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CD8αα and -αβ isotypes are equally recruited to the immunological synapse through their ability to bind to MHC class I

Vasily Rybakin[*], *Jean-Pierre Clamme*[*†], *Jeanette Ampudia, Pia P. Yachi*[‡] *& Nicholas R.J. Gascoigne*[+]
Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, California, USA

Bimolecular fluorescence complementation was used to engineer CD8 molecules so that CD8αα and CD8αβ dimers can be independently visualized on the surface of a T cell during antigen recognition. Using this approach, we show that CD8αα is recruited to the immunological synapse almost as well as CD8αβ, but because the kinase Lck associates preferentially with CD8αβ in lipid rafts, CD8αα is the weaker co-receptor. During recognition of the strong CD8αα ligand H2-TL, CD8αα is preferentially recruited. Thus, recruitment of the two CD8 species correlates with their relative binding to the available ligands, rather than with the co-receptor functions of the CD8 species.

Keywords: bimolecular fluorescence complementation; co-receptor; T-cell activation
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INTRODUCTION

CD8 is the co-receptor for major histocompatibility complex class I (MHC-I)-restricted recognition of antigen. It binds directly to non-polymorphic regions of the MHC-I heavy chain. Through its cytoplasmic tail, CD8 interacts with the Src-family protein tyrosine kinase Lck (Veillette *et al*, 1988; Barber *et al*, 1989). Antigen recognition by the T-cell receptor (TCR) brings CD8 and therefore Lck into close proximity with the TCR (Zamoyska, 1998; Yachi *et al*, 2005), allowing Lck to phosphorylate elements of the TCR–CD3 complex and to precipitate the signalling cascade. CD8 exists on the T-cell surface in two isoforms, CD8αα and CD8αβ. CD8αβ is expressed by mature T cells and developing thymocytes, whereas CD8αα is expressed in certain subsets of T cells, including TCRαβ-expressing gut intraepithelial cells, some γδ T cells and some natural killer and dendritic cells. Both CD8 dimers are expressed on CD8αβ$^+$ intraepithelial cells, and there is evidence for transient upregulation of CD8αα on activated CD8αβ$^+$ T cells (reviewed by Cheroutre & Lambolez, 2008).

CD8αβ is a much stronger co-receptor than CD8αα, the expression of CD8β enhancing TCR sensitivity about 100-fold over cells expressing only CD8α (Karaki *et al*, 1992; Renard *et al*, 1996; Yachi *et al*, 2005), and indeed CD8αα might be a negative regulator of T-cell activation (Cheroutre & Lambolez, 2008). CD8β is reported to mediate interactions between the CD8 and the TCR–CD3 complex (Doucey *et al*, 2003). Because the affinities of the two isoforms for MHC-I molecules are similar (Garcia *et al*, 1996; Kern *et al*, 1999; Wang *et al*, 2009), and because Lck interacts with the cytoplasmic domain of CD8α, the likely mechanism for this difference in activity is the location of CD8αβ in membrane rafts. The CD8β molecule, similar to the other T-cell co-receptor CD4, but unlike CD8α, can be palmitoylated, resulting in its localization in lipid rafts (Arcaro *et al*, 2000, 2001). Lck is also targeted to rafts through palmitoylation and myristoylation (Rodgers *et al*, 1994), so that the presence of Lck and CD8αβ in lipid rafts provides both a motif and an opportunity for their interaction. CD8α also mediates interaction with another lipid raft resident molecule, LAT (Bosselut *et al*, 1999).

At present there are few or no data on the differential roles of CD8αα and CD8αβ during antigen recognition. The findings noted above indicate that CD8αβ plus lipid rafts should be strongly recruited to the immunological synapse (IS). On the other hand, if CD8αα and CD8αβ bind equally well to MHC-I, there is no *a priori* reason why CD8αα should not be recruited to the IS as strongly as CD8αβ, even without lipid rafts. Moreover, there is some doubt about the relevance of lipid rafts in the formation of the IS (Bunnell *et al*, 2002). For this reason, we decided to test the differences, if any, that are present in the molecular dynamics of CD8αα and αβ on the surface of T cells before and during stimulation.

