Essential Role of Ubiquitin and TSG101 Protein in Formation and Function of the Central Supramolecular Activation Cluster

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SUMMARY

Agonist MHC-peptide complexes in the immunological synapse (IS) signal through T cell receptor (TCR) microclusters (MCs) that converge into a central supramolecular activation cluster (cSMAC). The determinants and function of the cSMAC remain unknown. We demonstrate an essential role for ubiquitin (Ub) and TSG101, but less so for HRS, in signal processing events at the cSMAC. Using siRNA in primary T cells, we show that Ub recognition by TSG101 is required for cSMAC formation, TCR MC signal termination, TCR downregulation, and segregation of TCR-MHC-peptide from PKC-θ-enriched signaling complexes. Weak agonist MHC-peptide induced CD80-dependent TCR MCs that dissociated in the center of the IS without recruiting TSG101. These results support TSG101-dependent recognition of CD80-independent TCR MCs as a molecular checkpoint for TCR downregulation.

INTRODUCTION

Complex signal integration in cells involves responses to diverse stimuli that often utilize overlapping signal transduction machinery. Appropriate responses to such stimuli may benefit from spatial segregation of ligand-receptor interactions. In T cells, immunological synapses (ISs) are specialized cell-cell junctions that combine cell polarization and positional stability with spatial segregation of interacting elements (Dustin, 2002; Dustin et al., 1998, 2008; Huppa and Davis, 2003; Krogsgaard et al., 2003). IS formation is critical for signal integration, as well as coordination of migration, directed secretion, asymmetric cell division, and differentiation (Davis et al., 2007; Dustin, 2008; Huppa and Davis, 2003; Krogsgaard et al., 2003). In CD4+ T cells, the IS is composed of three primary subdomains: a central supramolecular activation cluster (cSMAC) rich in proximal signaling components such as T cell receptor (TCR)-major histocompatibility complex (MHC)-peptide and PKC-θ kinase, a peripheral supramolecular activation cluster (pSMAC) dominated by intercellular adhesion molecule-1 (ICAM-1)-lymphocyte function associated-1 (LFA-1) interactions, and a distal supramolecular activation cluster (dSMAC) rich in dynamic actin (Grakoui et al., 1999; Monks et al., 1998). The cSMAC is generated and maintained by microclusters (MCs) of 10–20 TCRs that continuously form in an actin-dependent manner in the dSMAC but that become actin independent as they translocate to the IS center and fuse to form the cSMAC (Krummel et al., 2000; Varma et al., 2006; Yokosuka et al., 2005). TCR MCs are the sites of signal initiation based on recruitment and phosphorylation of the signaling molecules Lck, ZAP-70, and LAT, as well as SLP-76 and Grb-2 recruitment (Bunnell et al., 2002; Campi et al., 2005; Huse et al., 2007; Yokosuka et al., 2005). In contrast, the cSMAC has 1/20 amount of tyrosine phosphorylation compared with MCs and cannot independently sustain Ca2+ signaling (Campi et al., 2005; Varma et al., 2006). The molecular basis of signaling differences between MCs and the cSMAC is unknown.

