2     | [48]       |
chain was fused to the Fc region, whereas the β-chain was not part of a fusion. In general, the secretion levels were decreased relative to those obtained in mammalian cells (Figure 5B). As for the corresponding proteins produced in HEK 293E cells, both V and C region mutations increased anti-TCR Ab recognition, and the effects were additive (Figure 5C). In conclusion, correctly folded fusion molecules were obtained in insect cells after introduction of V and C region mutations, and the production levels were the same as that obtained in mammalian cells 2 days after transient transfection.

**The stability engineered fusion proteins bind specifically to pMHC on cells**

The molecules 4 Vmut and 7 Vwt (TCRV-IgC) as well as 4 mutSS and 7 wtSS (cTCR-cIg) were tested for binding to A20 cells in flow cytometry. A20 is a B cell lymphoma line that expresses the I-E^d^ molecule. We loaded the cells with synthetic λ2^315^ peptide and found that 4 mutSS and 7 wtSS bound (Figure 6), whereas 4 Vmut and 7 Vmut, neither of which bound anti-TCR Abs, did not (data not shown). For both 4 mutSS and 7 wtSS, binding was clearly peptide-specific, as pMHC was detected only after addition of peptide. The concentration of fusion protein used, was 100 μg/ml for 4 mutSS and 17 μg/ml for 7 wtSS, respectively. When reducing the concentration of 4 mutSS to 17 μg/ml, the staining intensity was comparable to that of 7A10B2 (data not shown). Binding was also compared to that of a recombinant isotype-matched mAb with I-E-specificity [49]. For both TCR fusion proteins, staining intensities were comparable to those of the mAb (Figure 6).
cells are A20 transfected with λ2315. We have previously demonstrated that F9 presents the λ2315 peptide on I-Ed and induces proliferation of specific T cells [50].

We tested whether the four molecules could distinguish between A20 and F9. Binding was not observed to either without addition of exogenous peptide. This shows that the reagent requires further affinity maturation to detect physiological concentrations of agonist peptide.

Discussion
Fusion to Ig might facilitate expression, purification, as well as recognition of soluble TCRs bound to target pMHC. Furthermore, fusion of one TCR onto each “arm” of the Ig molecule ensures TCR dimer formation in the final TCR-Ig molecule, and consequently increased pMHC binding strength due to avidity effects.

Recent advances in TCR engineering include the identification of stabilizing mutations in both V- and C regions. We therefore investigated how such engineering affected production and ligand binding properties of two well characterized TCRs fused to Ig.

The best domain pairing when the TCR V regions substitute the Ab V regions in the TCRV-IgC format, Vβ(CH)/Vα(CL) or Vα(CH)/Vβ(CL), may not easily be predicted, since the TCR Cα domain deviates from the Ig fold and has no clear homology with any of the Ig C domains [51]. We found Vα(CH)/Vβ(CL) to be secreted at the highest level when the TCRV-IgC format was investigated for production yield.

The TCR V regions were then optimized by amino acid replacements introduced into this TCRV-IgC fusion format, as shown in Figure 7A for Vα and 7B for Vβ. TCR 2C and 7A10B2 share Vα segment, and the two Vα replacements that were beneficiary for 7A10B2 were
first characterized in the context of a scTCR version of the 2C TCR. For 4B2A1Vα, the wt sequence was selected, which already contains one of these. 4B2A1 and 2C share Vβ segment, and all three Vβ replacements previously selected in the context of 2C were also selected for 4B2A1. 7A10B2 utilizes another Vβ gene segment, and again, this already contains the beneficiary amino acids.

The effect of the V domain mutations may be studied at the atomic level in the crystal structure of a mutated scTCR version of the 2C TCR (2C-T7) [52] and was recently thoroughly analyzed by Richman et al. [53]. Figure 7C shows a ribbon representation of 2C TCR with the five mutated positions highlighted. The Vβ segment was clearly well stabilized by the mutations, while the Vα segment was not. This emphasizes the need for further stability engineering. Such engineering by yeast surface display has been reported for several TCRs of both mouse and human origin [24,54]. The widely versatile phage display technology could develop the engineering beyond its present state by selecting for features such as resistance to aggregation after acid or heat exposure as described for antibodies [55,56]. Following initial selection, pools of V domains may be transferred from the displayed scTCR format into a TCR-Ig format by the method described here.

