Simultaneous TCR and CD244 Signals Induce Dynamic Downmodulation of CD244 on Human Antiviral T Cells

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J Immunol published online 2 August 2013
http://www.jimmunol.org/content/early/2013/08/01/jimmunol.1300435
Simultaneous TCR and CD244 Signals Induce Dynamic Down modulation of CD244 on Human Antiviral T Cells

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Various cosignaling molecules on T cells can contribute to activation, inhibition, or exhaustion, depending on context. The surface receptor signaling lymphocytic activation molecule (SLAM) family receptor CD244 (2B4/SLAMF4) has been shown to be capable of either inhibitory or enhancing effects upon engagement of its ligand CD48 (SLAMF2). We examined phenotypes of CD8 T cells from HIV+ and HIV− human donors, specific for HIV and/or respiratory syncytial virus. Cultured and ex vivo CD8 T cells expressed PD−1, CD244, and TIM−3. We found that ex vivo CD8 T cells downregulated CD244 in response to superantigen. Furthermore, cognate peptide induced rapid downregulation of both CD244 and TIM−3, but not PD−1, on CD8 T cell clones. CD244 downregulation required simultaneous signaling via both TCR and CD244 itself. Using a pH-sensitive fluorophore conjugated to avidin–Ab tetramers, we found that CD244 crosslinking in the presence of TCR signaling resulted in rapid transport of CD244 to an acidic intracellular compartment. Downregulation was not induced by PMA–ionomycin, or prevented by PI3K inhibition, implicating a TCR-proximal signaling mechanism. CD244 internalization occurred within hours of TCR stimulation and required less peptide than was required to induce IFN−γ production. The degree of CD244 internalization varied among cultured CD8 T cell lines of different specificities, and correlated with the enhancement of IFN−γ production in response to CD48 blockade in HIV+, but not HIV−, subjects. Our results indicate that rapid CD244 internalization is induced by a two-signal mechanism and plays a role in modulation of antiviral CD8 T cell responses by CD48–CD244 signaling. The Journal of Immunology, 2013, 191: 000–000.

To protect against infections and tumors while avoiding autoimmune and inflammatory pathological changes, T cells are regulated by complex networks of activating and inhibitory costimulatory/cosignaling molecules. The activity of each cosignaling receptor is regulated by factors such as posttranslational modification, expression of secondary signaling molecules, concentration of cognate ligands, and coexpression of other cosignaling receptors. Chronic viral infection can lead to progressive exhaustion of T cells. Exhausted T cells, including those found in HIV+ subjects, are defective in many antiviral functions and express progressively higher levels of markers, including PD−1, CD160, LAG−3, TIM−3, and CD244 (1). In some cases, blockade of one or more of these receptors can reverse the effects of exhaustion and restore immune function (2), thus proving that PD−1, for example, is not only a marker but also a mediator of T cell dysfunction in chronic infection. CD244 (2B4/SLAMF4) presents an interesting case of “dual functions,” as blockade of CD244 signaling has been shown to enhance or inhibit T cell responses in different contexts. The surface receptor signaling lymphocytic activation molecule (SLAM) family of receptors (3, 4) consists of eight type I transmembrane proteins and one GPI-linked receptor (CD48/SLAMF2). With the exceptions of CD48 and CD244, which in humans are thought to be the unique cellular ligands for each other, SLAM family receptors bind in a homophilic manner, such that each receptor is the unique cellular ligand for itself. SLAM family receptors play many roles in regulating the differentiation and effector functions of hematopoetic cells (3). Blockade of CD244–CD48 interactions in vitro has been variously shown to enhance or inhibit (5) antiviral T cell responses in the PBMCs of subjects with different viral infections.

All of the transmembrane SLAM family receptors for which intracellular signaling pathways have been identified can signal via the SLAM-associated protein (SAP) (3). SAP is encoded by SH2D1A, which is located on the X chromosome, and various mutations of SH2D1A cause X-linked lymphoproliferative disease (XLP) in male children, the hallmark of which is an inability to control EBV infection (6). The failure to control EBV infection is due to an inability of CTLs to lyse EBV-infected B cells, which express high levels of ligands for SLAM family receptors expressed by CTLs. Schlaphoff et al. (7) reported that the degree of enhancement or inhibition of specific clonotypes of antiviral T cells by CD244–CD48 blockade correlated with the respective degree of SAP expressed by those clonotypes. This result implies that the effect of CD244 expression on T cell function is controlled both by the expression of CD48 and by the expression of an internal signaling molecule (SAP). CD244 can be recruited to the immune synapse in NK cells (8) and CD8 T cells (9). Recently, Zhao et al. (10) reported that SLAMf6 (also known as Ly109 in

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Received for publication February 13, 2013. Accepted for publication June 29, 2013.

