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MMTV Superantigens Coerce an Unconventional Topology between the TCR and MHC Class II

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Mouse mammary tumor virus superantigens (vSAGs) are notorious for defying structural characterization, and a consensus has yet to be reached regarding their ability to bridge the TCR to MHC class II (MHCII). In this study, we determined the topology of the T cell signaling complex by examining the respective relation of vSAG7 with the MHCII molecule, MHCII-associated peptide, and TCR. We used covalently linked peptide/MHCII complexes to demonstrate that vSAG presentation is tolerant to variation in the protruding side chains of the peptide, but can be sensitive to the nature of the protruding N-terminal extension. An original approach in which vSAG was covalently linked to either MHCII chain confirmed that vSAG binds outside the peptide binding groove. Also, whereas the C-terminal vSAG segment binds to the MHCII α-chain in a conformation-sensitive manner, the membrane-proximal N-terminal domain binds the β-chain. Because both moieties of the mature vSAG remain noncovalently associated after processing, our results suggest that vSAG crosslinks MHCII molecules. Comparing different T cell hybridomas, we identified key residues on the MHCII α-chain that are differentially recognized by the CDR3β when engaged by vSAG. Finally, we show that the highly conserved tyrosine residue found in the vSAG TGXY motif is required for T cell activation. Our results reveal a novel SAG/MHCII/TCR architecture in which vSAGs coerce a near-canonical docking between MHCII and TCR that allows eschewing of traditional CDR3 binding with the associated peptide in favor of MHCII α-chain binding. Our findings highlight the plasticity of the TCR CDRs. The Journal of Immunology, 2014, 192: 000–000.

Superantigens (SAGs) are bacterial or viral proteins that have the ability to stimulate up to 20% of the total T cell population, bypassing conventional MHC class II (MHCII) Ag processing and presentation (1). SAG-activated T cells undergo a strong proliferation phase that is followed by either anergy or deletion (2). In contrast to canonical MHCII/TCR docking, which  

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site of c-vSAG includes the TCR Vβ FR3 and HV4 (13–17). Interaction with MHCIIIs, in contrast, is puzzling and has been reasoned from competition experiments in which vSAG and the staphylococcal enterotoxin A (SEA) compete for binding on both the α- and β-chains (10, 18).

The molecular basis for successful T cell stimulation by vSAG7 is an interesting conundrum. Our group and others have described vSAG presentation to be influenced by the peptide repertoire (19, 20). However, it is unknown whether this characteristic is linked to vSAG binding to MHCII or TCR recognition of the vSAG-bound MHCII complex. Conflicting data of vSAG binding to the MHCII α-chain, β-chain, or peptide-binding groove exist in the literature and none provided a consensual model on how they successfully generate the T cell signaling complex (10, 18, 21, 22). In the present study, we sought to determine the topology of the vSAG/MHCII/TCR complex by examining the respective relationship of vSAG7 with the MHCII chains, associated peptide, and TCR. To this end, we used a panel of covalently linked peptide/MHCII molecules, point mutants, and MHCII/vSAG single chain (SC) constructs. Altogether, our results define a novel MHCII/vSAG/TCR architecture.

Materials and Methods

Plasmids and mutagenesis

The vSAG7 sequence was codon optimized using GeneOptimizer (Invitrogen). pBUD-DRα and -DRβ have been described elsewhere (23). Lumenal portions of DRα (aa 1–191) and DRβ (aa 1–199) were linked to the lumenal part of vSAG7 (aa 67–322) or II (aa 72–297) by the PCR overlap extension method (24). The chains were joined by a small glycine-serine linker (G3-S-G3) introduced in DR chains, II and vSAG7 using complementary overlapping primers. DRα mutants, peptide-linked DRβ (25) hemagglutinin SC trimer (HASCT) and SC dimer (SCD)ACI were generated by the PCR overlap extension method using the Phusion polymerase (New England BioLabs). The resulting cDNAs were cloned into pCDNA3 vector (Invitrogen) and sequenced. The IFNAR, CLIP-HADIR, and GSPS-HADIR peptide-linked DRβ coding sequences were obtained by chemical cloning (Life Technologies) and cloned in pCDNA3.

Abs and reagents

The VS7 (IgG1) mouse mAb is specific to the C-terminal end of MMTV SAG (26), L243 (mouse IgG2a mAb) and ISCR3 (mouse IgG1 mAb) bind specific DRs conformational epitopes (27). XD5 (mouse IgG1 mAb) binds a linear epitope in the β1 domain of all HLA-DR molecules (28), BU45 (mouse IgG1 mAb) binds the C-terminal portion of human II (29), and CεCLP1 (mouse IgG1 mAb) recognizes the N-terminal portion of CLIP (30). The anti-DRα (antiserum against the DRα-chain) and anti-DRβ (antiserum against the DRβ*0101 chain) were obtained following the immunization of rabbits with proteins purified from SDS-PAGE (R.P.S., unpublished). The anti-SEA mAb was a gift of Dr. W. Mourad (University of Montreal) (31). SEA was purchased from Sigma-Aldrich (St. Louis, MO).

