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[1]Fine tuning of CD8+ T-cell effector functions by targeting the 2B4-CD48 interaction

Running title: Role of the 2B4-CD48 axis in CD8+ T-cell function

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Abstract

Polyfunctionality and cytotoxic activity dictate CD8$^+$ T-cell efficacy in the eradication of infected and malignant cells. The induction of these effector functions depends on the specific interaction between the TCR and its cognate peptide-MHC class I complex, in addition to signals provided by co-stimulatory or co-inhibitory receptors, which can further regulate these functions. Among these receptors, the role of 2B4 is contested, as it has been described as either co-stimulatory or co-inhibitory in modulating T-cell functions. We therefore combined functional, transcriptional and epigenetic approaches to further characterize the impact of disrupting the interaction of 2B4 with its ligand CD48, on the activity of human effector CD8$^+$ T-cell clones. In this setting, we show that the 2B4-CD48 axis is involved in the fine-tuning of CD8$^+$ T-cell effector function upon antigenic stimulation. Blocking this interaction resulted in reduced CD8$^+$ T-cell clone-mediated cytolytic activity, together with a subtle drop in the expression of genes involved in effector function regulation. Our results also imply a variable contribution of the 2B4-CD48 interaction to the modulation of CD8$^+$ T-cell functional properties, potentially linked to intrinsic levels of T-bet expression and TCR avidity. The present study thus provides further insights into the role of the 2B4-CD48 interaction in the fine regulation of CD8$^+$ T-cell effector function upon antigenic stimulation.

Introduction

CD8^{+} T-cells are key players in the fight against viruses and tumors. In recent years, a number of studies have emphasized the importance of functional cellular properties, like cytolytic capacity and the ability to secrete soluble effector molecules (i.e. cytokines and chemokines), in dictating CD8^{+} T-cell efficacy in infectious or malignant settings [2-4]. The induction and regulation of potent effector functions is directly related to the level of activation received by T-cells upon engagement of their cognate antigen [5]. T-cell activation in turn depends on the multiple molecular interactions that occur between a T-cell and its cognate, epitope-bearing target. These interactions include: i) the binding of the TCR and CD8 co-receptor to the peptide-MHC class I complex (pMHCI) (reviewed in [6]); ii) engagement of co-stimulatory or co-inhibitory receptor/ligand pairs (reviewed in [7]); and, iii) the effect of soluble factors, such as cytokines [8-10], which collectively influence T-cell function. These different inputs, also known as Signal 1 (pMHCI antigen), Signal 2 (co-stimulation/co-inhibition) and Signal 3 (inflammatory cytokines), determine the choice between full T-cell activation and tolerance (reviewed in [11]).

While the specificity and strength (i.e. avidity) of the TCR/pMHCI interaction constitute the major features that determine the outcome of CD8^{+} T-cell activation and function, the contribution of TCR/pMHCI independent factors is less clear. It is therefore important to provide further insight into the regulation of CD8^{+} T-cell effector functions, including cytolysis and cytokine production, by various immunomodulatory cell surface molecules (Signal 2). CD8^{+} T-cells express multiple co-stimulatory/co-inhibitory receptors, designed to regulate their function [12-14]. In recent years, much work has focused on clarifying the role of co-inhibitory molecules such as PD-1 and CTLA-4 [15-17], but the exact role of a number of other cell surface receptors remains to be fully understood. In this report, we focused our attention on 2B4 (CD244). 2B4 is a member of the signaling lymphocyte activation molecule (SLAM) family, and has been implicated in the regulation of co-stimulation,

cytokine production and cytotoxic activity of CD8^{+} T-cells [18-20]. Its only known ligand in humans is CD48, a molecule broadly expressed on hematopoietic cells [21-23]. Interestingly, blockade of the 2B4-CD48 interaction has been shown to both enhance [24-26] and inhibit [27, 28] CD8^{+} T-cell responses in virally infected human subjects and various murine disease models. Thus, the 2B4-CD48 axis presents an intriguing case of "dual functionality" [29, 30], with both co-stimulatory or co-inhibitory effects on modulating T-cell function.

The interaction of co-stimulatory and co-inhibitory receptors with their cognate ligands culminates in the expression of genes specific to T-cell effector function. In addition, gene transcriptional activity is likely to be regulated at the epigenetic level. Recent evidence indicates that the distinct expression patterns of T-cell functional genes are indeed controlled by epigenetic modifications at the chromatin level [31-35]. Chromatin structure is dynamic and can be found in two basic states: an open chromatin (euchromatin) state that is accessible to DNA-binding proteins (e.g. transcription factors, transcriptional activators or repressors, and RNA polymerase) and thus facilitates active gene transcription; and a closed chromatin (heterochromatin) state that is non-permissive to transcriptional machinery and is therefore associated with gene silencing [36]. Several epigenetic markers (i.e. chemical modifications of DNA and histones) have been identified in association with specific chromatin states and transcriptional levels. For instance, covalent modifications of the amino-terminal tails of histones (e.g. histones H2A, H2B, H3 and H4) have been shown to regulate the chromatin state and consequently gene transcription [37-40]. Assessing the contribution of epigenetic changes to the control of genetic transcriptional profiles is thus very important in broadening our understanding of CD8^{+} T-cell effector function.

