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# Anti-coreceptor antibodies profoundly affect staining with peptide-MHC class I and class II tetramers

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The T cell coreceptors CD8 and CD4 bind to invariable regions of peptide-MHC class I (pMHCI) and class II (pMHCII) molecules, respectively, and facilitate antigen recognition by a number of mechanisms. It is established that some antibodies (Ab) specific for the CD8 molecule, which stabilizes TCR/pMHCI interactions, can alter the binding of pMHCI tetramers to cell surface TCR. In contrast, the extremely weak pMHCII/CD4 interaction does not stabilize TCR/pMHCII interactions or contribute to cognate tetramer binding; consequently, it is assumed that anti-CD4 Ab do not affect pMHCII binding. Here, we used a panel of point-mutated HLA A2 molecules with a range of affinities for CD8 spanning over three orders of magnitude to demonstrate that anti-CD8 Ab-mediated inhibition of pMHCI tetramer binding and cognate T cell activation correlates directly with the strength of the pMHCI/CD8 interaction. Further, some anti-CD4 Ab were found to block pMHCII tetramer binding; these effects were also paralleled in T cell activation assays. In sum, these data challenge the assertion that anti-coreceptor Ab exert their effects on T cell activation and pMHC binding solely by blocking pMHC/coreceptor interactions.

**Key words:** CD4 · CD8 · Peptide-MHC tetramers · T cell activation · T cell receptors

## Introduction

T cell recognition is controlled by interaction between the highly variable complementarity determining regions of the T cell receptor (TCR) and the peptide-binding platform of the major histocompatibility (MHC) molecules. Peptide-MHC (pMHC) also interacts with the cell surface glycoproteins CD8 and CD4, which bind to invariable regions of the MHC class I and II molecules, respectively [1–5]. The binding sites for CD8 and CD4 are separate from the TCR-recognized [6], peptide-binding domains of MHC molecules. As a result, a single MHC molecule can be bound simultaneously by both TCR and CD8 or CD4 [2, 5].

The interaction between the TCR and its cognate peptide is intrinsically weak and lasts for just a few seconds at physiological temperatures. Multimerization of this interaction, using avidin-biotin-based 'tetramers' [7] or other means [8], can slow the composite dissociation rate by hundreds of fold [9] because disengagement of tetramer requires that all monomeric TCR/pMHC interactions are dissociated simultaneously. The greatly increased half-life of tetramerized pMHC or TCR provides a basis for the use of such reagents in

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Abbreviations: **pMHC:** peptide-MHC · **pMHCI:** peptide-MHC class I · **pMHCII:** peptide-MHC class II · **SPR:** surface plasmon resonance

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cellular binding assays. Indeed, the use of multimeric pMHC molecules has revolutionized T cell immunology by enabling the direct visualization, enumeration, phenotyping and sorting of T cells based on the antigen specificity of their TCR [7, 10, 11]. The pMHC class I (pMHCI)/CD8 and pMHC class II (pMHCII)/CD4 interactions allow the potential for coreceptor-mediated stabilization of pMHC tetramer binding at the cell surface. Several recent studies have examined this potential [12–21]. All reports examining peptide-MHC class II (pMHCII) show that CD4 does not aid the stabilization of the TCR/pMHCII interaction and plays no role in the binding of pMHCII tetramers [13, 16, 17]. No clear consensus has emerged from studies examining the role of CD8 in stabilizing the binding of pMHCI tetramers.

We have recently used a range of pMHCI molecules with abrogated, reduced, normal, enhanced and super-enhanced CD8 binding, but unaltered TCR binding, to examine the TCR/pMHCI/CD8 interaction at the cell surface [21]. These experiments allowed an assessment of cooperative binding not possible in previous biophysical and structural studies using soluble molecules [2, 22, 23]. Tetramer decay from the cell surface of $CD8^+$ cytotoxic T lymphocytes (CTL) stained with these reagents shows that TCR and CD8 bind to pMHCI cooperatively at the cell surface. Modeling for the monomeric TCR/pMHCI/CD8 interaction indicates that CD8 provides a maximum stabilization factor of $\sim 2$ to TCR/pMHCI interactions [21]. It would appear that the requirement for CD8 to stabilize tetramer binding is minimal when the TCR binds to its ligand strongly, but is increasingly apparent as the TCR/pMHCI half-life decreases [19, 20]. Indeed, these data point to a reconciliation of the apparently disparate findings regarding the requirement for the pMHCI/CD8 interaction in the stable binding of pMHCI tetramers [21]. Stable cell surface adhesion of pMHCI tetramers has an empirical requirement for the monomeric interaction to be of sufficient duration to allow a further monomer in the complex to interact with another TCR prior to dissociation of the original interaction [21] (Laugel *et al.*, unpublished). Presumably, strong TCR/pMHCI interactions, such as those of immunodominant human anti-viral CTL, exceed this minimal requirement *per se*. The ability of the pMHCI/CD8 interaction to delay the dissociation of the TCR/pMHCI interaction by a factor of $\sim 2$ [21] enables some weaker TCR/pMHCI engagements to attain the minimal half-life for stable binding of tetrameric reagents to the CTL surface (Laugel *et al.*, unpublished). This property is presumably responsible for the fact that wild-type tetramers bind to cell surface TCR on so-called high- and low-avidity T cells, whereas CD8-null tetramers only stain high-avidity CTL [19]. As a consequence, CD8-null pMHCI tetramers can be used to identify high-avidity CTL selectively in both humans and mice [19, 20].