Folding was analyzed employing a panel of anti-TCR Abs with V region specificity, and the results strongly suggested that all detected TCRs were correctly folded. In addition, the C region specific Ab H57 bound exactly the same molecules as the Abs with V region specificity. Thus, correct folding of the C domain could be used as an indicator of correct V domain folding, while erroneous V domain folding, when detected, was never a local event, but also affected the C domain. Secretion levels from producing cells were not a good indicator of

Figure 7 Structural alignment of the V domains from the 4B2A1, 7B2A1 and 2C TCRs. Alignments of the Vα (A) and Vβ (B) were made with MUSCLE [67] and annotated according to Hare et al. [68]. Amino acid positions mutated are indicated by colour code according to the source, orange [23] and green [24], and the resulting residues are indicated at each position. 4B2A1 was originally misaligned regarding α chain residue 82. Ribbon representation of the 2C crystal structure (PDB ID: 1TCR) with the positions of the five mutations selected in the 4Vmut and 7Vmut variants, as given by the alignments in A and B (shown as spheres). The figure was prepared using PyMol (C).
correct folding, however. The TCRV-IgC format with TCR V regions and antibody C regions were well secreted, but not properly folded and this was true for both 4B2A1 and 7A10B2. The finding is not dependent on the nature of the eukaryotic production system, as the same observations were made whether the molecules were secreted from mammalian cells or from insect cells.

Native-like fold was readily reached upon reconstitution of the TCR V-C interphase for 4B2A1. This underscores the importance of this interphase whenever C domains are present in TCRs produced in eukaryotic cells. This also presented the opportunity for introduction of a disulfide bridge linking $\alpha_\text{V}$ and $\beta_\text{V}$. The corresponding bridge was first introduced with a positive effect on stability of human TCRs produced in *E. coli* [53]. Here we demonstrate that the bridge improved secretion of correctly folded cTCR-clg molecules two (7A10B2) and five (4B2A1) fold, respectively, for murine TCRs. The V-C domain interphase analysis by Richman *et al.* [53] points to important differences in solvent exposure of the V domains in Abs and TCRs. In the native 2C TCR, $V_aW82$ is buried in the V-C interphase, but is exchanged with a hydrophilic residue in the exposed scTCR version (W82R). This hydrophilic substitution also appeared beneficial in 7Amut selected in the TCRV-IgC format which has the V-C interphase of an Ab. In the cTCR-clg format, however, the selected W82R mutation appeared counterproductive.

The fusion proteins were produced in different eukaryotic cells that secrete large proteins in a functional form, with disulfide bridges and glycosylation. HEK 293E cells were then chosen for large scale production and functional testing. The vectors used contain the oriP sequence that supports increased protein production and handling of cellular parts of TCRs on the other hand, are difficult to produce and handle as recombinant soluble molecules, due to low intrinsic stability. Recent advances in TCR engineering include the identification of stabilizing mutations in both V- and C regions. We therefore investigated how such engineering affected production of stable recombinant Ig molecules is well established in a number of systems, and a large panel of reagents for detection of Ig exists. The extracellular parts of TCRs on the other hand, are difficult to produce and handle as recombinant soluble molecules, due to low intrinsic stability. Recent advances in TCR engineering include the identification of stabilizing mutations in both V- and C regions. We therefore investigated how such engineering affected production of stable recombinant Ig molecules.

Specific binding to pMHC on cells was verified by flow cytometry after addition of the specific $\lambda_2^{2315}$ peptide. This underscores that the reagents produced are correctly folded and retained specificity, which was the major goal in this study. The reagents, like soluble TCR tetramers, will be very useful for the detection of kinetic stability of complexes of peptide and MHC [58]. Importantly, a difference in peptide-MHC stability was recently found to be related to autoimmune disease susceptibility [59].