We therefore studied the role that the 2B4-CD48 interaction plays in regulating antigen specific CD8^{+} T-cell activity at the transcriptional, functional and epigenetic levels, using CD8^{+} T-cell

clones. This approach enabled us to investigate the impact of blocking the 2B4-CD48 pathway on the induction of cytolysis and cytokine production by CD8^{+} T-cells upon cognate antigen stimulation, while controlling for TCR diversity. Chromatin immunoprecipitation in combination with high-throughput sequencing (CHIP-Seq) was also performed to assess histone modifications. Specifically, we analyzed the trimethylation of H3K4 (H3K4me3) and H3K27 (H3K27me3), which are associated with an open chromatin state (active gene expression), and a closed chromatin state (repressed gene expression), respectively. This comprehensive study revealed a subtle positive fine-tuning of CD8^{+} T-cell effector functions by the 2B4-CD48 interaction at the clonal level.

Results

Expression of 2B4 on CD8^{+} T-cells with potent effector functions *ex vivo* and *in vitro*

Based on the expression of diverse cell surface receptors, such as CD45RA, CCR7, CD28 and CD27, the CD8^{+} T-cell population can be divided into distinct subsets, referred to as naïve, central memory (CM), and early, intermediate or late effector/memory (E-EM, I-EM, L-EM, respectively) cells [41]. Although there is ongoing debate as to the stimulatory or inhibitory influence of 2B4 on CD8^{+} T-lymphocyte function, its expression is known to be particularly strong on highly differentiated effector/memory CD8^{+} T-cells, which exhibit potent effector functions [13, 42, 43]. In comparison to other co-inhibitory (i.e. CD160, PD-1, Tim-3, BTLA, LAG-3 and CTLA-4) or co-stimulatory receptors (i.e. LIGHT, 4-1BB, CD28 and CD38), the expression of 2B4 on these cells is particularly striking (Figure 1a and [13, 43]). Of the receptors mentioned, 2B4 is the most abundant on the CD8^{+} T-cell surface (Figure 1a), and its expression is stable upon activation (Figure 1b). Based on this expression pattern, highly differentiated CD8^{+} T-cells may be a particularly relevant setting to study the influence of the 2B4-CD48 interaction on effector function upon TCR stimulation. However, evaluating the role of such interactions in *ex vivo* PBMC samples encounters difficulties relating to the heterogeneity of this population, in terms of gene expression and clonotypic composition. We therefore decided to study antigen-specific CD8^{+} T-cell clones expanded *in vitro*, that were representative of highly differentiated effector/memory CD8^{+} T-cells both in terms of phenotype and co-stimulatory and co-inhibitory receptor expression. For this purpose, we selected three previously characterized CD8^{+} T-cell clones, namely G12C, G10A and H8B [5, 44], specific for the HLA-B27-restricted HIV-derived epitope KK10 (p24 Gag$_{263-272}$; KRWIILGLNK). These clones displayed an intermediate effector/memory phenotype (Figure 1e), and a co-stimulatory and co-inhibitory receptor (in particular 2B4) expression pattern similar to that of intermediate/late effector/memory CD8^{+} T-cells (Figures 1c, 1d and 1f). These tools enabled us to assess the influence of the 2B4-CD48 interaction on the induction of effector CD8^{+} T-cell functions upon

stimulation with a cognate antigen at a clonal level.

Negative impact of blocking 2B4-CD48 signaling on effector function gene expression