The role of the CD8 and CD4 coreceptors in T cell activation has often been studied using anti-coreceptor antibodies (Ab) [24, 25]. More recently, several studies have shown that anti-CD8 Ab can have either positive or negative effects on the binding of pMHCI tetramers to cell surface TCR [12, 14, 15, 18]; parallel Ab-mediated effects enhance or disrupt T cell activation in both humans and mice [18]. Interaction between pMHCI and CD8 is not obligatory for these effects [18], and the mechanism by which anti-coreceptor Ab function to alter T cell activation or tetramer binding remains unclear. Here, we use point-mutated pMHCI molecules to dissect the effects of anti-CD8 Ab on the binding of pMHCI tetramers and cognate T cell activation. We also examine the effects of anti-CD4 Ab on pMHCII tetramer binding and antigen-induced activation. The results rule out the notion that anti-coreceptor Ab block the binding of multimerized pMHC to cell surface TCR by inhibiting coreceptor-mediated stabilization of TCR/pMHC interactions. They also have important implications for tetramer-based flow cytometry studies.

## Results

### The pMHCI/CD8 interaction is not critical for pMHCI tetramer binding

Several studies have shown that anti-CD8 Ab can block staining with pMHCI tetramers in both human and murine systems [12, 14, 15, 18]. These observations suggested that CD8 played an obligatory or critical role in the binding of pMHCI tetramers [12, 14, 15]. However, our own studies have discounted this conclusion by showing that some human CTL stain equally well with wild-type and CD8-null pMHCI tetramers that have been mutated to abrogate the pMHCI/CD8 interaction without affecting the integrity of the TCR/pMHCI interaction [26]. Further, we challenged the notion that anti-CD8 Ab block tetramer binding through disruption of the pMHCI/CD8 interaction by showing that these reagents were also able to exert an inhibitory effect on the binding of CD8-null tetramers [18]. In contrast, a recent study has determined that anti-CD8 Ab only inhibit the binding of murine pMHCI multimers to the surface of 2C CTL when CD8 is capable of binding pMHCI [27]. Thus, it seems that the difference between our studies [18] and those with 2C CTL [27] may reflect either an inter-species difference or a peculiarity of 2C transgenic T cells.

We have tested the effects of five different anti-CD8 Ab on human pMHCI tetramer binding [18]. Three Ab clones were able to block the binding of pMHCI

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tetramers [18]. Clone DK-25 was the most potent in this respect [18]. All inhibitory Ab were able to reduce the binding of wild-type and CD8-null reagents [18]; however, the degree of inhibition was more pronounced with wild-type pMHCI tetramers than with the corresponding CD8-null reagents ([18] and Fig. 1A). This effect was also seen in the murine system [18]. Anti-CD8 Ab CT-CD8a completely inhibited binding of A2/$K^b$ tetramers to murine Melan-A CTL line m3a, but had a lesser effect on the binding of the corresponding HLA A2 tetramer [18]; HLA A2 did not bind to murine CD8 (mCD8) [26], whereas there was a relatively strong interaction between mCD8 and A2/$K^b$ folded with human β2 M ($K_D$ ∼10 μM) [18, 19].

The observation that the anti-CD8 Ab-mediated inhibition of tetramer binding is greater when pMHCI and CD8 are able to interact (Fig. 1A) suggests that the pMHCI/CD8 interaction might act to recruit and enhance the inhibitory effects of anti-CD8 Ab on tetramer binding.

### Inhibition of tetramer binding by anti-CD8 Ab correlates with the strength of the pMHCI/CD8 interaction

We have previously used surface plasmon resonance (SPR) to determine that the $K_D$ of the pMHCI/CD8αα interaction with DT227/8KA HLA A2, A245V HLA A2, wild-type HLA A2, Q115E HLA A2 and A2/$K^b$ folded around the HTLV-1 $Tax_{11–19}$ epitope (LLFGYPVYV) is >10 000 μM, 498 μM, 137 μM, 98 μM and 10.9 μM, respectively [21]. The $K_D$ of the pMHCI/CD8αα interaction for DT227/8KA HLA A2, wild-type HLA A2, Q115E HLA A2 and A2/$K^b$ folded around the HIV-1 p17–8 Gag epitope SLYNTVATL was shown to be >10 000 μM, 128 μM, 87 μM and 9 μM, respectively [21]. These substitutions in the α3 or α2 domain of the pMHCI molecule do not affect TCR binding [21]. CD8αα and CD8αβ bind to pMHCI with similar affinity [28] and the cubed root of the apparent off-rate of tetramerized forms of the reagents described above from the surface of several $CD8\alpha\beta^+$ CTL showed a linear relationship with the affinities for CD8αα measured in three-dimensions by SPR [21].