B lymphoma cells that had been transfected with the $\lambda_2^{315}$ gene were not detected. We have previously found that this particular transfectant stimulates cloned [50,60] or TCR-transgenic versions [61] of $\lambda_2^{315}$-specific T cells. A likely explanation is that the T cell based readout, with aggregation of pMHCII/TCR in immunological synapses, is rather more sensitive than binding of soluble TCRs as detected in flow cytometry. In addition, coreceptors and costimulatory molecules on T cells do indeed contribute to the former - but not the latter - assay. In previous *in vitro* experiments, we found that soluble 4B2A1 TCRs displayed polyvalently on phage at 3-5 copies per particle bound A20 loaded with the same specific $\lambda_2^{2315}$ derived peptide as that used here, but not F9 [29]. Thus, increasing avidity beyond the dimeric Ig was not sufficient to detect physiological concentrations of pMHC. It is well known that the intrinsic affinity of a TCR for its cognate pMHC is most often in the lower micromolar range. The affinities of Fabs are also low in the primary humoral response. Ab binding to antigen occurs due to polymerization to pentamers (in reality 10 binding sites) in IgM in the primary response or affinity maturation in the secondary response. In the case of antigen presentation, the specific pMHC levels are so low that avidity is not going to operate. Thus, affinity maturation will be necessary to increase the sensitivity of the soluble TCR-Ig fusions to make them useful probes for physiological presentation of pMHC in *in vitro* and *in vivo*.

**Conclusions**

Manufacturing of stable recombinant Ig molecules is well established in a number of systems, and a large panel of reagents for detection of Ig exists. The extracellular parts of TCRs on the other hand, are difficult to produce and handle as recombinant soluble molecules, due to low intrinsic stability. Recent advances in TCR engineering include the identification of stabilizing mutations in both V- and C regions. We therefore investigated how such engineering affected production and ligand binding properties of two well characterized TCRs fused to Ig. Without engineering, the molecules were secreted at very low levels from different eukaryotic cells. However, improving the thermodynamic properties by TCR V region mutagenesis and the introduction of a disulfide bridge between the TCR C domains greatly improved yields. Most significantly, the engineered molecules bound specifically to pMHC on cells. The reagents will be very useful for the detection of kinetic stability of complexes of peptide and MHC.

**Methods**

**Cells and antibodies**
The 4B2A1 and 7A10B2 T cell clones have previously been described [43,62]. Both recognize amino acids 91-101 from the $\lambda_2$ light chain of Ab produced by the
MOPC315 plasmyctoma (λ<sup>2</sup>) when presented on the MHC class II molecule I<sub>E<sup>d</sup></sub>. The official IMGT (http://imgt.cines.fr/) gene segment nomenclature is used throughout. Thus, the following TCR gene segment re-designation of the murine T cell clones 4B2A1 [V<sub>α1</sub>, J<sub>α19</sub>/V<sub>β8.2</sub>, D<sub>β1</sub>, J<sub>β1.2</sub>] and 7A10B2 [V<sub>α3</sub>, J<sub>α11</sub>/V<sub>β6</sub>, D<sub>β0</sub>, J<sub>β0.11</sub>] is [TRA7V7D-3*01, TRAJ40*01/ TRBV13-2*01, TRBD1*01, TRBJ1-2*01] and [TRA7V9-3*01, TRAJ58*01/ TRBV19*01, TRBD1*01, TRBJ1-1*01], respectively.

S9, A20 and HEK 293E cells were from ATCC (Manassas, VA). F9 is A20 transfected with the λ<sup>2</sup> light chain gene [50]. IgG-specific Abs used in ELISA where from Sigma-Aldrich (St. Louis, MO): two anti-human IgG<sub>3</sub> (h IgG<sub>3</sub>) mAbs (HP6047 and HP6050), the latter being biotinylated, goat anti-hlgG Ab (I2136), as well as HRP-conjugated goat anti-hlgG Ab (A9544). TCR-specific mAbs were GB113 [44] (clonotype-specific for 4B2A1), F23.2 [45,46] (recognizes TRBV13-2), 44-22-1 [47,63] (recognizes TRBV19), RR4-7 [48] (recognizes TRBV19), and H57 [64] (recognizes murine C<sub>β</sub>). F23.2, H57, 44-22-1 were kind gifts from Dr. Uwe D. Staerz (Department of Medicine, National Jewish Medical and Research Center, Denver, USA), Dr. Ralph T. Kubo (Cytel Corporation, San Diego, USA), and Dr. Hans Hengartner (Institute for Experimental Immunology, University Hospital Zurich, Zurich, Switzerland), respectively. RR4-7 was purchased from BD Pharmingen (San Diego, CA, USA). Abs used for flow cytometry were recombinant anti NIP- or I-Ed MHC class II molecule I-Ed.