Bimolecular fluorescence complementation (BiFC) allows identification of molecules that interact with each other in live cells. In this technique, the two proteins of interest are expressed as chimaeras with either the N-terminal or C-terminal half of a green fluorescent protein-related molecule. If the two proteins

Department of Immunology and Microbial Science, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037-1092, USA
*These authors contributed equally to this work
†Present address: Nitto Denko Technical, 501 Via Del Monte, Oceanside, California 92058, USA
‡Present address: Applied Molecular Evolution, 3520 Dunhill Street, San Diego, California 92121, USA
+Corresponding author. Tel: +1 858 784 9876; Fax: +1 858 784 8486;
E-mail: gascoigne@scripps.edu

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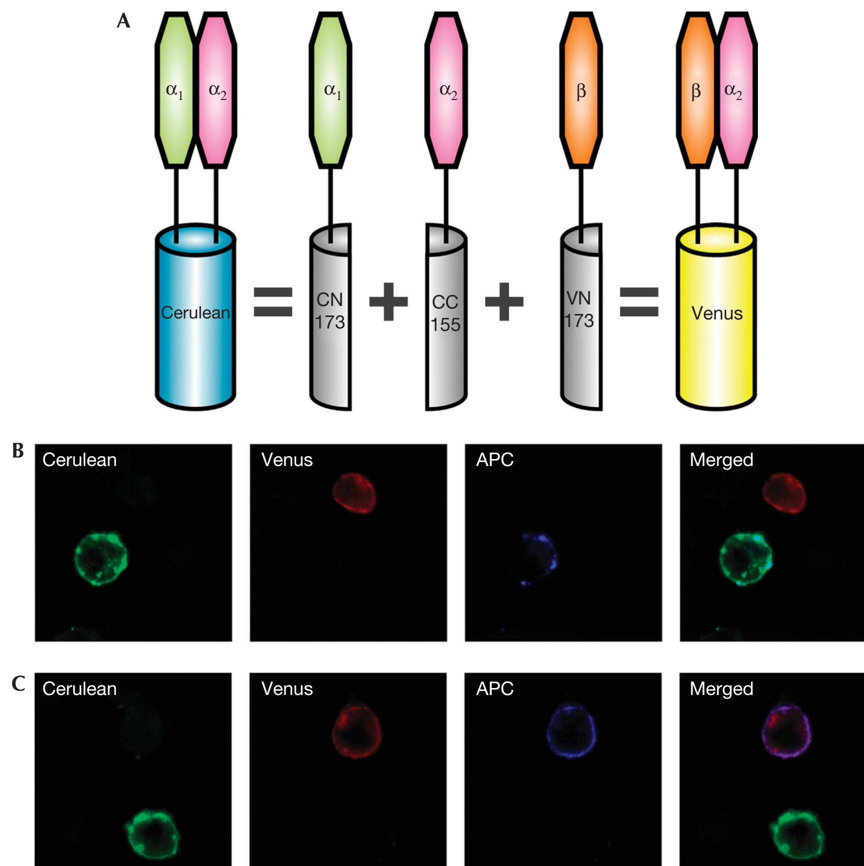

Fig 1 | Principle of the bimolecular fluorescence complementation (BiFC) assay and specific CD8α1 and CD8β detection. (**A**) CD8α2–CC155 forms CD8αα when expressed with CD8α1–CN173, or CD8αβ when expressed with CD8β–CN173. Note that non-fluorescent CD8αα or CD8αβ can also be formed from CD8α1 or CD8α2 homodimers, or from CD8α1–CD8β heterodimers. (**B,C**) Cells expressing only the fluorescent molecules CD8α2α1 (Cerulean: shown as green) or CD8α2β (Venus, shown as red) were mixed at a 1:1 ratio and stained with allophycocyanin (APC)–anti-CD8α1 (**B**) or APC–anti-CD8β (**C**). The antibody staining is shown in blue.