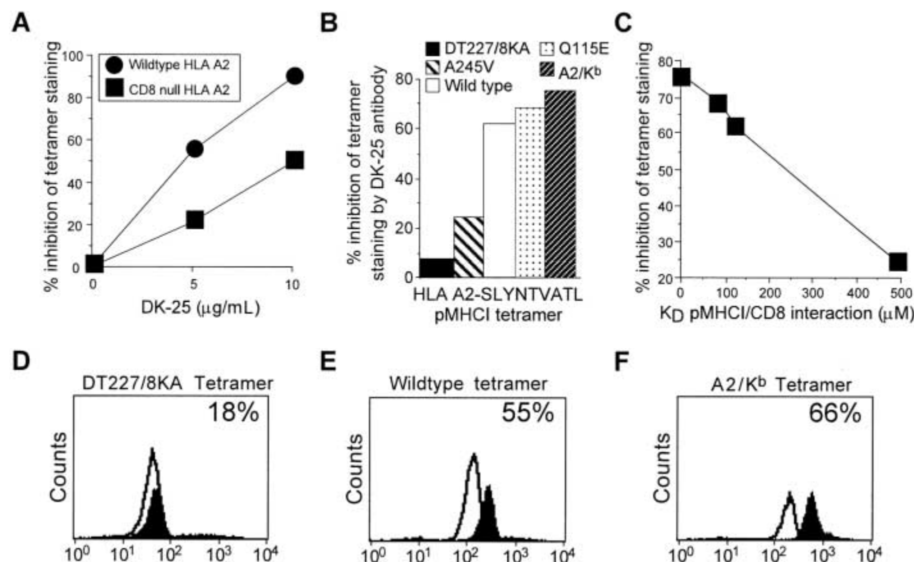


**Figure 1.** Inhibition of tetramer binding with DK-25 anti-CD8 antibody directly correlates with the strength of the pMHCI/CD8 interaction. (A) HLA A2 SLYNTVATL-specific 003 CTL ($10^5$ cells) were pre-incubated with the indicated concentrations of DK-25 Ab for 20 min and subsequently stained with 1 μg DT227/8KA HLA A2-SLYNTVATL or wild-type HLA A2-SLYNTVATL tetramer for 30 min. (B) HLA A2 SLYNTVATL-specific 003 CTL ($10^5$ cells) were pre-treated for 20 min with 5 μg/mL DK-25 in 20 μl PBS for 20 min and then stained with 1 μg DT227/87KA HLA A2, A245V HLA A2, HLA A2, Q115E HLA A2 or HLA A2/$K^b$ tetramer folded around the SLYNTVATL peptide for 45 min on ice. (C) The % inhibition for each mutant tetramer used in (B) following pre-incubation with DK-25 plotted against the 3D pMHCI/CD8 dissociation constant. CTL clone 003 is a so-called 'high-avidity' CTL and stains almost equally well in the presence or absence of CD8 engagement [19, 21, 26, 39]. (D–F) As (B), but $10^5$ GLCTLVAML-specific lower avidity EBV-A CTL were stained with 1 μg DT227/8KA HLA A2-GLCTLVAML (D), wild-type HLA A2-GLCTLVAML (E), or HLA A2/$K^b$-GLCTLVAML (F) tetramer following pre-incubation with DK-25 Ab. The white plots and black plots show staining with and without DK-25 inhibition, respectively. The numbers in the top right of each plot indicate the percentage inhibition of the mean fluorescence intensity (MFI) afforded by addition of anti-CD8 antibody in each case. The $K_D$ of binding of CD8 to the HLA A2 molecules DT227/8KA, A245V, wild-type, Q115E and A2/$K^b$ folded with the HTLV-1 $Tax_{11–19}$ epitope (LLFGYPVYV) is >10 000 μM, 498 μM, 137 μM, 98 μM and 10.9 μM, respectively [21].

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The spectrum of HLA A2 molecules described above, which exhibit normal TCR/pMHCI interactions but a range of pMHCI/CD8 interactions exceeding 1000-fold, was used to study the role of the pMHCI/CD8 interaction in the inhibition of pMHCI tetramer binding by inhibitory anti-CD8 Ab. Our previous studies have indicated that anti-CD8 Ab clone DK-25 is a potent inhibitor of pMHCI tetramer binding and CTL activation [18]. This effect titrates with DK-25 Ab concentration (Fig. 1A). Maximal inhibition occurs at >10 μg/mL DK-25 ([18] and data not shown). There is some variation (± 2–3-fold for a given clone over time) in the amount of anti-CD8 Ab required to cause maximal inhibition of tetramer binding (data not shown). This difference may reflect differences in the cell surface expression level of CD8. As described above, DK-25-mediated inhibition of CD8-null tetramer binding was less than that observed for the corresponding wild-type reagents (Fig. 1A). To confirm and extend these findings, we examined the ability of a fixed level of DK-25 Ab to inhibit the binding of HLA A2 tetramers that exhibit a range of binding affinities to CD8 (Fig. 1B). Inhibition of tetramer binding showed a good correlation with the 3D affinity of the pMHCI/CD8 interaction as measured by SPR (Fig. 1C). This effect was also seen with CTL clone EBV-A (Fig. 1D–F).