We began by assessing the impact of blocking 2B4-CD48 signaling on the expression of genes relating to T-cell function, differentiation, and transcription factors, following TCR mediated activation. For this purpose, G12C, G10A and H8B were stimulated for 3 hours in the presence of KK10 peptide-pulsed HLA-matched targets (722.221; a human MHC class I-deficient B-cell line, transfected with HLA-B27 and verified for CD48 expression), before being purified by FACS sorting. The shorter 3 hour (as opposed to the conventional 6 hour) activation period was chosen to minimize mRNA degradation. Blockade of the 2B4-CD48 interaction was achieved by pre-incubating both CD8^{+} T-cell clones and antigen presenting cells with a saturating concentration of an anti-CD48 blocking antibody [13, 42, 43]. Gene expression profiles of FACS-sorted CD8^{+} T-cell clones were then generated using the Fluidigm BioMark™ platform technology, allowing for a high-throughput assessment of the expression of up to 96 genes by RT-PCR [45]. The 96 gene targets were selected based on their relevance to T-cell function [45]. Cluster analyses showed that, as expected, the transcription of a series of genes relating to effector function was triggered in all three clones upon antigenic stimulation (Figure 2a). Meanwhile, the expression levels of these genes were lowered upon 2B4-CD48 blockade (Figure 2a). The degree of gene upregulation upon stimulation (expressed as ΔE_t values, equivalent to 40-Ct) was then calculated in the presence or in the absence of the anti-CD48 antibody (Figure 2b). Overall, blocking the 2B4-CD48 interaction resulted in a modest reduction in effector molecule gene expression (e.g. IFNγ, TNF, MIP-1β, perforin and granzyme B), but without overriding the expression of genes following TCR signaling. Of note, while H8B upregulated fewer genes compared to G12C and G10A upon cognate antigenic stimulation (Figure 2a), blocking 2B4-CD48 signal transduction resulted in a more apparent decrease in the expression of active genes, particularly those encoding effector molecules and

receptors (Figure 2a and 2b). Overall, these data suggest that in our clonal system, 2B4 acts as a co-stimulatory receptor, by contributing to the optimal induction of effector functions in CD8^{+} T-cells.

Reduced CD8^{+} T-cell cytolytic capacity upon 2B4-CD48 interaction blockade

We also analyzed the effect of 2B4-CD48 blockade directly on the functional attributes of the three CD8^{+} T-cell clones upon antigenic stimulation, assessing their ability to produce effector cytokines and degranulate. Although blocking 2B4-CD48 had hardly any effect on the degranulation capacity of the clones (i.e. upregulation of CD107a upon activation; Figure 3a), it significantly reduced their cytolytic activity (i.e. lysis of peptide loaded target cells in FATAL assays; Figure 3b and 3c). The effect was especially apparent at the earlier time-point of 6 hours (Figure 3c). The negative impact on cytolytic capacity of 2B4-CD48 blockade was also clearer with H8B, and was still found at 12h (Figure 3b and 3c). The influence of 2B4-CD48 blocking on the production of the cytokines IFNγ, TNF, MIP-1β and IL-2 (assessed by intracellular cytokine staining upon antigenic stimulation), had negligible effect (Figure 3b). Taken together, these transcriptional and functional data denote a moderate but steady effect of blocking the 2B4-CD48 signal, and suggest that the 2B4-CD48 interaction contributes positively to the fine-tuning of CD8^{+} T-cell effector functions. This was more obvious with H8B, which was apparently more sensitive to 2B4-CD48 blockade compared to G12C and G10A, especially at the level of effector gene transcription and killing activity.

Epigenetic regulation of CD8^{+} T-cell effector function

We next used the ChIP-Seq approach to determine if clues relating to the subtle effects of blocking the 2B4-CD48 interaction seen at the functional level could be obtained from epigenetic analysis (i.e. by monitoring chromatin structure). Recent studies have shown a close relationship between gene expression and the distribution of chromatin histone methylation sites [31-35]. In particular, the trimethylation of H3K4 (H3K4me3) and H3K27 (H3K27me3) correlate positively and negatively

with gene transcription, respectively [31, 34]. In order to achieve a high level of accuracy, the general ChIP-Seq technique requires a high starting cell number (i.e. 1 to 10 x 10^6 depending on the affinity of Abs and efficiency of ChIP), which emphasizes the need to use *in vitro* expanded CD8$^+$ T-cell clones. Genomic H3K4me3 and H3K27me3 ChIP libraries (composed of ~270 bp DNA fragments) were constructed for the three CD8$^+$ T-cells clones in a resting state (due to the difficulty of predicting chromatin remodeling kinetics on activation) and sequenced using the Miseq system (Illumina). Non-IP libraries, obtained from each of the clones, were used for reference. In all clones, we found a high degree of H3K4me3 associated with the promoter regions of effector molecule-encoding genes, including *PRF1*, *GZMB*, *LAMP1*, and *TBX21* (Figure 4a), while there was little sign of H3K27me3 in these regions (data not shown). Our data showing the active transcription of these genes is similar to observations previously made in the human effector/memory CD8$^+$ T-cell subset [1, 31, 34, 35, 37]. In addition, we analyzed the promoter regions of genes related to the 2B4 co-stimulatory pathway. Besides genes encoding the 2B4 receptor (*CD244)* and ligand (*CD48),* we also focused on other genes implicated in 2B4-mediated signal transduction and its co-stimulatory function. Namely, we looked at the epigenetic regulation of *SH2D1A* and *FYN*, which encode the intracellular adaptor molecule SLAM-associated protein (SAP) and the Src-related kinase Fyn, respectively [46]. The promoter regions of these genes were enriched in H3K4me3 ChIP-Seq samples, indicating an open chromatin state and high levels of transcriptional activity (Figure 4b). In contrast, we observed no H3K27me3 in these regions (data not shown). In line with these observations, the clones had high basal levels of mRNA transcripts for these functional molecules, including *TBX21* and *SH2D1A* (Figure 4c). These results suggest that the effector functions of all CD8$^+$ T-cell clones used in our study, are poised for induction through both TCR and 2B4 mediated pathways.