Experimental Immunology, University Hospital Zurich, Switzerland, respectively. RR4-7 was purchased from BD Pharmingen (San Diego, CA, USA). Abs used for flow cytometry were recombinant anti NIP- or I-Ed MHC class II molecule I-Ed.

**Generation of TCR-Ig fusion constructs**

Cloning of TCR V genes and fusion to the C region of h IgG<sub>3</sub> (TCRV-IgC in Figure 1A) has been described previously [57] as has cloning of complete TCR ectodomains (cTCR) and fusion to complete hlgG<sub>3</sub> (cTCR-clg in Figure 1B) [29]. In short, TCR α- and β-chain genes (V or V + C) were PCR amplified from 4B2A1 and 7A10B2 cDNA. PCR primers had restriction sites for cloning of TCR genes into the pLNOH2 and pLNO<sub>α</sub> vector systems (66). The TCR V genes were introduced upstream from the C<sub>β</sub> C region in pLNOH2 and the C<sub>α</sub> C region in pLNO<sub>α</sub>, whereas the complete TCRs were cloned upstream from a complete hlgG<sub>3</sub> heavy chain in pLNOH2 and a complete α light chain in pLNO<sub>α</sub>. For both formats and TCRs, TCRα was fused to either Ig heavy or light chain, as was TCRβ. In the case of the cTCR-clg constructs, the primers were designed to introduce a segment encoding a GS-linker of six amino acids between the TCRs and the Ig.

For in vitro mutagenesis and selection of mutants

Introduction of a disulfide bridge between the murine TCR C domains and replacement of an unpaired β-chain cysteine, have been described previously [29]. All other in vitro mutagenesis reactions were performed using the QuikChange Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA). Mutagenesis with multiple sets of primers was performed using the same protocol as for single site mutations, except for the presence of more than one primer set in each tube. The mutations were S82R in 4B2A1 V<sub>α</sub>; G17E, H47Y, and L80S in 4B2A1 V<sub>β</sub>; L43P and W82R in 7A10B2 V<sub>α</sub> and Q17E in 7A10B2 V<sub>β</sub>. The primers are listed in Additional file 1.

For selection, up to five culture aliquots were combined in pools, from which plasmid DNA were isolated. HEK 293E cells were then transiently transfected (2.4) with these plasmid preparations such that all possible combinations of pooled DNA for the α- and β-chains were tested. Protein production after each transfection was measured using the hlgG-specific ELISA (2.7), and wells with increased TCRV-IgC production identified. Plasmid DNA preparations were then made from the cultures initially pooled, and these individual DNA
preparations again combined in a new HEK 293E cell transfection to finally detect the best pair.

Transfection of HEK 293E cells
HEK 293E cells were transfected using Lipofectamine 2000 transfection reagent from Invitrogen (Carlsbad, CA) essentially as described [57]. For small scale testing, cells were seeded in 24 well plates at 2 × 10^5 cells/well, and supernatants were tested three days later. For larger scale protein production, cells were seeded in 750 ml bottles at 2 × 10^7 cells/bottle. In these, medium was harvested and replaced with fresh medium every 2-3 days for three weeks.

Baculovirus production and infection of insect cells
Generation of recombinant baculovirus and infection of SF9 insect cells was performed using the BaculoGold Transfection Kit from BD Biosciences (San Diego, CA). Briefly, samples of pAc vectors with TCR-Ig genes and baculovirus DNA were co-transfected into SF9 cells to generate recombinant baculovirus carrying TCR-Ig genes. After the initial transfection, virus titers were increased by three repeated SF9 cell infections.