Ligand-mediated TCR downregulation occurs via routing of internalized receptors to lysosomes (Valitutti et al., 1997), and enrichment of the cSMAC in multivesicular body (MVB) markers suggests a role for receptor degradation at the cSMAC (Varma et al., 2006). However, not all ligands induce cSMAC formation, and it has recently been proposed that avoidance of cSMAC formation and TCR downregulation may underlie the elevated stimulatory potency of certain weak agonist ligands (Cemerski et al., 2007). TCR degradation may occur via ubiquitin (Ub) recognition based on involvement of ubiquitin ligases such as Cbl-b (Naramura et al., 2002). Degradation of ubiquitinated substrates via MVBs is a stepwise process coordinated by multiple family members of the endosomal sorting complex required for transport (ESCRT) (Williams and Urbé, 2007). There are four ESCRT complexes (0, I, II, and III) with unique roles in signal termination and receptor degradation of epidermal growth factor receptor (EGFR). ESCRT-0 and -I directly recognize Ub (Williams and Urbé, 2007). ESCRT-0 can also associate with ubiquitinated cargo through the Ub interaction motif (UIM) of hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) (Bache et al., 2003b; Hirano et al., 2006) in parallel with inositol-3-phosphate bearing lipids present in endosomes (Raiborg et al., 2001). ESCRT-I recognizes Ub through the ubiquitin E3 variant (UEV) domain of tumor suppressor gene 101 (TSG101) and is required for sorting of EGFR into MVBs (Porillos et al., 2002; Sundquist et al., 2004; Teo et al., 2004). In the absence of TSG101, both MVB formation and sorting of proteins into MVBs is inhibited, resulting instead in persistent EGFR signaling...
Unresolved roles for the cSMAC in promoting proximal signaling still exist, particularly with respect to PKC-θ, whose enrichment at the cSMAC has been described (Monks et al., 1998; Monks et al., 1997). Activated PKC-θ leads to downstream activation of NF-κB and AP-1 transcription factors (Isakov and Altman, 2002; Sun et al., 2000). CD28 is dispensable for PKC-θ signaling to the IS, but is critical for its concentration in the cSMAC (Huang et al., 2002). Recent reports have suggested discrete sites of CD28, PKC-θ, and TCR enrichment within the IS (Sims et al., 2007; Tseng et al., 2008; Yokosuka et al., 2008), but the molecular basis for this segregation is as yet unclear. Understanding mechanisms of segregation may provide insight into how cells simultaneously process multiple signals.

To address the role of Ub and ESCRT-mediated Ub recognition in cSMAC formation and function, we have applied siRNA-mediated suppression and high-resolution fluorescence microscopy of IS formed between primary T cells and supported bilayers. We determined that Ub was required for cSMAC formation in the IS in response to agonist MHC-peptide complexes. Ub recognition by ESCRT-I component TSG101, but not ESCRT-0 component HRS, was required for cSMAC formation, TCR MC signal termination, and segregation of TCR-agonist MHCp complexes from PKC-θ-enriched domains within an outer cSMAC subdomain. We also demonstrated that TSG101 was rapidly recruited to the plasma membrane after T cell activation by fluorescence resonance energy transfer. Finally, signaling MCs formed in response to weak agonist ligands remained actin dependent and dissipated at the cSMAC-pSMAC boundary without recruiting ESCRT-I complexes. The IS thus engages in a conditional utilization of ESCRT-I and spatially defined checkpoints to regulate T cell signaling and downregulation.

RESULTS

Ubiquitination Is Required for cSMAC Formation

Prior observations that MVBs are enriched at the cSMAC led us to investigate whether ubiquitination is involved in IS patterning (Varma et al., 2006). Treatment of cells with MG132 inhibits the proteosome, resulting in accumulation of poly-ubiquitinated substrates and depletion of the free ubiquitin pool (Melikova et al., 2006). We therefore incubated MG132-treated AND TCR transgenic (Tg) T cells on supported planar bilayers presenting I-E^k-MCC + ICAM-1. Control treated T cells formed TCR MCs, which accumulated in the cSMAC in response to bilayers presenting agonist MHC-peptide (Figure 1A and Movie S1A and Movie S1 available online). In contrast, we found that MG132-treated T cells were able to efficiently form TCR MCs, but were unable to translocate MCs from the site of formation to the IS center. The amount of TCR accumulating at the interface was increased significantly in the MG132-treated cells (Figure 1B). Furthermore, MG132 treatment significantly impeded termination of TCR signals, given that phosphorylation of peripherally accumulated TCR MCs was 5- to 10-fold higher in MG132 treated T cell IS as compared to control T cell IS (Figures 1A and 1B). Although MG132 treatment may have a number of additional effects, these results suggest that ubiquitination is required for cSMAC formation and encouraged us to further pursue this line of experimentation.