## Inhibition of CTL activation by anti-CD8 Ab is largely independent of the pMHCI/CD8 interaction

We next examined the effects of anti-CD8 Ab on CTL activation. Anti-CD8 Ab DK-25 was able to inhibit activation induced by peptide-pulsed target cells bearing antigen in the context of all HLA A2 molecules, regardless of their CD8 binding properties (Fig. 2). Results with the telomerase-specific CTL clone ILA-1 (Fig. 2) and CTL clones with different peptide specificities (data not shown) confirmed that anti-CD8 Ab DK-25 was able to inhibit the activation of CTL by CD8-null antigen-bearing targets. Similar levels of anti-CD8 Ab did not inhibit the activation of MHC class II-restricted T cells (Fig. 3, 4). The degree of CTL activation, and consequently the absolute inhibitory effects of anti-CD8 Ab, increased as the affinity of the pMHCI/CD8 interaction increased from >10 000 to 85 μM. Interestingly, however, the super-enhanced pMHCI/CD8 interaction of A2/$K^b$ ($K_D$ ~ 10 μM) did not improve the recognition of antigen by ILA-1 CTL beyond that observed with the wild-type molecule.

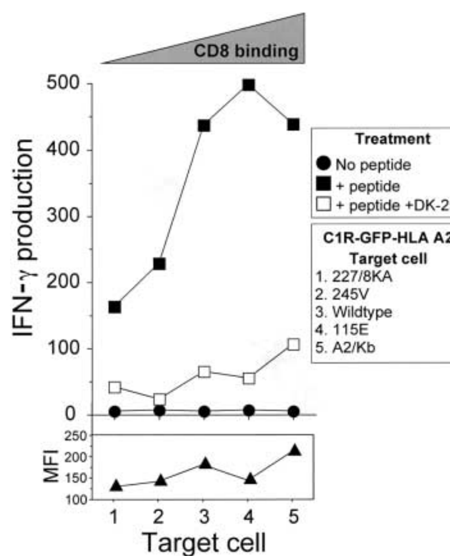


**Figure 2.** Inhibition of CTL activation by anti-CD8 antibodies. Production of IFN-γ (MFI by cytometric bead array) by 25 000 ILA-1 CTL exposed to 25 000 C1R-GFP-A2 targets with differing HLA A2/CD8 interaction affinities. Activation is shown for targets without peptide (filled circles), targets pulsed with 100 nM ILAKFLHWL peptide (filled squares) and targets pulsed with 100 nM ILAKFLHWL peptide in the presence of 10 μg/mL anti-CD8 antibody DK-25 as described in the *Materials and methods*. The bottom panel (filled triangles) shows the level of GFP expressed by the targets on the day of experimentation. Each target looked the same by fluorescence microscopy, with >95% of the green fluorescence being associated with the plasma membrane (data not shown). The level of GFP staining correlated with the level of staining observed with the HLA A2 conformation-specific antibody BB7.2 (data not shown). Symbols are joined to indicate that CD8 binding increases from left to right.

## Anti-CD4 Ab block the binding of pMHCII tetramer

All studies of pMHCII binding to cell surface TCR have concluded that, unlike the CD8 coreceptor, CD4 does not play a significant role in stabilizing the TCR/pMHCII interaction [13, 16, 17]. Indeed, the binding of pMHCII tetramers was equally efficient whether or not CD4 was available for binding [13, 16]. Despite this, we noticed that the staining intensity with pMHCII tetramers varied depending on the anti-CD4 Ab clone used in multiple systems (Fig. 3, 4 and data not shown). These unexpected effects were apparent when anti-CD4 Ab was added to the cells either before, or simultaneously with, tetramer but not when Ab was added 20 min after tetramer staining (data not shown). Experiments with the HLA DR1-restricted tetanus toxoid-specific $CD4^+$ T cell line JF-TTox, the HLA DR4-restricted HIV-1 p24 Gag-specific $CD4^+$ T cell line K37-p24.4, the HLA DR1-restricted HIV-1 p24 Gag-specific $CD4^+$ T cell clone Ox97-clone10 and the HLA DR1-restricted influenza

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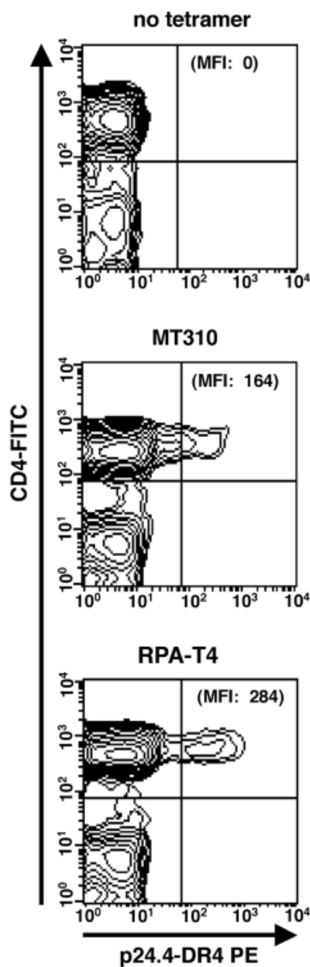