interact, the two halves of the fluorescent protein (FP) refold to form a complete FP (Hu *et al*, 2002; Hu & Kerppola, 2003). By using halves from different FPs, different spectra are obtained, allowing multicolour experiments to determine how and where one protein interacts with two different partners (Hu & Kerppola, 2003; Shyu *et al*, 2006). We adapted this technique to CD8, such that CD8αα and CD8αβ would be separately visible on the same cell. We then used fluorescence deconvolution microscopy to follow CD8αα and αβ dimers to investigate whether there is differential recruitment of the two forms of CD8 to the IS during antigen recognition.

RESULTS AND DISCUSSION

Expression of CD8αα-Cerulean and CD8αβ-Venus

We prepared constructs for BiFC to allow expression of CD8αα as a chimaera with the cyan (C)FP Cerulean and CD8αβ as a chimaera with the yellow FP Venus (Shyu *et al*, 2006). The strategy for expressing CD8 as different coloured chimaeric proteins is shown in Fig 1A. The CD8α attached to the C terminus of CFP (which folds with both Cerulean and Venus N termini) was the CD8α2 allele recognized by anti-Ly2.2 monoclonal antibody, whereas that attached to the N terminus of Cerulean was CD8α1 (epitope recognized by anti-Ly2.1). When stably expressed in the OT-I T-hybridoma line (Yachi *et al*, 2005), the CD8αα and CD8αβ fluoresced as predicted (Fig 1B,C). This is a new use of BiFC, which was originally developed to show the formation of dimers between different molecules (Hu *et al*, 2002; Hu & Kerppola, 2003).

The relative efficiency of formation of the two CD8 complexes was compared by flow cytometry, with expression of comparable amounts of fluorescent CD8αβ and CD8αα proteins, as well as the ovalbumin (OVA)-specific TCR (supplementary Fig S1 online). This does not take into account the formation of non-fluorescent CD8αβ and CD8αα dimers, which also occurs. The surface concentration of TCR was 7–9% of that of the mature T cells, similar to the difference between $CD4^+8^+$ and $CD4^+8^-$ or $CD4^-8^+$ thymocytes, or between $CD4^+8^+$ thymocytes and mature T cells (Crispe *et al*, 1987). Although the hybridomas stained ~2- to 3-fold brighter than primary T cells for CD8α1 and CD8β, the surface concentration of CD8 was 2- to 3.5-fold lower than the concentration on the mature T cells (supplementary Fig S1 online). We investigated the partitioning of the different molecules in membrane fractions on the basis of detergent solubility. As expected, CD8αα was primarily in the detergent-soluble non-raft fraction and CD8αβ primarily in the detergent-resistant raft fraction (supplementary Fig S2 online).