A potential link between T-bet expression and CD8$^+$ T-cell TCR avidity

T-bet (also known as Tbx-21) is an important transcription factor, responsible for the regulation of gene expression levels of effector molecules such as granzyme B, perforin and IFNγ, upon TCR stimulation [47]. We thus explored the possibility of the subtle reduction in CD8^{+} T-cell function, observed following 2B4-CD48 blockade, being related to changes in T-bet expression. Similarly to *ex vivo* PBMC-derived effector/memory CD8^{+} T-cells (Figure 5a), all three CD8^{+} T-cell clones expressed constitutively high levels of T-bet (Figure 5b). We observed no impact of 2B4-CD48 blocking on *TBX21* (Figure 2b) and T-bet (Figures 5b and 5c) expression levels in the clones, indicating that 2B4 mediated co-stimulatory effect most likely does not operate by increasing T-bet expression. However, upon antigenic stimulation, T-bet expression was barely increased in H8B, contrasting with G12C and G10A (Figure 5b), in line with the *TBX21* transcriptional data (Figure 2b). Overall, poor T-bet upregulation, together with weak effector gene expression and cytokine secretion upon antigenic stimulation in H8B, imply a suboptimal TCR mediated activation of this clone compared to G12C and G10A. In line with its low responsiveness to the cognate antigen, H8B displays indeed a considerably lower TCR avidity than the two other clones, as assessed using tetramer dilution assays (Figure 5d)[44]. Collectively, these data suggest that the higher susceptibility of H8B to 2B4-CD48 blockade may be related to its lower TCR avidity and consequently ability of initiate T-bet expression. Whether 2B4-CD48 signaling may partly compensate for a weak upregulation of T-bet due to suboptimal activation upon cognate antigen engagement will therefore merit further investigation.

Discussion

The 2B4-CD48 interaction holds a complex biological role in T-lymphocytes. It has been reported to mediate both activatory and inhibitory effects on the induction of T-cell functions in mouse and human studies [24-30]. Highly differentiated CD8^{+} T-cells express high levels of 2B4 [13, 42, 43], which may be crucial for regulating the potent effector functions deployed by these cells. To gain further insight into the role of the 2B4-CD48 interaction in such effector/memory CD8^{+} T-cells, we have used CD8^{+} T-cell clones of defined specificity, TCR avidity and phenotype to study the impact of blocking the 2B4-CD48 signal upon stimulation with a cognate antigen. Changes in CD8^{+} T-cell effector activity were subsequently monitored at the transcriptional, functional and epigenetic levels. We report a subtle co-stimulatory effect of the 2B4-CD48 interaction on CD8^{+} T-cell function, in terms of effector molecule gene expression and in particular, cytotoxicity. Since the observed reduction in specific target cell lysis (following the disruption of the 2B4-CD48 interaction) could not be attributed to changes in either perforin expression (data not shown) or degranulation, it is possible that the effect can be explained by changes in signaling. It has been previously shown that the co-stimulatory role of 2B4 on T-cells is related to the levels of SAP expressed within these cells [46, 48], an intracellular signaling molecule associated with the control of cytotoxicity in CD8^{+} T-cells [49]. Furthermore, defective 2B4-SAP signaling was reported to result in the diminished capacity of CD8^{+} T-cells to lyse cognate target cells infected with Epstein-Barr virus (EBV), due in part to the suboptimal recruitment of perforin to lipid rafts and improper T-cell/target cell association [49]. The work presented here is in agreement with these reports, as our system of CD8^{+} T-cell clones not only presented high levels of *SH2D1A* SAP-encoding transcripts (together with an open chromatin state associated with the *SH2D1A* promoter), but also exhibited substantially reduced on-target cytotoxicity following the disruption of the 2B4-SAP signaling network. Interestingly, SAP-deficient CD8^{+} T-cells have been shown to express normal levels of

lytic molecules (including perforin) and effector cytokines (including IFNγ and IL-2), also consistent with our own findings on blocking the 2B4-CD48 interaction.