**Figure 3.** Anti-CD4 antibodies have differential effects on the binding of pMHCII tetramer. Contour plots of $CD4^+$ T cell line K37-p24.4, which was pre-incubated with the indicated anti-CD4 Ab for 20 min and subsequently stained with the cognate p24.4-DR4 tetramer for 30 min as described in the *Materials and methods*. The MFI of cells falling into the upper right quadrant is indicated for each plot. Inhibition of pMHCII tetramer binding was also observed with anti-CD4 Ab clones 13B8.2 and M-T466 (Fig. 4 and data not shown). Anti-CD8 Ab clone DK-25 did not have a major effect on pMHCII tetramer binding or activation of MHC class II-restricted T cells (Fig. 4).

HA-specific $CD4^+$ T cell clone HA1.7 showed that these effects applied to tetramer staining with different antigens and different HLA DR molecules (Fig. 4A, B). Three of four anti-CD4 Ab tested (clones MT310, M-T466 and 13B8.2) were able to block the binding of cognate tetramer to JF-TTox, Ox97-clone10 and HA1.7 T cells (Fig. 4A, B). These three anti-CD4 Ab clones also inhibited the binding of cognate tetramer to K37-p24.4 T cells, although to a lesser extent. Clone RPA-T4 exerted minimal inhibitory effects (Fig. 4A, B). We have previously shown that the effects of anti-CD8 Ab on tetramer binding are paralleled by their effects on CTL activation [18]. This was also true for anti-CD4 Ab (Fig. 4B). The two Ab that inhibited pMHCII tetramer binding to cell surface TCR most efficiently, MT310 and M-T466, were also the most potent at blocking T cell activation induced by antigen-pulsed targets. Anti-CD4 Ab clone RPA-T4 that exhibited the smallest effect on pMHCII tetramer binding did not inhibit activation. Ab were used at a 1:5 dilution as recommended by the manufacturer to mimic normal staining practice. As a result, the non-inhibitory Ab clone RPA-T4 was used at a higher final concentration (20 μg/mL) than the inhibitory Ab, thus ruling out the possibility that inhibition by MT310, M-T466 and 13B8.2 was due simply to differential concentration effects. Anti-CD8 Ab clone DK-25 did not inhibit the binding of any pMHCII tetramer (Fig. 4A, B), but severely impeded the binding of pMHCI tetramer to CTL clones ILA1 and 003 (Fig. 4C). Conversely, none of the anti-CD4 Ab clones used impeded binding of pMHCI tetramers to these CTL clones (Fig. 4C). Furthermore, none of the anti-CD4 Ab clones affected CTL activation, thereby ruling out any general toxicity effects (data not shown). Pre-incubation of $CD4^+$ T cell clones with each of the anti-CD4 Ab clones used had no effect on the surface expression level of TCR (data not shown). Thus, anti-CD4 Ab exhibit differential effects on pMHCII tetramer binding. Ab that do not inhibit pMHCII tetramer binding do not inhibit T cell activation, whereas those that inhibit tetramer binding also inhibit activation. These results suggest that anti-CD4 Ab-mediated T cell inhibition might be due to an inhibition of TCR engagement at the cell surface rather than to a simple blocking of the pMHCII/CD4 interaction.

## Discussion

It has long been known that anti-coreceptor Ab can inhibit T cell activation [24, 29–32]. Indeed, it was this very inhibition that first hinted that these molecules might play a significant role in T cell activation. T cells exhibit a spectrum of sensitivities to anti-coreceptor Ab [33]. This has led to the definition of T cells as being coreceptor dependent or coreceptor independent [25]. It has been suggested that this dependence may correlate with the strength of the TCR/pMHC interaction [25]. As the CD8 and CD4 coreceptors bind to invariant regions of pMHCI and pMHCII, respectively, and act to recruit essential signaling molecules to the cytoplasmic side of the TCR/CD3/ζ complex, it was naturally assumed that the anti-coreceptor Ab that blocked T cell activation functioned by blocking the interaction between coreceptor and MHC. The pMHCI binding sites for TCR and CD8 are spatially distinct and allow the binding of both molecules simultaneously [2]. This fact enables the assembly of trimeric TCR/pMHCI/CD8 complexes at the T cell surface and allows for the