Protein purification
TCR-Ig fusion proteins were purified from transfected HEK 293E cell supernatants. Dead cells were removed by centrifugation, and the supernatant filtered through a 0.45 μm filter and run on a Protein G Sepharose 4 Fast Flow column from GE Healthcare (Uppsala, Sweden). Bound protein was eluted with Tris-HCl, pH 2.7, and the pH in each 1 ml fraction rapidly neutralized with 40 μl Tris-HCl pH 9. Fractions with TCR-Ig (as determined in ELISA) were concentrated on Amicon Centrifugal Filter Devices with MWCO 100 000 from Millipore (Billerica, MA). After a 40-70-fold concentration, four rounds of PBS were added to and spun through the filter column to replace the elution buffer.

Quantification of TCR-Ig fusion protein
A hlgG3-specific ELISA used to quantify TCR-Ig fusion protein has been described previously [57]. Briefly, wells were coated with mouse anti-hlgG3 (clone HP6047), and TCR-Ig fusion protein added. Biotinylated mouse anti-hlgG3 (clone HP6050) followed by streptavidin-coupled alkaline phosphatase (ALP) was used for detection. TCR-Ig fusion proteins on hlgG1 Fc were quantified with goat anti-hlgG Fc (at 1:1000) as coat and ALP-conjugated goat anti-hlgG (1:2000) as detection reagent.

TCR-Ig binding to TCR specific antibodies
For TCR-specific ELISAs, wells were coated with 3 μg/ml anti-TCR Ab (GB113, F23.2, 44-22-1, RR4-7 or H57) before addition of TCR-Ig fusion proteins. Detection was with ALP-conjugated goat anti-hlgG (1:5000). All ELISAs were developed using phosphatase substrate dissolved in diethanolamine buffer, pH 9.8. Streptavidin-ALP was from Amersham Biosciences (Uppsala, Sweden) whereas phosphatase substrate tablets were from Sigma-Aldrich.

Flow cytometry
A20 and F9 cells were incubated ON with synthetic λ2315 peptide (amino acid 89-107) from Biopeptide Co (San Diego, CA) at a final concentration of 50 μM. Then, additional peptide was added to a final concentration of 100 μM. After two hours, the cells were stained with TCR-Ig (at 17 or 100 μg/ml) or control Abs, followed by PE conjugated goat anti-hlgG F(ab)2 at 7.5 μg/ml. Recombinant I-E- and NIP-specific hlgG3 were positive and negative isotype-matched controls, respectively. 30-50 000 cells were run on a FACSCalibur flow cytometer from Becton Dickinson (Mountain View, CA) and analyzed.

Additional material

**Additional file 1:** Primers for cloning into pAc-κ-Fc

**Additional file 2:** Primers for V region mutagenesis

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Authors’ contributions
EL participated in the study design, performed all the experimental work and drafted the manuscript. GÅL participated in the SS bridge design, result interpretation and helped drafting the manuscript. BB participated in the study design and provided key reagents. IS conceived the project, organized funding, supervised the study and finalized the manuscript. All authors read and approved the final manuscript.

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References
1. Davenport MP, Fazou C, McMichael AJ, Callan MF: Clonal selection, clonal senescence, and clonal succession: the evolution of the T cell response to infection with a persistent virus. J Immunol 2002, 168(7):3309-3317.
2. O’Herrin SM, Slansky JE, Tang Q, Markiewicz MA, Gajewski TF, Pardoll DM, Schneck JP, Bluestone JA: Antigen-specific blockade of T cells in vivo using dimeric MHC peptide. J Immunol 2001, 167(5):2555-2560.
3. Devire M, Gupta K, Paulaitis ME, Schneck JP: Detection of antigen-specific T cells on pMHC microarrays. J Mol Recognit 2007, 20(1):32-38.
4. Murphy DB, Lo D, Rath S, Brinster RL, Flavell RA, Slanetz A, Janeway CA Jr: A novel MHC class II epitope expressed in thymic medulla but not cortex. Nature 1989, 338(6218):765-768.
Stable, soluble, high-affinity, engineered T cell receptors: novel reagents for clinical cancer immunology and immunotherapy. Expert Rev Anticancer Ther. 2005, 5(5):523-536.