Although the co-stimulatory impact of the 2B4-CD48 interaction remained relatively modest in the present setting, our findings argue against the definition of 2B4 as a marker of T-cell dysfunction [30, 50, 51], especially in terminally differentiated CD8+ T-cells. We previously showed that the co-expression of 2B4 and PD-1 on HIV-specific CD8+ T-cells was associated with suboptimal cellular functionality [13, 42, 43, 52]. The CD8+ T-cell clones used in the present study expressed mainly 2B4, but not PD-1, like CMV-specific CD8+ T-cells, which display a more differentiated phenotype compared to HIV-specific CD8+ T-cells [53]. The co-expression of various co-stimulatory or co-inhibitory molecules certainly complicates the study of the contribution of a given receptor, and may explain divergent outcomes and conclusions. Our present findings are also in agreement with a recently published study, pointing towards the potential benefits of elevated 2B4 expression on the surface of cytolytic HIV-specific CD8+ T-cells [54]. Even though the number of clones studied in the present work was limited, our data point towards the possibility that strong TCR signaling and T-bet upregulation, for instance exhibited by high avidity CD8+ T-cells, may override the need for 2B4-CD48 mediated co-stimulation. The superior contribution of a strong TCR signal may in part explain the subtle effects on T-cell function observed upon blockade of the 2B4-CD48 interaction, but the more pronounced effect on the low TCR avidity clone with a weak capacity to upregulate T-bet upon activation. It is thus tempting to speculate that the co-stimulatory effect mediated by the 2B4-CD48 interaction may serve to compensate for a suboptimal TCR activation signal, for instance helping to optimize the functional response of effector CD8+ T-cells displaying low TCR avidity.

While the use of CD8^{+} T-cell clones presents obvious advantages over PBMC-derived *ex-vivo* samples when it comes to performing detailed assessment of T-cell functional activity, it also has a number of drawbacks. Firstly, the clones are not representative of the diverse CD8^{+} T-lymphocyte subsets encountered *in vivo*. Secondly, the rigorous *in vitro* propagation of CD8^{+} T-cells is likely to alter their intrinsic properties, compared to their *ex vivo* clonotypic equivalents. It would therefore be important to perform similar studies on *ex vivo* isolated effector/memory CD8^{+} T-cell subsets, differing in their surface 2B4 expression levels. Finally, the 2B4-CD48 interaction may exert a different influence over the function of CD8^{+} T-cells at an earlier stage of differentiation, as suggested by a previous study [30], which could further explain the subtle effect observed in a setting of highly differentiated CD8^{+} T-cell clones. Accordingly, the chromatin structure and permissive epigenetic signatures for given genes have also been shown to change with T-cell differentiation [31]. In summary, we used here a combination of functional, transcriptional and epigenetic approaches to create a comprehensive picture of the parameters affecting the functional activity of human CD8^{+} T-cell clones. The present study provides further insights into the co-stimulatory role of the 2B4-CD48 interaction, engaged to fine-tune CD8^{+} T-cell effector function upon antigenic stimulation.

Methods

PBMCs and CD8^{+} T-cell clones. Peripheral blood mononuclear cell (PBMC) samples were obtained from healthy individuals with the approval by the relevant local institutional (at the Hospital Pitie Salpetriere) review board and ethics committee, and informed consent in accordance with the Declaration of Helsinki. CD8^{+} T-cell clones (G12C, G10A and H8B) specific for the HLA-B*2705 (HLA-B27 from hereon)-restricted p24 Gag epitope KK10 (KRWIILGLNK, Gag$_{263-272}$) were generated and expanded as described previously [5, 44]. An HLA-B27^{+}, CD48^{+} Epstein–Barr virus (EBV)-transformed 722.221 B-cell line was used to present exogenous KK10 peptide antigen in activation assays.

Flow cytometry reagents. Directly conjugated monoclonal antibodies (mAbs) were purchased from commercial sources as follows: anti-CD8-allophycocyanin-cyanine7 (APC-Cy7), anti-CD45RA-V450, anti-CCR7-phycoerythrin (PE)-Cy7, anti-CD107a-PE-Cy5, anti-IFNγ-Alexafluor (AF)700, anti-TNF-PE-Cy7, anti-CTLA-4-APC, anti-BTLA-PE and anti-41BB-PE-Cy5 (BD Biosciences, Franklin Lakes, NJ, USA); (ii) anti-CD3-ECD and anti-2B4-PE (Beckman Coulter, Brea, CA, USA); (iii) anti-CD27-BV570, anti-2B4-APC and anti-CD28-AF700 (Biolegend, San Diego, CA, USA); (iv) anti-T-bet-AF647, anti-LIGHT-PE and anti-CD160-AF488 (eBiosciences, San Diego, CA, USA); (v) anti-MIP-1β-fluorescein isothiocyanate (FITC), biotinylated anti-PD-1, anti-Tim-3-AF700 and anti-LAG-3-FITC (R&D Systems, Abingdon, UK); anti-IL-2-APC (Miltenyi Biotec, Bergisch Gladbach, Germany); and, (vi) anti-CD38-ECD (Life Technologies, Carlsbad, CA, USA). The amine-reactive viability dye Aqua (Life Technologies) was used to eliminate dead cells from the analysis. Intracellular staining for T-bet was performed using the Transcription Factor Buffer Set (BD Pharmingen, Franklin Lakes, NJ, USA), according to the manufacturers instructions. Staining with all other reagents was conducted according to standard procedure [55]. Data were acquired using a Fortessa flow cytometer (BD Biosciences) and analyzed