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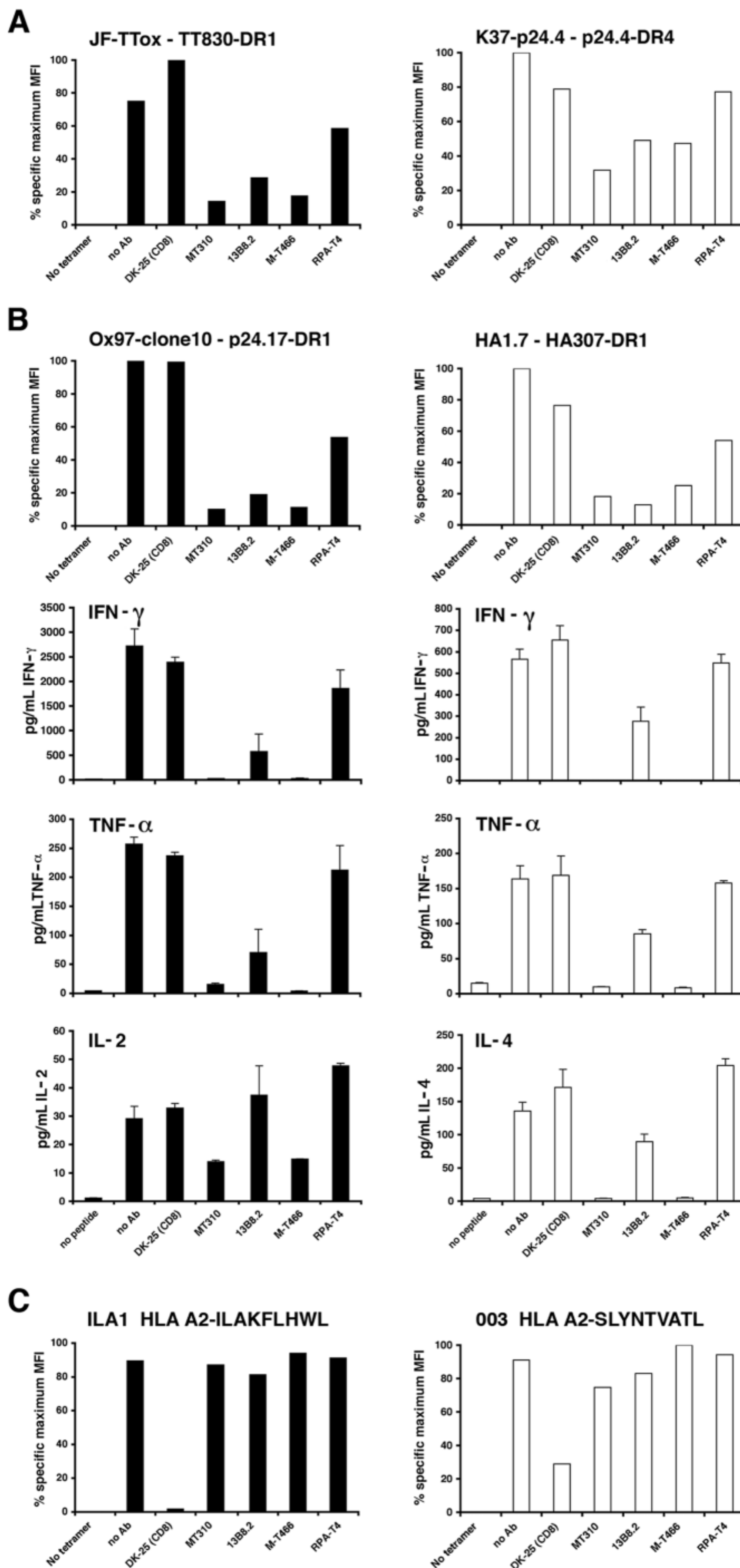


**Figure 4.** Anti-CD4 antibody-mediated inhibition of pMHCII binding correlates with anti-CD4 Ab-mediated inhibition of T cell activation. (A) The % maximum specific MFI of HLA class II tetramer staining of the DR1-restricted CD4⁺ T cell line JF-TTox (black bars) and the DR4-restricted CD4⁺ T cell line K37-p24.4 (empty bars) when these cells were stained with cognate tetramer after incubation with the indicated anti-CD4 Ab clones. The anti-CD8 Ab DK-25 acts as a nonspecific control. (B) Anti-CD4 Ab-mediated inhibition of HLA class II tetramer staining (upper plots) and activation (lower 3 plots) of $CD4^+$ T cell clones Ox97-clone10 (black bars) and HA1.7 (empty bars). For the lower three plots, the mean concentration of respective cytokine produced in response to immortalized B cell targets pulsed with 0.2 μM cognate peptide by Ox97-clone10 (black bars) and HA1.7 (empty bars) is plotted. Error bars represent SEM. Clone Ox97-clone10 produced IL-10 in addition to IFN-γ, TNF-α and IL-2 and an identical pattern of anti-CD4 Ab inhibition was seen (data not shown). Clone HA1.7 produced IL-2 and IL-10 in addition to IFN-γ, TNF-α and IL-4; identical patterns of anti-CD4 Ab inhibition were again observed (data not shown). (C) Anti-CD4 Ab do not inhibit HLA class I tetramer staining of cognate $CD8^+$ T cells. CTL clones ILA1 (black bars) and 003 (open bars) were pre-incubated with anti-CD4 Ab or the anti-CD8 Ab DK-25 and subsequently stained with HLA A2-ILAKFLHWL or HLA A2-SLYNTVATL tetramer, respectively. Anti-CD4 Ab were used at a 1:5 dilution. As a result, final concentrations were: 20 μg/mL (RPA-T4), 4.4 μg/mL (M-T466), 16 μg/mL (MT310) and 5 μg/mL (13B8.2). Thus, the Ab with the least inhibitory effect (RPA-T4) was used at the highest concentration. None of the anti-CD4 or anti-CD8 Ab altered cell surface TCR expression levels (data not shown).