12. Boulter JM, Jakobsen BK. Stable, soluble, high-affinity, engineered T cell receptors: novel antibody-like proteins for specific targeting of peptide antigens. Clin Exp Immunol 2005, 142(3):454-460.

13. Gregoire C, Lin ST, Mazza G, Rebai N, Luescher IF, Malissen B. Covalent assembly of a soluble T cell receptor-peptide-major histocompatibility class I complex. J Immunol 2006, 176(5):2807-2812.

14. Chang HC, Bao Z, Yao Y, Tse AG, Goyarts EC, Madsen M, Kawai K, Brauer PA, Sacchettini JC, Nathenson SG, et al. A general method for facilitating heterodimeric pairing between two proteins: application to expression of alpha and beta T-cell receptor extracellular segments. J Biol Chem 2005, 280(3):1882-1892.

15. Kappler J, White J, Kozono H, van der Bruggen P, Clemants J, Marrack P. Binding of a soluble alpha beta T-cell receptor to superantigen/major histocompatibility complex ligands. Proc Natl Acad Sci USA 1994, 91(14):6108-6112.

16. Chung S, Wucherpfennig KW, Friedman SM, Haffer DA, Strominger JL. Functional three-domain single-chain T-cell receptors. Proc Natl Acad Sci USA 1994, 91(26):12690-12695.

17. Zhu X, Belmont HJ, Price-Schiavi S, Liu B, Lee HI, Fernandez M, Wong RL. Characterization and quantitation of peptide-MHC complexes produced from hen egg lysozyme using a monoclonal antibody. Immunol 1997, 66(6):727-738.

18. Shusta EV, Holler PD, Kranz DM, Wittrup KD. Mimotopes for allorative and conventional T cells in a peptide-MHC display library. Proc Natl Acad Sci USA 2004, 101(9):3051-3056.

19. Davis SJ, Ikemizu S, Evans EJ, Fugger L, Bakker TR, van der Merwe PA. The nature of molecular recognition by T cells. Nature immunology 2003, 4(3):217-224.

20. Crawford F, Huseby E, White J, Marrack P, Kappler JW. Mimotopes for allorative and conventional T cells in a peptide-MHC display library. Proc Natl Acad Sci USA 2004, 101(9):3051-3056.

21. Davis SJ, Ikemizu S, Evans EJ, Fugger L, Bakker TR, van der Merwe PA. The nature of molecular recognition by T cells. Nature immunology 2003, 4(3):217-224.

22. Davis SJ, Ikemizu S, Evans EJ, Fugger L, Bakker TR, van der Merwe PA. The nature of molecular recognition by T cells. Nature immunology 2003, 4(3):217-224.
45. Staerz UD, Rammeensoe HG, Benedetto JD, Bevan MJ. Characterization of a murine monoclonal antibody specific for an alphabeta T cell antigen receptor. J Immunol 1985, 134(6):3994-4000.

46. Manning TC, Schlueter CJ, Brodnicki TC, Parke EA, Speir JA, Garcia KC, Teyton L, Wilson JA, Kranz DM. Alkaline scanning mutagenesis of an alphabeta T cell receptor: mapping the energy of antigen recognition. Immunity 1998, 8(4):413-425.

47. Acha-Orbea H, Zinkernagel RM, Hengartner H. Cytoxic T cell clone-specific monoclonal antibodies used to select clonotypic antigen-specific cytoxic T cells. Eur J Immunol 1985, 15(11):31-36.

48. Kanagawa O, Palmer E, Bill J. The T cell receptor V beta 6 domain imparts reactivity to the Mls-1a antigen. Cell Immunol 1989, 119(2):412-426.

49. Lunde E, Western KH, Rasmussen IB, Sandlie I, Bogen B. Efficient delivery of T-cell epitopes to APC by use of MHC class II-specific Troybodies. Journal of Immunology 2002, 168(5):2154-2162.

50. Weiss S, Bogen B. 8-lymphoma cells process and present their endogenous immunoglobulin to major histocompatibility complex-restricted T cells. Proc Natl Acad Sci USA 1989, 86:382-386.