This work was supported by the Terry and Susan Ragon Foundation; National Institutes of Health/National Institute of Allergy and Infectious Diseases Grants IR56AI095088 and 5R01AI130914 (to D.G.K.), P01 AI-080192 (to D.E.K. and D.G.K.), and P30 AI060354 (Harvard University Center for AIDS Research); and a Research Scholar Career Award of the Quebec Health Research Fund (Fonds de recherche du Québec—Santé) (to D.E.K.).

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Abbreviations used in this article: B-LCL, B lymphoblastoid cell line; ICS, intracellular cytokine staining; MFI, median fluorescence intensity; RSV, respiratory syncytial virus; SAP, SLAM-associated protein; SLAM, surface receptor signaling lymphocytic activation molecule; XLP, X-linked lymphoproliferative disease.
mice and NBTA in humans) can localize to the murine T cell synapse and modulate T cell signaling by differential recruitment of SHP-1 (Src homology region 2 domain–containing phosphatase-1).

For the current study, we investigated cosignaling receptor expression on CD8 T cells isolated from the blood of HIV+ or HIVneg subjects and specific for a nonpersistent pathogen [respiratory syncytial virus (RSV)] or a chronic persistent pathogen (HIV). We found that cultured antiviral CD8 T cells expressed varying levels of PD-1, TIM-3, and CD244. Upon recognition of cognate Ag, some T cells rapidly downregulated expression of TIM-3 and CD244. We found that CD244 downregulation in cultured or in ex vivo human CD8 T cells required simultaneous signaling both via the TCR and via CD244 itself and involved relocation of CD244 into an acidic intracellular compartment. Consistent with previous findings for other viruses, blockade of CD244–CD48 interaction had a dual effect of either enhancing or inhibiting various CD8 T cell clonotypes. On the basis of a comparison of CD244 expression on responding T cells in the presence or absence of CD48 blockade, we present evidence that the degree of enhancement or inhibition in the IFN-γ response to Ag correlated with the degree of CD244 downmodulation in response to TCR stimulation.

Materials and Methods

Human subjects

Peripheral blood was obtained by venipuncture with acid-citrate-dextrose anticoagulant from HIV-infected and uninfected individuals at the Massachusetts General Hospital (Boston, MA). Written informed consent was obtained from all volunteers prior to enrollment in the study. Buffy coats were obtained from normal human donors via the Massachusetts General Hospital blood bank. The study was approved by the Massachusetts General Hospital Institutional Review Board. All subjects were averse at the time of blood draw. PBMCs from HIV-infected individuals were isolated by Ficoll density gradient centrifugation within 6 h of blood draw and cryopreserved in the presence of 10% DMSO in a liquid nitrogen freezer. PBMCs from buffy coats were isolated by Ficoll density gradient centrifugation within 24 h of blood draw and were not frozen.

Abs

For flow cytometry the following directly conjugated mAbs were used: CD3–Alexa Fluor 700 (clone UCHT1; BD), CD8–Qdot 605 (clone 3B5; Invitrogen), CD244–PeCy5.5 (clone C1.7; BioLegend), PD1–V421 (clone EH12.2H7; BioLegend), IFN-γ–PeCy7 (clone B27; BD), CD19–APC.Cy7 (clone SJ25C1; BD), CD14–APC.Cy7 (clone MqP9; BD), CD4–APCCy7 (clone SK3; BD), TIM3–PE (clone 344823; R&D Systems). For blockade, and L-glutamine). IL-2 (50 IU/ml) was added on day 3. Specificity for cognate peptide was tested on day 10 by IFN-γ ELISA. Lines that produced IFN-γ >1000 U/ml were further tested by in vitro proliferation assay. Lines that did not produce IFN-γ were considered nonresponsive. Cells were sorted at one cell per well into 96-well plates using a FACSAria cell sorter (BD) in the Ragon Cytometry Core. Single cells were propagated by stimulation with anti-CD3 (clone 12F6) in the presence of irradiated allogeneic PBMCs.