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possibility of CD8-mediated stabilization of the TCR/pMHCI interaction. The line of logic that anti-CD8 Ab might serve to block the pMHCI/CD8 interaction was naturally extended to explain how anti-CD8 Ab are able to inhibit the binding of pMHCI tetramers [12, 14, 15]. However, our recent studies have suggested that anti-CD8 Ab do not inhibit the binding of pMHCI tetramers by blocking the interaction between pMHCI and CD8 [18]. In the present study, we have extended these findings to show that the pMHCI/CD8 interaction may serve to recruit a large proportion of the anti-CD8 Ab-mediated inhibition of tetramer binding (Fig. 1). It has been suggested that the binding of pMHCI ligands results in a subtle re-arrangement of CD8 with respect to TCR/pMHCI [34]. When CTL are co-stained with tetramer and anti-CD8 Ab, most CD8 molecules are likely to have an anti-CD8 Ab bound to them before tetramer engagement if the anti-CD8 Ab is added before, or simultaneously with, tetramer. pMHCI ligands with an increased capacity to interact with CD8 would recruit CD8-bound Ab more efficiently, thus increasing the capacity of anti-CD8 Ab to interfere with pMHCI tetramer binding. To some extent, the role of the pMHCI/CD8 interaction in recruiting anti-CD8 Ab-mediated effects were also paralleled in CTL activation assays (Fig. 2). However, in general, activation is inhibited more completely than pMHCI tetramer binding, perhaps indicating that higher order aggregation of interactions in the context of membrane-membrane dynamics might mask the "inhibition recruitment" role of the pMHCI/CD8 interaction that is apparent when anti-CD8 Ab are used in conjunction with soluble tetrameric forms of cognate antigen.

As part of our studies with pMHCII tetramers [35, 36], we noticed that the fluorescence intensity of staining with these reagents exhibited some dependency on the anti-CD4 Ab clone used in co-staining protocols (Fig. 3 and data not shown). Further analysis revealed that different CD4 Ab clones vary widely in their ability to inhibit pMHCII tetramer staining and T cell activation (Fig. 4). Anti-CD4 Ab clones MT310 and M-T466 were consistently effective at blocking both pMHCII tetramer binding and T cell activation (Fig. 4). Anti-CD4 Ab clone RPA-T4 exhibited the weakest effect on both tetramer binding and T cell activation (Fig. 4). Thus, we conclude that the choice of anti-CD8 or anti-CD4 Ab clone can profoundly affect staining with pMHCI and pMHCII tetramers, and that these effects are paralleled in T cell activation assays.

Previous rigorous studies have shown that the CD4 molecule does not contribute to the binding of pMHCII tetramers *per se* [13, 16]. At first glance, our finding that some anti-CD4 Ab can inhibit the binding of pMHCII tetramers might seem at variance with these studies. Our observation that the pMHCI/CD8 interaction serves to recruit a significant proportion of the inhibitory effects of anti-CD8 Ab (Fig. 1, 2) may help to explain this apparent discrepancy. In an analogous manner, interaction between CD4 and pMHCII might ensure that CD4 is in close proximity to cognate TCR/pMHCII interactions even if this weak interaction fails to make a significant contribution to TCR/pMHCII stabilization. The pMHCII/CD4 interaction presumably also acts to recruit anti-CD4 Ab to the site of the TCR/pMHCII interaction when cells are pre-treated with anti-CD4 Ab or it is added at the same time as tetramer. This recruitment may then result in steric interference with the TCR/pMHCII interaction for some, but not all, CD4 Ab. These effects are likely to vary depending on the site of the Ab epitope within the CD4 molecule.

This study has several implications. First, it further instructs us to be wary of conclusions based solely on the use of anti-coreceptor Ab. Co-receptors are known to have a number of important roles in T cell activation. These roles include, but may not be limited to, the recruitment of essential signaling molecules to the cytoplasmic side of the TCR/CD3/ζ complex, stabilization of TCR/pMHC interactions at the cell surface (in the case of CD8) and the topographical organization of cell surface TCR via direct TCR/coreceptor interactions. Anti-coreceptor Ab may profoundly affect all roles of the coreceptor, thus making it impossible to attribute their effects to any one function. Second, our data further question the notion that anti-coreceptor Ab function by blocking interactions between the coreceptor and pMHC. It seems more likely that these reagents interfere with TCR/pMHC interactions, and that the extracellular coreceptor interactions with pMHC serve to recruit these inhibitory effects. Finally, our results have implications for the use of pMHCII tetramers to characterize antigen-specific $CD4^+$ T cells. The fact that the pMHCII/CD4 interaction does not affect the stability of TCR/pMHCII interactions suggested that anti-CD4 Ab would not affect the binding of pMHCII tetramers. The present study shows that this is not necessarily the case. Thus, anti-CD4 Ab staining should be conducted after incubation with pMHCII tetramer for optimal resolution of antigen-specific $CD4^+$ T cell populations by flow cytometry; alternatively, anti-CD4 Ab clones that do not inhibit pMHCII tetramer binding should be selected for this purpose. Indeed, we have recently used human pMHCII tetramers for direct *ex vivo* phenotyping of Th cells in peripheral blood with a frequency as low as 1:250 000 CD4 cells [35]; all of these stainings were performed with the non-inhibitory anti-CD4 Ab clone RPA-T4.