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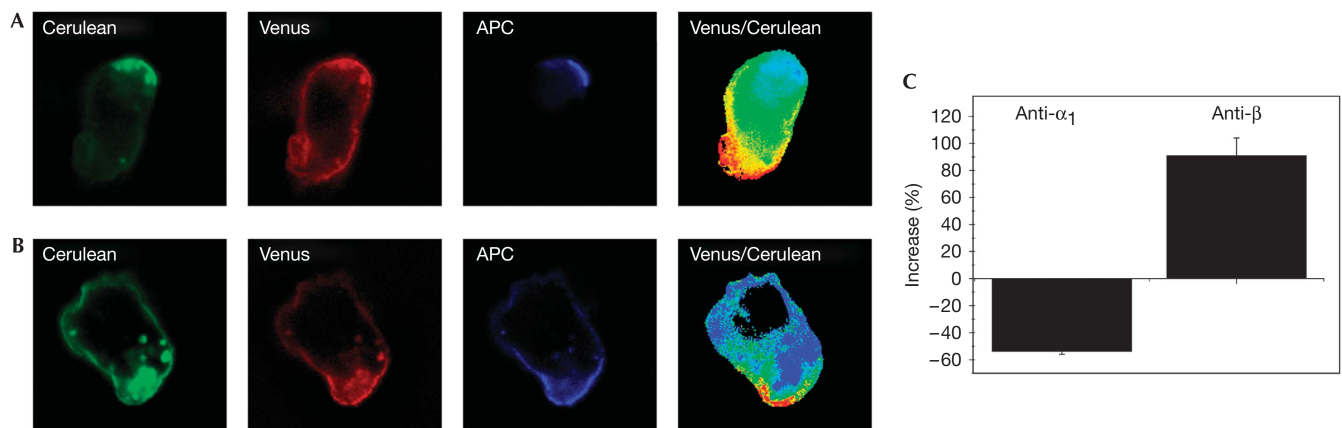

Fig 2 | Separate capping of CD8αα and CD8αβ. (**A,B**) Representative images of co-capping with anti-CD8α1 (anti-Ly2.1) (**A**) or anti-CD8β (**B**) monoclonal antibodies on cells expressing all three bimolecular fluorescence complementation (BiFC) constructs. On panels labelled Venus/Cerulean, the intensity ratio of Venus/Cerulean is represented using a high/low scale in which blue indicates a lower ratio value (more CD8α2α1) and red a higher ratio value (more CD8α2β). (**C**) Shows quantification of the differential capping of CD8α2α1 and CD8α2β as percentage of increase. Negative values reflect preferential recruitment of CD8α2α1 and positive values the preferential recruitment of CD8α2β. Error bars show s.e.m., $n = 16$ for anti-α1 and $n = 30$ for anti-β. APC, allophycocyanin.

Co-receptor-dependent TCR–MHC binding

We used MHC-peptide (MHCp) tetramers (K^b-OVA, recognized by OT-I TCR) to investigate the ability of CD8αα-Cerulean and CD8αβ-Venus to enhance TCR binding to MHCp. Cells expressing fluorescent CD8αα, CD8αβ or both were mixed and allowed to bind phycoerythrin-labelled K^b-OVA tetramers (supplementary Fig S3A online). Cells expressing OT-I TCR but not CD8 rapidly bound a small quantity of K^b-OVA. Cells coexpressing OT-I TCR and either CD8αβ-Cerulean or CD8αα-Venus bound labelled tetramer more effectively, whereas expression of both CD8αα-Cerulean and CD8αβ-Venus resulted in the highest rate of tetramer accumulation. CD8αα + αβ-expressing hybridoma cells grown for several weeks showed a gradual loss of expression of either co-receptor. Tetramer binding to cells that had lost one of the co-receptors showed tetramer binding rates similar to those of the single co-receptor-expressing cells (supplementary Fig S3B online). Cells expressing more co-receptor molecules showed stronger tetramer binding (supplementary Fig S3C online). Surprisingly, tetramer binding to TCR plus CD8αα—the weaker co-receptor—was stronger than that binding to TCR plus CD8αβ. When the cells were subgated for increasing ratio of one CD8 species to the other, tetramer binding increased as the proportion of CD8αα increased relative to CD8αβ. This can be explained because, similarly to CD8αα, unstimulated TCRs are not raft associated (Montixi *et al*, 1998). Earlier data showing increased binding of multimeric MHCp to CD8αβ compared with CD8αα used a T hybridoma in which CD8αβ was reported to be constitutively associated with TCR in lipid rafts (Arcaro *et al*, 2001; Doucey *et al*, 2003). This is not always the case (Yachi *et al*, 2005), which might explain the discrepancy in the results. Thus, initial binding to TCR, shown by the CD8-independent, small, rapid rise in binding seen with all of the cell populations, is followed by a stage in which the proximity and/or mobility of the CD8 molecules makes a difference to the ability to increase tetramer binding.