51. Halaby DM, Poupon A, Momon J. The immunoglobulin fold family: sequence analysis and 3D structure comparisons. Protein Eng 1999, 12(7):563-571.

52. Coll LA, Bankovich AJ, Hanick NA, Bowerman NA, Jones LL, Kranz DM, Garcia KC. How a single T cell receptor recognizes both self and foreign MHC. Cell 2007, 129(1):135-146.

53. Richman SA, Aggen DH, Dossett ML, Donemeyer DL, Allen PM, Greenberg PD, Kranz DM. Structural features of T cell receptor variable regions that enhance domain stability and enable expression as single-chain ValphaBeta fragments. Molecular Immunology 2008.

54. Weber KS, Donemeyer DL, Allen PM, Kranz DM. Class II-restricted T cell receptor engineered in vitro for higher affinity retains peptide specificity and function. Proc Natl Acad Sci USA 2005, 102(52):19033-19038.

55. Jespers L, Schon O, Farm K, Winter G. Aggregation-resistant domain antibodies selected on phage by heat denaturation. Nat Biotechnol 2004, 22(9):1161-1165.

56. Christ D, Farm K, Winter G. Repertoires of aggregation-resistant human antibody domains. Protein Eng Des Sel 2007, 20(8):413-416.

57. Berntsen G, Lunde E, Flobak M, Andersen JT, Laurvak V, Sandlie I. Prolonged and increased expression of soluble Fc receptors, IgG and a TCR-Ig fusion protein by transiently transfected adherent 293E cells. J Immunol Methods 2005, 298(1-2):93-104.

58. Bowerman NA, Crafts TS, Chlewicki L, Do P, Baker BM, Christopher Garcia K, Kranz DM. Engineering the binding properties of the T cell receptor: peptidemHC ternary complex that governs T cell activity. Molecular Immunology 2009, 46(15):3000-3008.

59. Fallang LE, Bergseng E, Hotta K, Berg-Larsen A, Kim CY, Sollid LM. Differences in the risk of celiac disease associated with HLA-DQ2.2 or HLA-DQ2.5 are related to sustained gluten antigen presentation. Nature Immunology 2009, 10(10):1096-1101.

60. Weiss S, Bogen B. MHC class II-restricted presentation of intracellular antigen. Cell 1991, 64:767-776.

61. Launtenzen GF, Weiss S, Dembic Z, Bogen B. Naive idiotyp-specific CD4 + T cells and immunosurveillance of B-cell tumors. Proc Natl Acad Sci USA 1994, 91:5700-5704.

62. Snodgrass HB, Fisher AM, Bruyns E, Bogen B. Restricted alpha/beta receptor gene usage of idiotyp-specific major histocompatibility complex-restricted T cells: selection for CDR3-related sequences. European Journal of Immunology 1992, 22:169-2172.

63. Payne J, Huber BT, Cannon NA, Schneider R, Schilham MW, Acha-Orbea H, MacDonald HB, Hengartner H. Two monoclonal rat antibodies with specificity for the beta-chain variable region V beta 6 of the murine T-cell receptor. Proc Natl Acad Sci USA 1998, 85(20):7695-7698.

64. Kubo RT, Born W, Kappler JW, Marrack P, Pigeon M. Characterization of a monoclonal antibody which detects all murine alpha beta T cell receptors. Journal of Immunology 1989, 142(8):2736-2742.

65. Lunde E, Bogen B, Sandlie I. Immunoglobulin as a vehicle for foreign antigenic peptides immunogenic to T cells. Mol Immunol 1997, 34:1167-1176.

66. Norderhaug L, Olafsen T, Michaelesen TE, Sandlie I. Versatile vectors for transient and stable expression of recombinant antibody molecules in mammalian cells. Journal of Immunological Methods 1997, 204:77-87. 67. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 2004, 32(5):1792-1797.

68. Hare BJ, Wyss DF, Osborne MS, Kern PS, Reinherz EL, Wagner G. Structure, specificity and CDR mobility of a class II restricted single-chain T-cell receptor. Nat Struct Biol 1999, 6(6):574-581.