Intracellular cytokine staining

Cells were incubated with peptide or with Ag-loaded B lymphoblastoid cell line (B-LCL) for 1 h in R10, and then overnight in R10 plus 10 μg/ml brefeldin A. Cells were stained for surface markers (CD3, CD8, CD14, CD4, CD19, CD244, TIM-3, PD-1), fixed, permeabilized, and stained for internal IFN-γ. Flow cytometry data were acquired using a BD LSR II cytometer and were analyzed with FlowJo software (TreeStar).

Internalization assay

The fluorogenic pH sensor pHrodo Red Avidin was purchased from Invitrogen. The pHrodo Red Avidin was mixed with specific or control Ab at a molar ratio of 1:4 in PBS/1% BSA, then incubated overnight at 4˚C. The mixture was briefly centrifuged to remove any precipitates and then added to the cell suspension at the indicated dose in R10 medium plus 10 μg/ml brefeldin A in the presence or absence of PHA. Cells were incubated at 37˚C for the indicated time, and pHrodo fluorescence was determined by flow cytometry. Flow cytometry data were acquired using a BD Fortessa cytometer with a 561-nm excitation laser, and were analyzed with FlowJo software (TreeStar).

ELISA

ELISA was used to screen Ag-specific CD8 T cell lines and clones, as described. Soluble IFN-γ production by T cell lines was quantified using the Human IFN-γ OpELISA ELISA Set (BD). CTL lines were stimulated with either medium alone or 5 μM cognate peptide overnight, and supernatants were harvested and tested for IFN-γ content, according to the manufacturer’s instructions.

Statistical methods

Paired quantitative variables were compared by the Wilcoxon signed-rank test. Correlation was calculated using the Spearman correlation test. All calculations were performed using GraphPad software version 6, and a difference or a correlation was considered significant when p < 0.05.

Results

In light of published reports of activation-induced internalization of CD244 on NK cells, and incorporation of SLAM family receptors into the T cell synapse, we decided to investigate dynamic changes in surface marker expression on human T cells. When ex vivo PBMCs were stimulated with the superantigen SEB in an IFN-γ ICS assay, we observed that the SEB-responsive cells expressed less CD244 than did the nonresponding cells (not shown), consistent with preferential response to SEB by CD244low cells and/or with CD244 downregulation in conjunction with T cell activation.

To investigate further, we endeavored to stimulate T cells under conditions in which the TCR stimulus was delivered by APC expressing high levels of the CD244 ligand, CD48. CD48 was highly expressed on B-LCLs (not shown). Therefore, we stimulated PBMCs from HIV+ and HIVneg donors in the presence or absence of CD48 blockade, with autologous B-LCLs that were loaded or not with SEB, and washed to remove unbound superantigen (Fig. 1). After overnight stimulation, we assessed specific IFN-γ production and CD244 expression by ICS. The majority of CD8 T cells that produced IFN-γ in response to SEB were CD244low in the absence of blockade, but CD244high in the presence of CD48 blockade (Fig. 1A), consistent with CD48-dependent down-regulation on SEB-reactive T CD8 cells. We repeated the SEB stimulation, using PBMCs from six different HIV+ donors, and four HIVneg donors, and determined the level of CD244 expression in the presence or absence of CD48 blockade (Fig. 1B). In each case, the level of CD244 expression on SEB-reactive CD8 T cells was higher in the presence of CD48 blockade than without blockade, and the difference was significant (p < 0.02, Wilcoxon signed-rank test). No effect of TCR stimulation on CD244 expression was observed for CD4 T cells (not shown). The degree of...
CD244 downregulation in this assay was independent of the amount of PD-1 expression, and PD-1 expression was not affected by SEB stimulation. Ex vivo TIM-3+ cells failed to produce IFN-γ in this assay (not shown).

We considered that the observed change in median fluorescence intensity (MFI) of CD244 on IFN-γ+ T cells in the presence of CD48 blockade might be due to disinhibition of SEB-reactive CD244+ T cells and subsequent recruitment of CD244high cells into the IFN-γ+ population. Therefore, we compared the percent IFN-γ+ SEB-reactive CD8 T cells in the presence or absence of CD48 blockade. As shown in Fig. 1B, no significant change was observed in the percent of CD8 T cells responding to SEB in the presence or absence of blockade. Overall, these results are consistent with CD244 downmodulation being induced by combined signaling via SEB-reactive TCR and CD244 itself.