Separate capping of CD8αα-Cerulean and CD8αβ-Venus

To induce patching and capping of the CD8αα or -αβ molecules, cells expressing both species were incubated at 4 °C with monoclonal antibodies recognizing CD8α1 (anti-Ly2.1) or CD8β, followed by crosslinking reagents. After further incubation at 37 °C, cells were fixed and analysed. Both CD8αα-Cerulean (Fig 2A) and CD8αβ-Venus (Fig 2B) were preferentially capped by their cognate antibody, although there was some co-capping of the other species. We calculated the percentage of increase (Fig 2C; see Methods) to show the preferential recruitment of one species over the other in a defined region of interest. A positive value represents preferential recruitment of CD8αβ, whereas a negative value represents preferential recruitment of CD8αα. The anti-CD8α1 preferentially capped CD8αα-Cerulean, whereas the anti-CD8β preferentially capped CD8αβ-Venus, indicating that these species were mostly physically independent of each other. Lck associated with both CD8αα and CD8αβ. Capping of CD8αα or CD8αβ with specific antibodies co-capped Lck. In cells expressing both CD8αα and CD8αβ, Lck was co-capped with either species (supplementary Fig S4 online).

CD8αα and CD8αβ recruitment to the IS

The interaction of a $CD8^+$ T cell with an antigen-presenting cell (APC) results in recruitment of CD8αβ to the IS (Yachi *et al*, 2005, 2006; Gascoigne *et al*, 2010). Both CD8αα-Cerulean and CD8αβ-Venus were recruited to the IS during the interaction of the T cells with APC (Fig 3A,B), with only a slight bias towards recruitment of CD8αβ. The comparable recruitment of both species was surprising given that CD8αβ is a much better co-receptor. It suggests that the enhanced co-receptor function is not because of any preferential ability of CD8αβ to go to the IS, nor is it because of its stronger ability to bind to MHC-I. One potential reason could be that the strong recruitment of both molecules is due to some interaction between CD8 molecules. To test this directly, we

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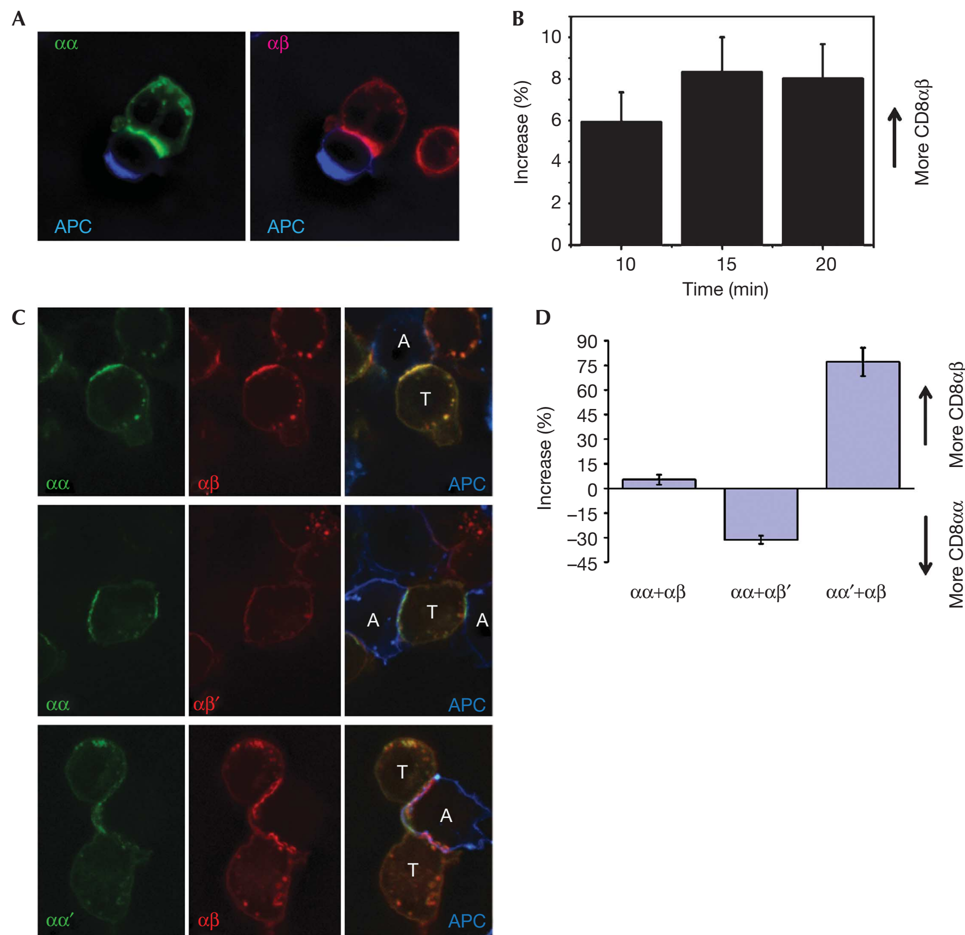