TSG101 Is Required for cSMAC Formation and MC Dephosphorylation

The suggested role for ubiquitination in cSMAC formation led us to investigate the role of ubiquitin recognizing ESCRT complex components in regulating TCR signaling within the IS. We chose to target HRS and TSG101, the respective critical components of the ESCRT-0 and ESCRT-I complexes with established ubiquitin binding (Figure 1C) (Williams and Urbé, 2007). We developed a procedure to efficiently suppress expression of HRS or TSG101 in 100% of activated AND TCR transgenic (Tg) T cells, by nucleofection of either specific siRNA duplexes or scrambled versions of the same sequence as controls. We were able to reduce HRS and TSG101 expression more than 90% during primary expansion of AND TCR Tg T cells (Figure 1D).

In EGFR systems, ESCRT-0 is initially recruited to ubiquitin-positive cargo within clathrin-coated pits and is required for efficient receptor degradation (Bache et al., 2003a; Bilodeau et al., 2003; Katzmann et al., 2003). However, HRS knockdown (KD) T cells formed a cSMAC containing dephosphorylated TCR (Figures 1E and 1F). In addition, T cells transfected with GFP-tagged clathrin light chain showed no colocalization of clathrin-coated pits with TCR MCs in the IS (Figure S1B and Movie S2). However, clathrin-coated pits were present in distinct puncta emerging in the IS periphery and accumulated in the IS center (Figure S1B and Movie S2). Furthermore, HRS KD resulted in accumulation of phosphotyrosine in the IS periphery that did not colocalize with TCR (Figures 1E and 1F). This leaves open the possibility that clathrin-HRS might be involved in regulation of downstream signaling complexes that are known to dissociate from TCR MCs and signal independently (Bunnell et al., 2002); however, because this phenotype is distinct from that of MG132 treatment, we did not pursue this further here.

In contrast to HRS, TSG101 KD generated a phenotype more similar to that of MG132 treatment. Control siRNA-treated cells formed TCR MCs, which accumulated in the cSMAC in response to bilayers presenting agonist MHCp. In contrast, TCR MCs failed to translocate to the cSMAC and instead accumulated more peripherally in TSG101 KD T cells (Figure 1G, Figures S1C and S1D, and Movie S3). TSG101 KD resulted in accumulation of phosphorylated TCR MCs in quantities and distribution that were similar to MG132-treated T cell IS (Figures 1G and 1H and Figures S1C and S1D), suggesting that TSG101 translocates ubiquitinated TCR MCs to the cSMAC for dephosphorylation and degradation. The elevated antiphosphotyrosine intensity in TSG101 KD ISs did not correlate directly with the TCR intensity in MCs, but all visible TCR MCs were intensely phosphotyrosine positive, except in rare cases in which TCR MCs penetrated into the IS center. A proportion of the elevated phosphotyrosine signal in TSG101 KD ISs did not colocalize with TCR. This may be due to phosphorylation of small clusters of TCR that are below the limit of detection. Alternatively, they may represent other surface proteins, such as LAT and associated proteins that are known to cluster independently after TCR signaling (Balagopalan et al., 2007). The accumulation of large TCR MCs in the periphery of TSG101 KD continued to engage agonist MHCp (Figure S1E) and resulted in slightly
To rule out this possibility, we imaged control and TSG101 KD T cells forming ISs with primary B cells by confocal microscopy. Reconstructed T-B cell interfaces displayed comparable peripheral TCR accumulation and elevated phosphotyrosine after TSG101 KD to those observed on bilayers (Figures 2A–2C).

We then investigated whether the recruitment of TSG101 to the IS seen in T cells incubated on supported planar bilayers was recapitulated in a cell-cell system. In particular, we wondered whether T cell activation resulted in recruitment of TSG101 to membrane compartments, as would be required in order to execute sorting processes within the IS. We therefore reconstructed T-B cell interfaces displayed comparable peripheral TCR accumulation and elevated phosphotyrosine after TSG101 KD to those observed on bilayers (Figures 2A–2C). Consistent with our imaging results, flow cytometry analysis of intact or permeabilized T-B cell conjugates demonstrated a significant defect in antigen-induced TCR surface downregulation and degradation, respectively, in the absence of TSG101 (Figure 2D).