To investigate dynamic changes in marker expression on CD8 T cell lines specific for viral peptides, we stimulated PBMCs from an HIV-infected subject with synthetic peptides corresponding to either of two epitopes: RSV-YY9 or HIV-YT9. After 10 d in culture, we performed ICS with autologous B-LCLs loaded with cognate peptide in the presence or absence of CD48 blockade, and measured the amount of CD244 expressed on specific CTLs (Fig. 2). In the absence of CD48 blockade, the RSV-YY9–specific CTLs expressed very low levels of CD244, whereas in the presence of CD48 blockade, they expressed higher levels of CD244, consistent with CD48-mediated CD244 downregulation. In contrast, HIV-YT9–specific CTLs expressed high levels of CD244 even in the absence of CD48 blockade, and CD48 blockade resulted in a small increase in CD244 expression compared with that observed in the YY9–specific line. PD-1 expression was not affected by peptide stimulation or by CD48 blockade. To investigate whether T cells of the same clonotype would respond consistently over repeated tests, we conducted seven independent experiments all using PBMCs from the same donor. The observed effects on CD244 expression for YY9- and YT9-specific cells were consistent in all experiments (Fig. 2B). As for Fig. 1 above, we determined that CD48 blockade did not have a consistent or significant effect on the percent of specific IFN-γ+ cells for YY9- or YT9-specific lines from this donor. Thus, Fig. 2 shows that antiviral T cells of different clonotypes/specificities differed in their tendency to downregulate CD244 in response to cognate peptide. Notably, this difference could not be attributed to differences in CD48 expression on the APC, because identical autologous B-LCLs were used as APCs for all stimulations.

To conclusively demonstrate CD48-dependent CD244 downmodulation on antiviral CD8 T cells, we investigated these effects at the level of an individual T cell clone. An RSV-YY9 line comparable to those shown in Fig. 2 was generated from the blood of an HIV+ subject. To produce a clone, the line was stimulated with cognate peptide and live sorted for peptide specificity, using IFN-γ capture at a concentration of one cell per well, and specific clones were propagated and screened for specificity (not shown). We used the specific clones to investigate the relationship between receptor downregulation and signaling via TCR and CD244.

When a representative clone specific for RSV-YY9 was stimulated with cognate peptide in an overnight ICS assay, expression of CD3 and that of CD8 were both reduced, as expected for activated T cells. In addition, surface expression of CD244 was almost entirely eliminated. Likewise, expression of TIM-3 was also strongly reduced. In contrast, expression of PD-1 was not reduced (Fig. 3A, 3B). We compared these parameters for a panel of autologous CTL clones of varying specificities (not shown) and found that some degree of downregulation of CD244, TIM-3, CD8, CD3 was apparent for all clones, although downregulation of CD244 on the RSV-YY9 clone was the most extreme.

To determine the respective roles of signaling through TCR and CD244 in receptor downmodulation, we stimulated an RSV-YY9

![FIGURE 1. Ex vivo TCR- and CD48-induced downregulation of CD244, but not PD-1. PBMCs from HIV+ and HIVneg donors were cocultured overnight with autologous B-LCL in the presence or absence of SEB and isotype IgG or CD48 blockade. Cells were stained for surface phenotype and IFN-γ expression by ICS. (A) Zebra plots show the levels of CD244 and PD-1 on CD8 T cells for a representative subject. (B) The SEB stimulation was repeated for six HIV+ and four HIVneg donors. The respective MFIs of CD244 and PD-1 in the IFN-γ+ gate were determined. The percent of CD8 T cells responding to SEB in the presence or absence of CD48 blockade was also determined. Significance was assessed by the Wilcoxon signed-rank test; *p < 0.02. Data shown represent results of two independent experiments.](http://www.jimmunol.org/)