Fig 3 | CD8αα or αβ recruitment to the IS depends on the ability to bind to MHC-I. (**A**) T cell expressing CD8αα-Cerulean and CD8αβ-Venus interacting with a Cy5-labelled antigen-presenting cell (APC). For clarity, Cerulean and Venus channels merged with the Cy5 channel are presented separately. (**B**) Time course of differential recruitment of CD8αα-Cerulean and CD8αβ-Venus in cells responding to RMA-S cells presenting K^b-OVA. Graph shows the percentage of increase ± s.e.m., $n \geqslant 20$. (**C**) T cells expressing CD8αα + CD8αβ, CD8αα + CD8αβ′ or CD8αα′ + CD8αβ fluorescent proteins during interaction with ovalbumin (OVA) peptide-loaded EL4 cells. Cerulean and Venus channels are presented separately (left and centre) and merged with Cy5 (APC) channel in right panels. T cells and APCs are labelled on the merged image (T and A, respectively). (**D**) Preferential recruitment of wild-type co-receptor species compared with non-major histocompatibility complex-I-binding mutants to the immunological synapse (IS). Negative values reflect preferential recruitment of CD8αα and positive values the preferential recruitment of CD8αβ. Graph shows the percentage of increase ± s.e.m. $n = 17$, 18 and 15.

made mutations in the MHC-I-binding sites of CD8α and CD8β, and expressed them with CD8α2–CC155 such that binding-competent CD8αα was expressed with mutated CD8αβ (CD8αβ′; β-chain class I-binding mutation S101A (Devine *et al*, 2006)), or binding-competent CD8αβ was expressed with mutated CD8αα (CD8αα′; α-chain class I-binding mutation N107A (Devine *et al*, 2002)). These binding mutants of CD8 were not recruited to the IS in response to antigen recognition, and did not interfere with the recruitment of the non-mutant species (Fig 3C,D). We next measured tetramer binding using the same analysis strategy as in supplementary Fig S3B (Fig S5 online). We gated samples of cells expressing CD8αα + CD8αβ′ or CD8αα′ + CD8αβ, cells that had lost one of these species or non-fluorescent cells that had lost both. In both cell types, the addition of mutant CD8 did not substantially alter tetramer binding by the non-mutant species. Cells showing only the fluorescent signal characteristic of the mutant species bound significantly less tetramer. This binding was, however, higher than that of $CD8^-$ cells. This probably indicates the presence of non-fluorescent (non-mutant) CD8α2–CC155 homodimers, which were capable of binding to the tetramer.