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Figure 1. TSG101 Is Required for Central TCR Accumulation and Dephosphorylation
(A and B) AND T cells were pretreated with DMSO (vehicle) or the proteasomal inhibitor MG132 to deplete cells of free ubiquitin. They were then incubated on glass-supported planar bilayers containing 10 molec/µm² I-Ek-MCC and 200 molec/µm² ICAM-1 for 30 min at 37°C, fixed, and stained for TCR and phosphotyrosine (pY). In (B), bar graphs depict quantification in arbitrary units (AUs) of mean fluorescence at cell contact interfaces and represent >100 cells across three experiments. Standard errors are indicated. (C) A model for ESCRT recruitment to ubiquitinated substrates. HRS, the critical component of ESCRT-0, binds ubiquitin through its UIM domain but requires recruitment to PI-3-P-rich endosomes through its FYVE domain. TSG101, the critical component of ESCRT-1, binds ubiquitin through its UEV domain. (D) Cell lysates of 10⁶ control TSG101 knockdown (KD) and HRS KD T cells were separated by SDS-PAGE and blotted for TSG101 and HRS. Anti-alpha actin was used as a loading control. (E and F) Control and HRS KD T cells were incubated on glass-supported planar bilayers containing 10 molec/µm² I-Ek-MCC and 200 molec/µm² ICAM-1 for 30 min at 37°C, fixed, and stained for TCR and pY. In (F), bar graphs depict quantification in arbitrary units (AU) of mean fluorescence at cell contact interfaces, and represent >100 cells across three experiments. Standard errors are indicated. (G and H) Control and TSG101 KD T cells were incubated on glass-supported planar bilayers containing 2 molec/µm² I-Ek-MCC and 200 molec/µm² ICAM-1 for 30 min at 37°C, fixed, and stained for TCR and pY. In (H), bar graphs depict quantification in arbitrary units (AU) of mean fluorescence at cell contact interfaces, and represent >100 cells across three experiments. Standard errors are indicated.
dequenching after acceptor photo-bleaching, with GFP-tagged proteins as donors and the membrane dye DiI as acceptors (McLean et al., 2000). In order to use this method to probe membrane interactions of TSG101, we stably expressed a murine TSG101-GFP chimera in AND T cells by retroviral transduction and sorted them for uniform expression. We then briefly labeled transduced T cells with DiI before allowing them to form conjugates with MCC-loaded murine B cells. Changes in donor fluorescence after photobleaching of DiI revealed a low and diffuse FRET signal throughout the T cell, indicating loose coupling of TSG101 with plasma membrane and endomembranes in the steady state (Figures 2E and 2F) as previously reported (Welsch et al., 2006). Upon IS formation with antigen-pulsed B cells, TSG101 underwent a marked redistribution, polarizing strongly to the IS but also outside the IS (Figures 2E and 2F). The high apparent FRET efficiencies observed is consistent with direct apposition of membranes and tagged TSG101.

Notably, the FRET signal was most prominent at the cSMAC in the T-B cell contacts (Figure 2E). Therefore, T cell activation results in redistribution of TSG101 to membrane compartments concentrated in the IS.