CD244 downregulation in this assay was independent of the amount of PD-1 expression, and PD-1 expression was not affected by SEB stimulation. Ex vivo TIM-3+ cells failed to produce IFN-γ in this assay (not shown).
clone with cognate peptide or with PHA (both of which activate the clone via the TCR), or with PMA–ionomycin (which bypasses the TCR), or with PMA–ionomycin plus cognate peptide. As shown in Fig. 3B, CD244 and TIM-3 were both strongly downregulated in response to cognate peptide and weakly downregulated in response to PHA. TIM-3, but not CD244, was also lost from the cell surface in response to PMA–ionomycin treatment. Although PMA–ionomycin treatment did not enhance or inhibit CD244 expression, it also did not inhibit downregulation, as shown by the fact that simultaneous stimulation with PMA–ionomycin and cognate peptide did induce CD244 downregulation (Fig. 4A). Loss of surface expression of CD244 was observed whether cognate peptide was added directly to CTL clones or whether peptide was presented by autologous B-LCLs (data not shown). As expected, downregulation of CD244, but not TIM-3, was specifically prevented by CD48 blockade (Fig. 4B). These results prove that CD244 was downregulated in response to CD8 T cell activation and that simultaneous signals transmitted by TCR and CD244 itself were required.

We next performed a timecourse experiment to determine the kinetics of CD244 downregulation on a CTL clone specific for RSV1-YY9. As shown in Fig. 5A, significant CD244 downregulation occurred during the first 2 h of peptide exposure, with the majority of downregulation occurring within the first 6 h of stimulation with cognate peptide. CD244 expression was not restored

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**FIGURE 2.** Ag-specific TCR- and CD48-induced downregulation of CD244 in cultured T cell lines. (A) PBMCs from an HIV+ donor were stimulated with optimal peptides RSV-YY9 or HIV-YT9 and expanded for 10 d in culture. On day 10, lines were stimulated with autologous B-LCL loaded with cognate peptide in the presence or absence of CD48 blockade, as shown. Cells were stained for surface phenotype and IFN-γ expression by ICS. The experiment was repeated seven times with PBMCs from the same donor. A representative experiment is shown. (B) Fold decrease in expression of CD244 and PD1 among IFN-γ+ cells in seven repeated experiments was calculated as (MFI with CD48 blockade/MFI with isotype control), and fold change in IFN-γ+ CD8 T cells induced by CD48 blockade was calculated as (IFN-γ+ with CD48 blockade/IFN-γ+ with isotype control). *p < 0.02, Wilcoxon.

**FIGURE 3.** Downregulation of CD244 in an RSV-specific CD8 T cell clone. (A) A CD8 T cell clone specific for RSV-YY9 was incubated overnight in the presence or absence of cognate peptide. The expression of CD244, TIM-3, PD-1, CD8, and CD3 was determined by ICS. The experiment shown in (A) was repeated four times, and the MFI of the markers shown was compared in the presence or absence of cognate peptide. *p < 0.02, Wilcoxon.
within the first 48 h of stimulation (Fig. 5B), although CD244 expression is high after 10 d in culture post-stimulation (Figs. 2, 3).

When CTLs were stimulated by increasing doses of peptide, the markers CD244, CD3, and CD8 were downregulated concomitantly, whereas IFN-\(\gamma\) expression was induced at higher peptide doses (Fig. 5C). This result is consistent with previously observed differences in the concentration of peptide required to induce CD3 internalization versus IFN-\(\gamma\) production (14, 15).

To further examine involvement of TCR signaling pathways, we stimulated the same CTL clone in the presence of cognate peptide and increasing doses of the PI3K inhibitor LY294002 (Fig. 5D). Downregulation of both CD244 and TIM-3 was independent of PI3K signaling at all doses of LY294002, even though IFN-\(\gamma\) expression was strongly inhibited in a dose-dependent manner. Overall, the results in Figs. 4 and 5 demonstrate that downregulation of CD244 is dependent on a TCR-proximal signal in response to cognate peptide. In contrast TIM-3 downregulation is induced by either TCR-proximal signals (as observed in the presence of both cognate peptide and LY294002) or TCR-distal signals, as induced by the 1,2-diacylglycerol analog PMA.

Downmodulation of CD244 in the above experiments was determined by quantification of surface CD244 expression. Those results do not address the mechanism: in theory, loss of CD244 detection might be due to binding of a ligand that competes with the detecting Ab, or to shedding, or to sequestration in membrane ruffles, or to internalization. We hypothesized that CD244 downmodulation was a result of internalization and developed an assay to measure CD244 internalization using the pH-sensitive indicator dye pHrodo (Fig. 6). The pHrodo dyes are minimally fluorescent at neutral pH and fluoresce brightly in acidic environments such as late endosomal/lysosomal compartments (16–18).