These results show that the recruitment of both CD8 species in response to antigenic stimulation is due to each species independently interacting with the MHC-I molecule, and not due to interactions between CD8 molecules. Thus, the co-receptor prowess of CD8αβ is not related to a stronger ability to bind to MHC-I, nor to a stronger ability to be recruited to the IS during antigen recognition, nor even to its association with lipid rafts *per se*, leaving only its ability to preferentially bind to Lck. This is in accord with a recent modelling study indicating that the

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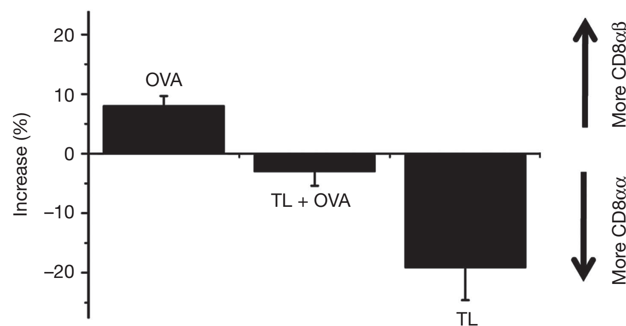

Fig 4 | Ligand dependence of the differential recruitment of CD8αα and CD8αβ. Data are presented for T cells expressing CD8αα-Cerulean and CD8αβ-Venus responding to RMA-S cells presenting K^b-OVA (OVA), RMA-S cells expressing thymus leukaemia antigen (H2-TL or TL) and also presenting K^b-OVA (TL + OVA) and RMA-S cells expressing TL alone (TL). Error bars represent s.d. $n = 25$ for each group.

most important function of co-receptors is to target Lck to the TCR-recognizing antigen (Artyomov *et al*, 2010).

Preferential CD8αα recruitment by H2-TL

The non-classical MHC-I molecule thymus leukaemia antigen (H2-TL) binds to CD8αα with much higher affinity than to CD8αβ (Leishman *et al*, 2001; Liu *et al*, 2003; Attinger *et al*, 2005). We therefore tested whether CD8αα or CD8αβ recruitment to the T-cell–APC (T–APC) interface would be altered in the presence of H2-TL, owing to the non-cognate interaction of CD8αα with H2-TL. We stimulated the OT-I hybridomas expressing the two fluorescent forms of CD8 with RMA-S cells or with RMA-S cells transfected with H2-TL (RMA-S–TL; Attinger *et al*, 2005). These RMA-S cells were first incubated at 32 °C in the presence or absence of OVA peptide. This peptide binds to H2-K^b expressed on the cell surface at this temperature, thus stabilizing the H2-K^b molecule when the temperature is later raised to 37 °C (Ljunggren *et al*, 1990; Yachi *et al*, 2005, 2006, 2007). Because H2-TL does not bind to peptides (Weber *et al*, 2002; Liu *et al*, 2003), it does not require Tap2 for its cell-surface expression. The RMA-S–TL cells therefore express H2-TL constitutively (Attinger *et al*, 2005).

CD8αβ was recruited slightly more effectively than CD8αα when OVA was presented on K^b on RMA-S cells (Fig 4). Cells presenting both H2-TL and OVA-K^b recruited CD8αα and CD8αβ to the interface at the same rate. Finally, presentation of H2-TL alone caused preferential recruitment of CD8αα to the T–APC interface. Thus, recognition of H2-TL enhanced CD8αα recruitment, and recognition of H2-K^b enhanced CD8αβ recruitment. The presence of both H2-TL and OVA-K^b removed the difference in the preferential recruitment of the two CD8 species.