**Sorting of TCR MCs into the IS Center Depends on Ub Recognition by TSG101**

We then sought to confirm the specificity of TSG101 in inducing cSMAC formation and signal termination. We observed similar defects in cSMAC formation and dephosphorylation when TSG101 was suppressed with three different siRNA oligomers (Figures S3A and S3C). Restoration of wild-type IS patterning and cSMAC formation and dephosphorylation when cSMAC formation and signal termination. We observed similar defects in cSMAC formation and signal termination. We observed similar defects in cSMAC formation and signal termination. We observed similar defects in cSMAC formation and signal termination. We observed similar defects in cSMAC formation and signal termination.
abrogate Ub binding (Pornillos et al., 2002) was completely unable to restore cSMAC formation or dephosphorylation after TSG101 KD (Figure 3). Cell lysates from TSG101 KD T cells demonstrated a marked accumulation of ubiquitinated species as compared to control siRNA-treated T cells (Figure S3D), and ubiquitination was further increased by agonist MHCp stimulation. These higher-molecular-weight species were also identifiable by antibodies to CD3ζ (Figure S3E). This suggests that CD3ζ may be a ubiquitinated protein recognized by TSG101. In combination with the equivalent defects observed in TSG101 KD and MG132-treated T cells forming ISs, these data support the model that TSG101 specifically interacts with ubiquitinated components of TCR MCs to induce cSMAC formation and TCR signal termination.

**Differential Sorting of Receptors by TSG101**

Numerous receptors are engaged within the IS that are regulated by diverse mechanisms. A recent example of sorting of such receptors in the IS is the observation that CD28 and TCR traffic together in peripheral MCs upon engagement and are then segregated from each other in the cSMAC (Yokosuka et al., 2008). In light of our finding that Ub-mediated TSG101 recognition is necessary for its incorporation of TCR into a cSMAC, we wondered whether TSG101 might also be involved in segregating TCR from CD28. We evaluated these sorting events by observing control and TSG101 KD T cells interacting with bilayers containing MHCp, ICAM-1, and CD80 and following PKC-θ enriched compartments. Consistent with prior reports, we found that in control cells, PKCθ, and TCRs colocalized in peripheral MCs but became segregated in the cSMAC (Figure 4A and Figure S3E) (Yokosuka et al., 2008). In TSG101 KD T cells, however, PKC-θ and TCR failed to segregate and remained colocalized in large MCs in the pSMAC region, with a significantly elevated Pearson’s correlation coefficient (Figure 4A and Figure S4A). Therefore, TSG101 functions at the cSMAC–pSMAC interface to generate distinct TCR- and PKC-θ-enriched domains of the cSMAC.

We then investigated the mechanism by which TSG101 selectively terminates TCR MC signals at the IS center. Normally, TCR MCs become relatively immobile as they accumulate at the IS center (Varma et al., 2006). The association of immobile central TCR with lysobisphosphatidic acid (LBPA) staining is consistent with incorporation of TCR into MVBs (Varma et al., 2006). In control T cells, TCR MCs became immobilized as they became colocalized with LBPA+ structures (Figure 4C). In contrast, we observed continued jostling movements of large TCR MCs in the periphery of TSG101 KD IS (Figure 4B and Movie S3). This jostling movement is represented in Figure 4B by overlaying three sequential images at 2 min intervals with red, green, and blue coding such that immobile structures appear white and mobile structures appear colored. The continued jostling of TCR in TSG101 KD IS correlated with a striking segregation of TCR from LBPA+ structures (Figure 4C). This segregation was confirmed by a significant decrease in the Pearson’s correlation coefficient between TCR and LBPA in TSG101 KD IS (Figure S4B). Thus, TSG101 is required for TCR colocalization with LBPA+ compartments.

**TSG101 Suppression Leads to Chronic TCR Signaling**

The striking persistence of phosphorylated TCR MCs after TSG101 KD (Figures 1G and 1H) suggested to us that the duration of signaling through engaged TCR might be extended...
in TSG101 KD T cells. To test this, we allowed control and TSG101 KD T cells to be activated with agonist MHCp ligand on bilayers for 10 min, sufficient to induce stable TCR engagements, prior to treatment with blocking I-E<sup>k</sup>-MCC antibodies (D4) to prevent new TCR MC formation. Signaling was monitored with intracellular Ca<sup>2+</sup>. In control siRNA-treated T cells, the Ca<sup>2+</sup> signal decayed over 2 min (Figures 5A and 5B and Figure S5), as previously described (Varma et al., 2006). In contrast, Ca<sup>2+</sup> elevation decayed only partially and was then sustained at an intermediate level in TSG101 KD T cells after D4 treatment because TSG101 is required for the abscission step of cellular cytokinesis (Morita et al., 2007); as such, we noted that TSG101 KD consistently inhibited T cell proliferation. Thus, TSG101 plays an important role in limiting downstream T cell signals and effector responses after productive proximal signaling in response to TCR engagement.