To prepare avidin–Ab tetramers, we complexed biotinylated anti-CD244 Ab (or isotype control) to pHrodo avidin conjugate at a molar ratio of 4:1. We then added increasing doses of pHrodo avidin–Ab complexes to cultured CD8 T cells. Cells were washed and stimulated with medium alone or with PHA for 6 h. The pHrodo fluorescence was determined by flow cytometry. We used pHrodo fluorescence as an indicator of complete internalization and transport to a late endosomal compartment.

For a readout dependent on simple receptor attachment, signal would be expected to follow a monotonic curve and be directly proportional to ligand or Ab dose. In contrast, for a readout that depends on receptor crosslinking, signal is expected to follow a biphasic curve—increasing to an inflection point, and then decreasing at supraoptimal doses as receptor is saturated and cross-linking is lost. On the basis of our results shown above, we predicted that receptor internalization as indicated by pHrodo fluorescence would be 1) dependent on CD244 crosslinking (biphasic with respect to the dose of Ab–avidin complex) and 2) enhanced by TCR signal (i.e., PHA). Fig. 6 shows that observed results were consistent with our predictions: pHrodo red fluorescence is biphasic with respect to tetramer concentration, and it is enhanced by PHA stimulation. Tetramers formed with nonspecific isotype control Ab did not produce any significant pHrodo signal. Importantly, the fact that pHrodo fluorescence decreases at high tetramer concentration proves that fluorescence is dependent on a crosslinking signal and is not the product of tetramer loading at the cell surface. These results demonstrate that subsequent to simultaneous signals via TCR and CD244, the CD244 molecule is internalized and enters an acidic endosomal/lysosomal compartment inside the T cell.

We wanted to investigate how CD244 downregulation in response to Ag might relate to antiviral CD8 T cell function. Because most of our subjects did not have significant ex vivo RSV-specific CD8 responses (not shown), we generated antiviral CD8 T cell lines, as for Fig. 2 above, from HIV+ and healthy HIVneg donors (Fig. 7). PBMCs from each donor were stimulated with optimal viral peptides matched to the donor HLA type and expanded in culture for 10 d. Each Ag-responsive line was tested by ICS in the presence or absence of CD48 blockade. Consistent with the reported “dual functions” of CD244, we found that CD48 blockade...
FIGURE 5. TCR-induced CD244 downregulation is rapid, sensitive, and independent of PI3K. (A) RSV-YY9 clone was stimulated with cognate peptide for the indicated time, fixed, and stained for expression of CD244 and CD8. Expression was measured by flow cytometry. (B) (Figure legend continues)
Downmodulation of CD244 is mediated by endocytosis. (A) Schematic summary of tetramer assay. pHrodo red produces minimal fluorescence at neutral pH and fluoresces brightly at low pH. Specific or isotype control Ab was complexed with pHrodo Red Avidin at a molar ratio of 4:1 to form Ab tetramers. Tetramers were allowed to bind to CD8 T cells, and then CD8 T cells were stimulated with PHA or medium-only control. Internalization and transfer of tetramers to an acidic compartment was determined by fluorescent detection of pHrodo red signal on a flow cytometer. (B) pHrodo red detection in activated CD8 T cells. A cultured CD8 T cell line was stained with anti-CD244 or isotype control tetramers and stimulated with PHA for 5 h. Cells were fixed and stained for viability and T cell markers. Plots are gated on live CD8+ T cells. (C) Internalization of anti-CD244 tetramers induced by simultaneous signals via CD244 and TCR. A cultured CD8+ T cell line was loaded with specific or isotype tetramer at the indicated concentrations for 30 min, washed, and then stimulated with PHA or medium control. Cells were incubated at 37˚ for 5 h, and then stained for FACS, fixed, and analyzed by flow cytometry. Results are representative of two similar experiments. The percent of pHrodo-positive cells across multiple treatments was significantly greater for anti-CD244 tetramer compared with isotype control. (Fig. 6A, 6B, respectively).