with FlowJo software version 9.3.7 (TreeStar Inc, Ashland, OR, USA). Sorting of viable, CD8^{+} T-cells was performed on a FACSDiva (BD) software (v8.0). A representative example of the gating strategy is shown in Supplemental Figure 1.

Transcriptional analysis. Prior to assessing changes in gene expression, CD8^{+} T-cell clones and HLA-B27^{+} 722.221 target cells were treated separately with 5μg/ml purified anti-CD48 blocking mAb (eBiosciences) for 1 hour at 37°C/5% CO_2. Without removing the blocking antibody, anti-CD48 treated and untreated CD8^{+} T-cells and 722.221 target cells were combined in an effector to target (E:T) ratio of 1:5, and incubated for 3 hours at 37°C/5% CO_2. Upon completion of the activation period, viable, CD3^{+}, CD8^{+} T-cells were sorted by flow cytometry (100 cells/well) into duplicate assay wells containing reverse transcription (RT)-PCR reaction and cDNA synthesis mix. Transcripts were amplified using the SuperScript III Platinum One-Step qRT-PCR system (Invitrogen), according to manufacturer's instructions. Quantification of gene expression was performed as described in [45], using the Fluidigm BioMark™ platform. Information on the primers and probes that were used in this study is provided in supplemental table 1. For ease of data interpretation, "Et" (threshold expression) values were used in the manuscript; these values correspond to 40-Ct, and thus increase with greater expression of a transcript; they are proportional to log2 RNA abundance. The two ways hierarchical clustering analysis was performed using JMP version 10.

Intracellular cytokine staining (ICS). Prior to stimulation, a portion of CD8^{+} T-cells and 722.221 cells were subjected to CD48/2B4 blockade, as described in the gene expression assessment methods section. The CD8^{+} T-cell clone and HLA-B27^{+} 722.221 target cells, pulsed with 1μM KK10 peptide or media alone, were subsequently incubated together at an E:T ratio of 1:5 for the first 0.5 hours with an anti-CD107a-PE-Cy5 antibody and a further 2.5 hours in the additional

presence of monensin (2.5µg/mL; Sigma-Aldrich, St. Louis, MO, USA) and brefeldin A (5µg/mL; Sigma-Aldrich) at 37°C/5% CO_2. The cells were collected and the staining for intracellular markers (TNF, IFNγ, MIP-1β and IL-2) was performed as described previously [5]. Briefly, intracellular cytokine/chemokine expression and surface CD107a upregulation were determined on CD3^{+}, CD8^{+} T-lymphocytes, following exclusion of dead cells and doublets. Data were acquired using a Fortessa flow cytometer (BD Biosciences) and analyzed with FlowJo software version 9.3.7 (TreeStar Inc.).

Fluorometric assessment of T-lymphocyte antigen-specific lysis (FATAL) cytotoxicity assay. This assay is an adaptation of a previously published protocol [56]. In order to measure specific lysis by the CD8^{+} T-cell clones, target HLA-B27^{+} 722.221 cells were pulsed with 1µM KK10 peptide and labeled with 10µM Pacific blue succinimidyl ester (PBSE) dye to allow for flow cytometric separation from the PBSE^{-} control cells, which were incubated with media alone and left unstained. Target and control 722.221 cells, and CD8^{+} T-cell clones were treated separately with the anti-CD48 blocking antibody for 1 hour, or incubated with media alone. Pulsed PBSE^{+} and an equal number of unpulsed PBSE^{-} 722.221 cells were subsequently combined with specific CD8^{+} T-cells, giving a final E:T ratio of 5:1. Control assays containing either no effectors, or a non-cognate CD8^{+} T-cell population, cultured in equivalent conditions, were set up in parallel. Following a 6 or 12 hour assay period, the cells were harvested, stained with fluorescently-conjugated mAbs and acquired using a Fortessa flow cytometer (BD Biosciences). The flow cytometric assessment of lytic activity was based on the quantification of PBSE-labeled target cell elimination relative to internal PBSE^{-} control cells.