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## Materials and methods

### Cell lines and clones

The HLA A2-restricted HIV-1 p17–8 Gag-specific CTL clone 003 (epitope SLYNTVATL; residues 77–85) has been described previously [37]. The HLA A2-restricted EBV *BMLFI*-specific CTL clone EBV-A (epitope GLCTLVAML; residues 259–267) was generated by limiting dilution of a peptide-specific CTL line derived from a healthy HLA $A2^+$ donor. The ILA-1 clone specific for the human telomerase reverse transcriptase $(hTERT)_{540–548}$ peptide ILAKFLHWL was generated in a similar fashion. The C1R cells expressing full-length HLA A2 and HLA A2 mutants are described elsewhere ([26] and Wooldridge *et al*. submitted). $CD4^+$ T helper cell lines JF-TTox, K37-p24.4 and Ox97-clone10 were generated and maintained as previously described [37]. The influenza HA307–319-specific $CD4^+$ T cell clone HA1.7 [38] was kindly donated by Jonathan Lamb.

### Antibodies

The following mAb were used in this study: (i) anti-human CD8 SK1-peridinin chlorophyll protein (PerCP) (BD Biosciences), DK-25-fluorescein isothiocyanate (FITC) and DK-25 unconjugated (DAKO); (ii) anti-human CD4 MT310-FITC (DAKO), RPA-T4-FITC (Serotec), 13B8.2-FITC (Beckman Coulter) and M-T466-FITC (Miltenyi Biotech). According to the manufacturers, anti-CD4 clone 13B8.2 binds to the CDR3-like region of domain 1, MT310 binds to the HIV gp120-binding domain (amino acids 40–60) of domain 1 and RPA-T4 binds to the CDR1/CDR3-like regions of domain 1.

### pMHCI and pMHCII tetramers

Human pMHCI monomers were made as described previously [18]. The pMHCI tetramers used in this study were made fresh for the week of use from pMHCI monomers stored at –80°C to avoid previously reported [39] effects due to differences in protein stability. The HLA DRB1*0101 tetramers p24.17-DR1 (HIV p24 Gag peptide FRDYVDRFYKTLRAEQASQD; residues 294–313), HA307-DR1 (influenza HA peptide PKYVKQNTLK-LAT; residues 307–319) and TT830-DR1 (tetanus toxoid peptide QYIKANSKFIGITE; residues 830–843) and the HLA DRB1*0401 tetramer p24.4-DR4 (HIV p24 Gag peptide AFSPEVIPMFSALSEGATPQ; residues 164–183) were phycoerythrin (PE)-conjugated and supplied at a concentration of 100 μg/mL (Beckman Coulter, Marseilles, France).

### Flow cytometry

Flow cytometric analysis of $CD8^+$ CTL was performed as previously described [18]. For $CD4^+$ T cell analysis, $10^5$ cells were resuspended in 100 μL PBS/0.5% fetal calf serum (FCS) and preincubated with or without anti-CD4 Ab at a 1:5 dilution for 20 min at room temperature. Subsequently, HLA class II tetramers were added at a final concentration of 1 μg/mL and the cells were incubated for a further 30 min at 37°C. Samples were then washed in PBS/0.5% FCS, fixed in 1% paraformaldehyde in PBS and analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences). Anti-CD4 Ab were supplied by the manufacturers at 22 μg/mL (M-T466), 80 μg/mL (MT310), 25 μg/mL (13B8.2) and 100 μg/mL (RPA-T4).

### T cell activation assays

C1R targets expressing equal levels of green fluorescent protein-HLA A*0201 (GFP-A2) were incubated with 100 nM ILAKFLHWL peptide for 90 min at room temperature, washed twice and resuspended in RPMI medium supplemented with 2% FCS. CTL clone ILA-1 was incubated in RPMI medium supplemented with 10% FCS ± DK-25 Ab for 30 min. Subsequently, 25 000 target cells and 25 000 ILA-1 CTL ± DK-25 Ab were added to individual wells of a 96-well U-bottom plate in a total volume of 100 μL. $CD4^+$ T cells were washed and preincubated with or without anti-CD4 Ab at a 1:5 dilution in RPMI containing 10% human AB serum for 20 min at room temperature; 25 000 cells were then added to individual wells of a 96-well U-bottom plate together with 25 000 peptide-pulsed DRB1*0101-expressing EBV-transformed lymphoblastoid B cells in a total volume of 100 μL and incubated for 6 h at 37°C/5% $CO_2$. Supernatants were analyzed with a Th1/2 cytometric bead array kit II (BD Biosciences).

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