**Weak Agonist MHCp Do Not Involve TSG101**

Typical recognition of agonist pMHC results in both tyrosine phosphorylation-mediated T cell activation and Ub-mediated TCR downregulation (Naramura et al., 2002; Samelson et al., 2010).
However, T cell activation in the absence of TCR downregulation has recently been reported. The altered peptide ligand (APL) MCC-K99A, a variant of MCC88-103 with a single amino acid substitution that greatly accelerates I-E\textsuperscript{k}-based tetramer dissociation compared to the native sequence, effectively stimulates proliferation of AND Tg T cells when presented by splenic APCs despite inducing diminished TCR downregulation and central TCR accumulation (Cemerski et al., 2007). We began investigating IS formation by AND T cells in response to I-E\textsuperscript{k}-MCC-K99A in a planar bilayer system to understand the properties of this ligand-receptor interaction. Supported planar bilayers presenting I-E\textsuperscript{k}-MCC-K99A and ICAM-1 arrested T cell migration and induced sustained Ca\textsuperscript{2+} elevation in AND T cells only in the presence of CD80 (Figures S6A and S6B). This is consistent with reports in which CD28 engagement is required for response to weak agonist ligands (Bachmann et al., 1986). Real-time imaging of TCR and LFA-1-ICAM-1 interactions demonstrated that, in the presence of CD80, T cells responding to I-E\textsuperscript{k}-MCC-K99A and ICAM-1 arrested T cell migration and induced sustained Ca\textsuperscript{2+} elevation in AND T cells only in the presence of CD80 (Figures S6A and S6B). T cells spread symmetrically on K99A-presenting surfaces, and LFA-1-ICAM-1 interactions quickly organized into a pSMAC (Figure S6C). Tyrosine phosphorylation and proximal TCR signaling (indicated by recruitment and phosphorylation of LAT) were concentrated in small MCs in the periphery of the IS with a similar intensity and frequency to IS formed in response to I-E\textsuperscript{k}-MCC (Figures S6C and S6D). However, in striking contrast to I-E\textsuperscript{k}-MCC-induced ISs, TCR did not accumulate in the IS center even in response to 10 molecules/\mu m\textsuperscript{2} of I-E\textsuperscript{k}-MCC-K99A (Figures 6A and 6B, \(p < 0.0001\); Figures S6C and S6D). Time-lapse TIRFM imaging of IS formed in response to I-E\textsuperscript{k}-MCC-K99A revealed costimulation dependent formation and centripetal translocation of TCR MCs (Figure 6A and Movie S4); however, these MCs dissipated as they reached the center of the IS rather than accumulating in a cSMAC as they do in response to I-E\textsuperscript{k}-MCC.

In order to resolve this difference between I-E\textsuperscript{k}-MCC-K99A and I-E\textsuperscript{k}-MCC-induced ISs, we investigated the contribution of TSG101 to ISs formed in response to these respective ligands. Notably, TSG101 was recruited to the cSMAC of I-E\textsuperscript{k}-MCC-induced ISs, but not to I-E\textsuperscript{k}-MCC-K99A-induced ISs (Figure 6B). Furthermore, TSG101 KD had no impact on TCR MC formation, size, transport, or phosphotyrosine levels within MCC-K99A induced ISs (Figure 6C and Figure S6E). These results demonstrate that TSG101 is not required for early TCR signaling, TCR MC transport, pSMAC formation, or a radially symmetric IS in response to the MCC-K99A weak agonist MHCp. It has previously been demonstrated that TCR MCs form in an F-actin-dependent manner, but then become rapidly F-actin independent on the basis of their stability in the presence of latrunculin A, which sequesters G-actin (Varma et al., 2006). Weak agonist MCC-K99A-induced TCR MCs remained F-actin dependent, in contrast to many F-actin-independent MCs that accumulate in response to I-E\textsuperscript{k}-MCC (Figure S6F). The cSMAC is generally free or depleted of F-actin (Kaiuzka et al., 2007). Analysis of the F-actin distribution in the IS formed in response to MCC and MCC-K99A MHCp confirmed that I-E\textsuperscript{k}-MCC-K99A does not induce an F-actin-free central zone (Figure S6G).
DISCUSSION