ChIP-Seq. The standard ChIP-Seq procedure has been previously described [1, 31, 37]. Briefly, 10^5 CD8^{+} T-cells were sorted by flow cytometry on the basis of CD8 expression and viability, prior to

treatment with micrococcal nuclease (MN)ase enzyme (2 units; cat no. N3755, Sigma-Aldrich) and subjected to rounds of sonication by Branson 250 digital sonifier (Branson), in order to obtain mononucleosomes. The suspension of mononucleosomes was subsequently dialyzed to remove impurities and increase concentration, followed by immunoprecipitation (IP) with either an anti-H3K4Me3 antibody (ChIP Grade ab8580; Abcam) or H3K27Me27 (ChIP Grade ab6002; Abcam). A non-IP control was included to assess the level of background. The IP-DNA was separated from the histone proteins, chemically fragmented and used to construct barcoded Illumina Truseq libraries. Libraries were size-selected (~270 bp fragments, including adaptor), quantified, pooled, and sequenced on an Illumina Miseq in a 2×50-base paired-end, indexed run. The mapping statistics for ChIP were as follows: for ChIP-Seq with anti-H3K4Me3, total 6,612,833 reads for G10A, total 7,186,721 reads for G12C and total 6,526,057 reads for H8B, and uniquely mapped reads (5,408,938 reads for G10A (81.7%), 5,858,449 reads for G12C (81.5%) and 5,352,067 reads for H8B (82%)); for ChIP-Seq with anti-H3K27Me3, total 4,630,791 reads for G10A, total 6,265,493 reads for G12C and total 6,857,447 reads for H8B, and uniquely mapped reads (3,362,708 reads for G10A (72.6%); 4,974,573 reads for G12C (79.3%) and 4,642,052 reads for H8B (67.6%)). The SICER algorithm [57] was used for peak calling with window-size of 200 bps, gap-size of 600bps for H3K27me3 and 400bps for H3K4me3, and a FDR (false-discovery rate) of < 0.01 was used identify stringent H3K4Me3-binding or H3K27-binding peaks. After peak calling, the statistics were as follows: for ChIP-Seq with anti-H3K4Me3, total 1,682,404 (31% of the uniquely mapped) enriched reads for G10A, total 2,042,792 (34.9% of the uniquely mapped) enriched reads for G12C and total enriched 1,700,074 (31.76% of the uniquely mapped) reads for H8B; for ChIP-Seq with anti-H3K27Me3, total 588,407 (17.5% of the uniquely mapped) enriched reads for G10A, total 826,236 (16.6% of the uniquely mapped) enriched reads for G12C and total 855,751 (18.4% of the uniquely mapped) enriched reads for H8B. The sequence reads and

expression data have been deposited in the NCBI Short Reads Archive (Reference number: SRP051786).

Tetramer dilution assay. TCR avidity was measured using tetramer dilution assays. Briefly, CD8^{+} T-cell clones were incubated with cognate tetramer at a range of concentrations (10 μg/mL to 0.0015 μg/mL in 1/3 dilutions) for 30 minutes at 4°C, then stained for CD8 expression before fixation. The percentage and fluorescence intensity of tetramer^{+} CD8^{+} T-cells at each tetramer concentration was determined by flow cytometry.

Statistical analysis. Comparisons between groups were performed using the non-parametric Wilcoxon signed rank test in Prism 5 (GraphPad). P values <0.05 were considered significant.

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Supplementary information is available on the Journal of Immunology and Cell Biology website.

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Figure Legends

Figure 1. Immunomodulatory molecule expression on CD8^{+} T-cells. The pattern of co-inhibitory and co-stimulatory receptor expression on resting and activated PBMCs, averaged over three different donors (**a** and **b**) and CD8^{+} T-cell clones G12C (black), G10A (grey) and H8B (white) (**c** and **d**). Non-specific stimulation of PBMC samples was achieved using anti-CD3 and anti-CD28 antibody-coated microbeads for 3 hours. Immunomodulatory molecule expression was measured by flow cytometry, gating on the viable, single-cell CD3^{+}, CD8^{+} T-cell population. In parallel, CD8^{+} T-cell clones were stimulated for 3 hours with HLA-B27^{+} 722.221 target cells, presenting exogenously pulsed-on 1μM KK10 peptide. Phenotypic profiling of the CD8^{+} T-cells was achieved by evaluating the expression of the markers CD27, CD28, CD45RA and CCR7. (**e**) Representative flow cytometry plots showing the phenotype of one of the CD8^{+} T-cell clones (G12C, black dots) overlaid onto the phenotype of a reference CD8^{+} T-cell population within a healthy donor PBMC sample (grey density plot). (**f**) A staggered flow cytometry histogram plot showing the MFIs (indicated by numbers on the right) of surface 2B4 staining for clones G12C, G10A and H8B compared to reference PBMC-derived CD8^{+} T-cell population at various stages of differentiation. CM; central memory subset, E-EM, I-EM and L-EM; early, intermediate and late effector memory subsets, respectively. Representative line graphs from a single experiment out of five independent experiments shown (**a** and **b**). Error bars indicate SD from the mean of three independent experiments (**c** and **d**).