Because our results suggest that CD244 downmodulation may be related to involvement of CD244 in the immune synapse, we wondered whether the change in CD244 expression induced by TCR activation would correlate with enhancement of IFN-γ production in response to CD48 blockade. Consistent with our observations above, we found that Ag stimulation produced a variable decrease (but never an increase) in surface expression of CD244. When we compared the degree of enhancement in IFN-γ production versus the change in CD244 expression on responding cells in response to CD48 blockade, we found a significant positive correlation among RSV-specific CD8 lines from HIV+ donors (Fig. 6C; slope = +0.38, r_c = 0.37, p = 0.01). Intriguingly, for HIVneg donors we found the opposite relationship: a significant negative correlation (Fig. 7D; slope = −0.35, r_c = 0.41, p = 0.046). These results suggest a close connection between activation-induced CD244 downregulation and CD48-mediated modulation of CD8 T cell function.

Discussion

T cells are controlled by complex networks of signals. As one of many “switches” in these networks, CD244 may be of special interest because it has been demonstrated to have dual functions, with the ability to enhance or inhibit antiviral human CD8 T cell functions in different contexts (5). For example, CD244 is one of a suite of markers progressively upregulated on exhausted T cells (1, 20–26). Consistent with a role in exhaustion, in subjects chronically infected with hepatitis B virus, blockade of CD244 or CD48 resulted in enhancement of antiviral cytotoxicity and cytokine production (27). In contrast, for subjects with human T cell leukemia virus I–associated neurologic disease, blockade of CD244, or knockdown of the signaling molecule SAP, reduced human T cell leukemia virus–specific antiviral functions (28). In subjects with HIV infection, blockade of CD244 was found to enhance HIV-specific CD8 T cell proliferation from PBMCs (22). For subjects infected with hepatitis C virus, blockade of CD48 was found to variably enhance or inhibit different CD8 clonotypes, with opposite effects correlating with the degree of SAP expression (7). In the present report, we also find variable and opposing effects of CD48 blockade on different antiviral clonotypes. Further, we identify a mechanistic correlate of enhancement and inhibition: rapid dynamic downregulation of CD244 on the surface of the responding cell.

Recent studies have elucidated the molecular basis for dual action of CD244 (29). Human subjects with XLP disease, associated with mutations in SAP gene SH2D1A, are at high risk of lethal EBV infection owing to an inability to clear EBV-infected B cells (30, 31). Many aspects of XLP disease are recapitulated in SH2D1A knockout mice (32). Zhao et al. (10) demonstrated that...
T cell lines from HIVneg donors between fold change in CD244 expression and enhancement in IFN-γ. Overall CD244 expression on IFN-γ or absence of CD48, and the percent IFN-γ+ cells, as well as the MFI of CD244 and PD-1 on IFN-γ− cells, was determined by ICS. Lines did not express significant amounts of IFN-γ in the absence of cognate peptide (not shown). The fold enhancement of IFN-γ response in the presence of cognate peptide was calculated as (% IFN-γ+ cells in the presence of CD48 blockade/% IFN-γ− cells in the presence of isotype control). (A) Lack of correlation between overall CD244 expression on IFN-γ− cells in the presence of CD48 blockade and enhancement in IFN-γ− response induced by CD48 blockade in HIV+ donors. (B) A similar lack of correlation in HIVneg donors. (C) Correlation among 16 RSV-specific CD8 T cell lines from HIV+ donors between fold change in CD244 expression and enhancement in IFN-γ− response induced by CD48 blockade (p = 0.01; r = 0.37). (D) Correlation among eight RSV-specific CD8 T cell lines from HIVneg donors between fold change in CD244 expression and enhancement in IFN-γ− response induced by CD48 blockade (p = 0.046; r = 0.41). The fold change in CD244 expression on IFN-γ− cells was calculated as (MFI in the presence of CD48 blockade/MFI in the presence of isotype control). The rs and p values are based on the Spearman rank correlation.

Before developing the tetramer assay shown in Fig. 6, we attempted several other approaches to monitor CD244 dynamics at the cell surface (not shown). Labeling CD244 at the cell surface with monomeric fluorescent Ab prior to activation interfered with receptor downmodulation, presumably by blocking interaction with CD48 (not shown). To prove that CD244 was being internalized, we developed a strategy based on two features: the ability of an avidin molecule to crosslink four biotinylated Abs, and the pH-dependent fluorescence of the pHrodo red reagent (16–18). By mixing avidin and biotinylated Ab at a molar ratio of 1:1 or 1:2 (not shown); we presume that Ab tetramers are able to crosslink CD244 in a similar manner to an APC expressing CD48. As shown in Fig. 6, pHrodo avidin uptake increased with increasing dose of avidin, up to an inflection point, after which signal decreased. This is a classic dose–response curve for a crosslinking-dependent signal, such that supraoptimal doses of avidin lead to saturation of the receptor and reduced crosslinking. Receptor internalization is a feature of many lymphocyte signaling pathways; pH-sensitive Ab/ligand tetramers may be a useful tool for monitoring dynamic relocation of receptors in live primary T cells in multiple contexts.