We previously demonstrated that the cSMAC shows a striking enrichment of LBPA (Varma et al., 2006), a lipid associated with MVBs. This suggested that the cSMAC might be a site for degradation of TCR through an ESCRT-dependent pathway (Williams and Urbé, 2007). Other results in the field were consistent with this notion including earlier observations that CD2AP, an adaptor linking actin and Cbf1 family ubiquitin E3-ligases is required for normal TCR degradation and signal termination in the cSMAC (Lee et al., 2003). There was also ample evidence for a role of Cbf1 family E3 ligases in TCR signal termination and a role for lysosomes in TCR degradation (Liu et al., 2000; Naramura et al., 2002; Valitutti et al., 1997). It had also been demonstrated that TCR downregulation correlates with MHCp potency (Itoh et al., 1999) and that a subset of weak agonist ligands do not trigger TCR downregulation at all (Cemerski et al., 2007). Furthermore, the TCR ζ and δ chains are directly ubiquitinated upon strong TCR engagement (Cenciarelli et al., 1992). On the basis of these observations, we anticipated that ubiquitination and ESCRT machinery might be important for TCR signal termination and eventual degradation after engagement with agonist MHCp. We were surprised by our finding in this study that depletion of free ubiquitin with MG132 or knockdown of TSG101 not only blocked TCR signal termination and degradation but also blocked cSMAC formation in response to agonist MHCp. In contrast, a weak agonist MHCp could induce robust TCR MCs, Ca2+ elevation, and pSMAC formation without recruiting TSG101 or inducing a cSMAC in normal T cells. These results have important implications for signal integration in response to agonist MHCp and how this differs from weak agonist MHCp.

Our results suggest distinct roles for major Ub-recognition ESCRT components HRS and TSG101 in IS formation and TCR downregulation. HRS is part of ESCRT-0 and associates with ubiquitinated cargo through its ubiquitin interaction motif (UIM) (Bache et al., 2003a; Hirano et al., 2006), in parallel with inositol-3-phosphate-bearing lipids present in endosomes (Raiborg et al., 2001). In EGF systems, HRS promotes ubiquitin recognition by TSG101 by directing TSG101 to early endosomes containing ubiquitinated cargo (Katzmann et al., 2003). This process is dependent on the recruitment of HRS to receptors within clathrin lattices (Raiborg et al., 2002), and clathrin clustering of ubiquitinated cargo is required for efficient HRS recognition (Raiborg et al., 2006). Indeed, in EGF-R systems involving both clathrin-dependent and clathrin-independent endocytic mechanisms, HRS is involved only in degradation of clathrin-endocytosed receptors (Myromslien et al., 2006). The specificity of HRS for clathrin-coated pits and the failure of TCR MCs to intersect with these compartments may explain the finding that a cSMAC is formed in the absence of HRS (Razi and Futter, 2006). Furthermore, in non-EGF-R systems, the association of HRS and TSG101 is not so clear. Indeed, TSG101-mediated endosomal trafficking of Tyyp1 is independent of HRS function in melanocytes (Truschel et al., 2009). Furthermore, HRS is involved in alternate internal trafficking pathways, such as sequence-dependent recycling of the beta-2 adrenergic receptor (Hanyaloglu et al., 2005). Thus, prior models suggesting a strictly sequential transfer of ubiquitinated cargo from ESCRT-0 to ESCRT-I may not apply in all situations, as has been recently demonstrated (Shields et al., 2009). Down-regulation of engaged TCR complex appears to be clathrin independent (Monjas et al., 2004), whereas internalization of the proximal signaling molecule LAT has been shown to be clathrin dependent (Brignatz et al., 2005). We detected a weak but significant effect of HRS KD on non-TCR MC-associated phosphotyrosine. This suggests that HRS may act on distinct substrates from the TCR during T cell activation and this is worthy of further study.