Figure 2. The effect of blocking the 2B4/CD48 interaction on the transcriptional profile of CD8^{+} T-cell clones G12C, G10A and H8B. (**a**) Heat map representation of the transcriptional signature for the G12C, G10A and H8B clones. The black frame highlights the impact of blocking the 2B4/CD48 interaction on the expression of genes relating to CD8^{+} T-cell function, whose levels are altered upon antigenic stimulation (3 hour stimulation with HLA-B27^{+} 722.221 target cells

presenting 1μM KK10 peptide). (**b**) ΔEt graphs for selected genes, providing a quantitative measure for the impact of 2B4/CD48 blockade on the expression of genes associated with CD8^{+} T-cell effector function. Assays were set up in parallel using CD8^{+} T-cell clones and 722.221 target cells that had been pre-treated (for 1 hour) with an anti-CD48 blocking antibody, or left untreated. Transcriptional analysis was performed on duplicate FACS sorts of 100 cells/well. Error bars indicate SD from the mean of two replicates.

Figure 3. The influence of blocking the 2B4/CD48 interaction on CD8^{+} T-cell effector functions and cytotoxicity. (**a**) Degranulation (upregulation of CD107a) by the CD8^{+} T-cell clones G12C, G10A and H8B following a 3 hour stimulation with HLA-B27^{+} 722.221 cells presenting 1μM KK10 peptide, either following treatment with an anti-CD48 blocking antibody (administered individually to CD8^{+} T-cells and HLA-B27^{+} 722.221 target cells for 1 hour, prior to initiation of the activation assay) or media alone. (**b**) Representative FATAL cytotoxicity assay histograms (from a single experiment reproduced two times) showing the lysis of the 1μM KK10-pulsed HLA-B27[+] 722.221 target cells (PBSE[+] peak) relative to an internal unpulsed 722.221 control population (PBSE[-] peak), during a 6 hour incubation period, following pre-treatment with an anti-CD48 antibody or in the absence of CD48 blocking. Specific lysis by clones G12C, G10A and H8B was assessed against a reference assay containing no CD8^{+} T-cells. (**c**) Specific lysis (%) of the 722.221 target population by clones G12C, G10A and H8B, averaged over two separate 6 hour and two 12 hour experiments. (**d**) Effector cytokine and chemokine production by clones G12C, G10A and H8B (averaged over four independent assays), measured by ICS following a 3 hour period of stimulation with HLA-B27[+] 722.221 cells presenting 1μM KK10 peptide, with or without CD48 blocking. Error bars (for **a**, **c** and **d**) indicate SD from the mean of three independent experiments. Statistical comparisons between groups +/- anti-CD48 blocking (**a**, **c** and **d**) were performed using

the Wilcoxon signed rank test. P values <0.05 were considered significant. No statistical significance was achieved for data presented in **a** and **d**.

Figure 4. Epigenetic and transcriptional profiling for selected genes, performed on resting CD8^{+} T-cell clones G12C, G10A and H8B. H3K4Me3 ChIP-Seq data, showing an open chromatin state at the promoters of genes encoding molecules associated with (**a**) CD8^{+} T-cell effector function (*PRF1*; perforin, *GZMB*; granzyme B, *LAMP1*; LAMP1/CD107a and *TBX21*; T-bet) and (**b**) the 2B4/CD48 signaling pathway (*CD244*; 2B4, CD48, *SH2D1A*; SAP, and Fyn) (**c**) Basal expression of selected genes associated with CD8^{+} T-cell function. Error bars indicate SD from the mean of two replicates.

Figure 5. The effect of 2B4/CD48 blockade on T-bet expression by resting and activated CD8^{+} T-cells. (**a**) Representative flow cytometry histograms (showing data from one of two independent experiments), showing intracellular T-bet expression within phenotypically defined populations of CD8^{+} T-cells (in a PBMC sample) and CD8^{+} T-cell clones. EM; effector memory, L-EM; late effector memory, FMO; fluorescence minus one. (**b**) A flow cytometry plot (representative of two independent experiments) showing the increase in T-bet expression by a CD8^{+} T-cell clone (G12C used as an example) on activation with 722.221 target cells presenting cognate KK10 antigen. (**c**) Assessment of T-bet expression by resting or antigen-stimulated CD8^{+} T-cell clones G12C, G10A and H8B in the presence or absence of an anti-CD48 blocking antibody. (**d**) KK10/HLA-B27 tetramer titration curves for clones G12C, G10A and H8B, used as a measure of TCR avidity. Error bars indicate SD from the mean of two independent experiments.