To investigate whether the observed dynamic changes in CD244 expression were connected with T cell function, we compared the degree of enhancement or inhibition by CD48 blockade with the degree of CD244 downregulation in response to cognate peptide in cultured antiviral (RSV-specific) CD8 T cell lines. The fact that we observed a significant positive correlation in HIV+ donors suggests that differential CD244 incorporation into the synapse is intimately connected with modulation of TCR signaling. This study we observed CD244 downregulation on primary ex vivo CD8 T cells and in cultured CD8 T cell lines and clones in response to activation via the TCR (by cognate ligand, SEB, or PHA). Notably, downregulation was neither induced nor prevented by PMA–ionomycin, which activate T cells by bypassing TCR signaling, strongly suggesting that downregulation is mediated by signals proximal to the TCR and the immune synapse. TCR-dependent downregulation of CD244 also required signaling via CD244 itself, such that downregulation was prevented by CD48 blockade.

Previous studies showed that CD244 can localize to the immune synapse in NK cells (8, 35), that SLAMf6 can localize to the synapse in T cells (36), and that CD244 relocates to the T cell–B-LCL interface in a SAP-dependent manner (9). CD244 downregulation was previously demonstrated in response to activation of NK cells (37). CD229 (SLAMf3) was found to be downregulated on Jurkat T cells in response to artificial coligation of CD229 plus TCR (38), similar to our finding that CD244 is downregulated on specific T cells by combined signals from CD48 and cognate peptide–MHC. To our knowledge, the present article is the first to describe activation-induced downregulation of CD244 on T cells.

In this study we observed CD244 downregulation on primary ex vivo CD8 T cells and in cultured CD8 T cell lines and clones in response to activation via the TCR (by cognate ligand, SEB, or PHA). Notably, downregulation was neither induced nor prevented by PMA–ionomycin, which activate T cells by bypassing TCR signaling, strongly suggesting that downregulation is mediated by signals proximal to the TCR and the immune synapse. TCR-dependent downregulation of CD244 also required signaling via CD244 itself, such that downregulation was prevented by CD48 blockade.
fine-tuning function is consistent with the finding that CD244 modulation of antiviral T cell function correlates with the expression of a specific signaling molecule (SAP) (7). Compared with HIV+ subjects, T cell lines derived from HIV−/−/ subjects presented an opposite, negative correlation between IFN-γ production and CD244 downregulation. This finding suggests a model in which the context of signaling-molecule expression is determined by systemic factors such as chronic infection, which in turn drive some CTLs toward positive or negative responses to CD244 ligation. Clearly, many key questions about the role CD244 in T cell signaling and response remain to be answered in future studies.

The effect of TCR stimulation on TIM-3 downregulation on T cell clones requires further investigation. Whereas CD48 blockade facilitated our ability to observe ex vivo IFN-γ production by CD244+ cells in response to Ag and superantigen, we were unable to make a similar observation for TIM-3+ CD8 T cells ex vivo, as Ab against the TIM-3 ligand Gal-9 had no effect (not shown). Short-term lines propagated ex vivo did express varying levels of TIM-3, even on IFN-γ− Ag-specific cells, but there was no effect of TCR stimulation on TIM-3 expression (not shown). In contrast to PBMC and short-term lines, our antiviral clones did usually express high levels of TIM-3, which was downregulated in response to Ag or PMA (Fig. 4A). The mechanism of this modulation remains to be determined.

In conclusion, our results provide evidence that simultaneous ligation of CD244 by CD48, and of TCR by cognate peptide–MHC on an APC, results in variable downmodulation of CD244, presumably via incorporation into the synapse. For a particular clonotype, the degree of downregulation correlates with the degree of enhancement or inhibition in antiviral functions induced by CD48 notype, the degree of downregulation correlates with the degree of TIM-3+ CD8+ T cell exhaustion during chronic viral infection. Immunity 27: 670–684.