Our results provide insight into the distinct mechanisms of sustained signaling by agonist and weak agonist MHCp. TSG101 prevents chronic signaling by agonist MHCp induced TCR MCs. TCR-agonist MHCp and CD28-CD80 interactions are initially colocalized in MCs, but then are segregated within the cSMAC (Yokosuka et al., 2008). We show that this segregation requires TSG101. There are a number of possibilities for how TSG101 organizes this process. Two possibilities are polymerization of ESCRT III components to generate MVBs that are then translocated toward the center by microtubules (Wollert et al., 2009) or linkage to a final actin-dependent translocation step through Alix before later ESCRT steps are initiated (Pan et al., 2006; Strack et al., 2003). This sorting process, however mediated, led to formation of the PKC-δ-rich compartment, which is a hallmark of the IS formed with professional APCs (Monks et al., 1997; Tseng et al., 2008; Yokosuka et al., 2008). Weak agonist MHCp required CD80 in the bilayer to form TCR MCs and sustain Ca2+ signals, which is not the case for agonist MHCp. This result is consistent with earlier studies showing that responses to weak agonist MHCp are CD28 dependent (Bachmann et al., 1997). Weak agonist MCC-K99A induced TCR MCs that remained F-actin dependent and disappeared in the center of the IS without a requirement for an F-actin free central zone or TSG101. The mechanism by which weak agonist MHCp signals are terminated is not clear, but we speculate that the requirement for a dynamic contribution from CD80 and the continued requirement for F-actin may enable physical disengagement and recycling of TCR at the point of convergence of centripetal F-actin flow (Kaizuka et al., 2007). More stable MCs formed by agonist MHCp might persist in response to the same physical process, necessitating the use of TSG101 to mark irreversibly clustered TCR-MHCp complex for elimination. The other distinguishing feature of signaling mediated by agonist and weak agonist MHCp is the generation of the consolidated PKC-δ-rich compartment by agonist MHCp. Given that the cSMAC size is linearly related to agonist MHCp density (Varma et al., 2006), the formation of the PKC-δ-rich outer cSMAC may provide a mechanism to measure integrated TCR avidity in an IS as long as CD80 or CD86 are present on the APC.

These data lead us to a model in which ESCRT-I is required for sorting of TCR into MVB-like structures within the cSMAC and for signal termination in response to agonist MHCp. TCR and CD28 engagements occur primarily in the IS dSMAC, resulting in receptor phosphorylation, actin-driven centripetal movement of microclusters, and ubiquitination of TCRs but not CD28 complexes. Upon reaching the border between the pSMAC and cSMAC, TSG101 specifically sorts strong agonist-engaged ubiquitinated TCR into central MVB or lysosomal compartments, leaving CD28 along with associated PKCδ to accumulate and...
continue signaling from an annular cluster. Weak agonist-engaged TCR clusters are not subject to TSG101-mediated sorting and are thus not incorporated into the cSMAC. Our findings clarify the mechanism of cSMAC formation and the origin of recently described segregation of inner TCR and outer, PKCθ-enriched, cSMAC regions (Kaizuka et al., 2007; Varma et al., 2006; Yokosuka et al., 2008). Integrating TSG101 into our current picture of IS structure clarifies the inhibitory role of the cSMAC and the sorting function of TSG101 renews the coexistence of signaling and degradation compartments within this central structure in T cell activation (Cemerski et al., 2008; Lee et al., 2003; Varma et al., 2006).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, four movies, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.immuni.2010.04.005.

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