CONCLUDING REMARKS

BiFC was successfully used to engineer CD8αα and CD8αβ dimers to enable them to be independently visualized on the T-cell surface. Both αα and αβ dimers behaved independently when crosslinked by antibodies, but both were strongly recruited to the IS by recognition of antigen. The strong recruitment of CD8αα was surprising, as it is a much weaker co-receptor than CD8αβ. Synapse recruitment of either species was lost when its ability to bind to MHC-I was compromised, showing that this recruitment was a genuine effect of MHC recognition. Lck and CD8αβ were mainly present in lipid rafts, whereas CD8αα was excluded, although Lck was able to co-cap with both CD8 species. Thus, the ability of CD8αβ to function as a strong co-receptor is related to its ability to associate with Lck in the appropriate membrane domains, not to binding to MHC or to recruitment to the IS. However, we found that recognition of H2-TL separated CD8αα recruitment from that of CD8αβ, owing to the much stronger interaction of CD8αα with H2-TL. Thus, in this case, the recruitment of the two CD8 species correlated with the relative binding to the available ligands.

METHODS

DNA constructs and gene expression. CD8α1 (methionine at residue 78) was generated from the CD8α2 (Val78) construct by single point mutation. BiFC fragments CC155, Cr173 and Vn173 (Hu *et al*, 2002; Hu & Kerppola, 2003) were fused to the C terminus of CD8α2, CD8α1 and CD8β, respectively, with spacers between the proteins to allow proper folding and flexibility (Yachi *et al*, 2005). Mutations in the MHC-I-binding sites of CD8 were as follows: CD8α1 N107A to make CD8αα′. In the CD8αα structure, N107 on one α-subunit interacts with residues M228, E229 and L230 of K^b, and in the other α-subunit it interacts with K^b E222 (Devine *et al*, 2002). CD8β S101A was made for CD8αβ′ (Devine *et al*, 2006). S101 interacts with residues T225, M228 and L230 of K^b (Wang *et al*, 2009). Constructs cloned in pLN1 retroviral expression vector were transduced into hybridomas expressing the OT-I TCR but not CD8 (Yachi *et al*, 2005, 2006). Expressors were selected by FACS, sorted and cloned. The following antibodies were used: anti-Ly2.1 (that is, anti-CD8α1; clone 49-31.1, mouse IgG_3), anti-Ly2.2 (that is, anti-CD8α2; clone AD4(15), mouse IgM, Cedarlane) and anti-CD8β (YTS156.7.7, rat IgG_{2b}, BioLegend).

Imaging. Fluorescence imaging was performed on a Marianas system (Intelligent Imaging Innovations, Santa Monica, CA). To avoid crosstalk, images were acquired sequentially using the following filter sets: Cerulean, Ex: 430/25 nm, Em: 470/30 nm; Venus, Ex: 510/20 nm, Em: 550/50 nm, Cy5 or APC Ex: 622/36 nm, Em: 700/75 nm (Zal & Gascoigne, 2004; Yachi *et al*, 2005, 2006). Image analysis was performed using the Slidebook software. After background subtraction, the IS (or cap) and a non-IS (or non-cap) area were delimited manually on the image. Average fluorescence intensities for both areas were measured, and the area intensity ratio (AR) $= I_{synapse}/I_{non\text{-}synapse}$ was calculated for each channel. The percentage of increase was then calculated as $((AR_{Venus}/AR_{Cerluean})-1)\times 100$. This parameter defines the preferential recruitment of one fluorophore compared with the other in the region of interest, independently from their actual concentration or fluorescence intensity. The −1 term was introduced so that preferential recruitment of Cerulean (CD8αα) or Venus (CD8αβ) is easily identified by a negative or a positive value, respectively.

Statistics. The mean difference hypothesis of Student's two-tailed *t*-test assuming different variances and a confidence level of 95% was performed using Microcal Origin.

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

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Author contributions: N.R.J.G. and J.-P.C. designed the project; J.-P.C. made and transfected the BiFC constructs; and J.-P.C. and V.R. conducted the experiments, with contributions from J.A. and P.P.Y.; N.R.J.G. J.-P.C. and V.R. wrote the manuscript. Figures were prepared by J.-P.C. and V.R.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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