Cell lines and transfections

Human epithelial HEK 293T, HeLa DM.5 and HeLa CIITA cells (20), and murine DAP cells were culture in DMEM (Wisent) with 5% FBS (Wisent), whereas Knl1 13.11 and Knl1 12.6 T cell hybridomas (32) were cultured in DMEM with 10% FBS. CTL-2 (TIB-214; American Type Culture Collection) and HAI.7 TCRγ Jurkat cell line, CHTC17 (33), were cultured in RPMI 1640 (Wisent) with 10% FBS and 4 μM 2-ME. HeLa and DAP cells were transfected using Lipofectamine LTX reagent (Invitrogen) according to the manufacturer’s instructions. The cells were selected for resistance against genetin (500 μg/ml; Wisent), and MHCII alleles were sorted by flow cytometry on a FACS Vantage SE (BD Biosciences). HEK 293T cells were transfected using the calcium phosphate method as described previously (34) using 8 µg plasmid DNA. Cells were harvested and analyzed 48 h after transfection.

Flow cytometry

Cells were incubated in PBS with the primary Ab for 30 min on ice. Cells were washed twice with PBS and incubated a further 30 min with the Alexa Fluor 488–coupled goat anti-mouse IgG Ab (Invitrogen) for 30 min on ice. After two more washes, cells were analyzed by flow cytometry using a FACS Calibur (BD Biosciences). Alternatively, cells were incubated for 1 h with SEA on ice and washed twice with PBS prior to the addition of Abs. Intracellular stainings were performed as mentioned on fixed, permeabilized cells (4% paraformaldehyde, 0.05% saponin).

T cell stimulation assays

vSAG7-expressing APCs were cocultured at a 1:1 ratio with 5 × 10^5 T cell hybridomas for 18 h at 37°C. IL-2 production was determined by the ability of the coculture supernatant to sustain the proliferation of the IL-2–dependent T cell line CTL-2 and was measured by [3H]thymidine incorporation (35). Alternatively, the IL-2 concentration was determined by cytometric bead array (BD Biosciences). For indirect presentation, APCs (donors) were cocultured with BJAB cells (acceptors) and responder T cell hybridomas at a ratio of 1:1:1.

Immunoblots

Cells were lysed for 30 min on ice at a density of 2 × 10^7 cells/ml in 1% (v/v) Triton X-100 in lysis buffer (20 mM Tris-HCl, 150 mM NaCl) and complete protease inhibitor (Roche). Heated (65°C) or boiled total protein extracts were then separated by SDS-PAGE (10%) under reducing conditions, unless mentioned otherwise. Samples were transferred to Hybond-ECL nitrocellulose membrane (Amersham Biosciences). Membranes were blocked for 1 h with 5% (w/v) dry milk in TBST (TBS with 0.1% [v/v] Tween 20). The following steps were also performed in TBST with 5% dry milk. Primary Abs to DRα, DRβ, and vSAG7 were incubated for 1 h at room temperature. Membranes were washed three times for 10 min and incubated for 1 h with HRP-conjugated secondary Abs (Bio/Can Scientific) followed by three more 10-min washes. Bands were visualized by the BM chemiluminescence blotting substrate (Roche). For endo H sensitivity, 500 U endoglycosidase H (New England BioLabs) was added to the protein extract and incubated for 1 h at 37°C. Samples were analyzed on SDS-PAGE as above.

Results

MHCII-associated peptide influences vSAG7 presentation

It was previously shown that DM-deficient human cells could not present the toxic shock syndrome toxin-1 or vSAGs (20, 36). Similarly, presentation of these SAGs by cells from H2-DM–deficient mice was inefficient (37–39). DM edits the peptide repertoire by exchanging CLIP for a variety of peptides with better kinetic stability (40). Thus, we wondered whether the accumulation of CLIP/MHCII complexes observed in DMβ2 cells was responsible for the lack of vSAG7 presentation. We used the well-characterized DMβ2 T2-DR3 cell line (41). In these cells, >90% of the DR molecules are loaded with CLIP, a phenotype that is lost upon transfection of DM (30). As shown in Fig. 1A, whereas T2-DR3 cells failed to present vSAG7, T2-DR3-DMα cells led to strong T cell proliferation, similar to that observed with DM-deficient BJAB and COX B cell lines. Of note, in this experiment, vSAG was provided by coculturing APCs with the MHCIIα–DAP–vSAG7* cell line (10). These results suggest that CLIP/DR3 complexes do not support vSAG7 presentation.

We next aimed to determine how the CLIP peptide could affect vSAG7 presentation. Considering the MHCII-associated peptide, vSAG presentation can be influenced by 1) peptide flanking residues (PFRs) extending outside the groove, 2) peptide protruding side chains, or 3) peptide-induced MHCII conformational change. To discriminate between these possibilities, we generated cell lines displaying distinct homogenous pMHCII populations by transfecting DRs with various peptide/DRβ fusion proteins (42). The mouse DAP cells were chosen as model APCs because they are easily transfected and allow vSAG7-specific T cell stimulation upon cotransfection of MHCIIIs and vSAG (43). Once stable peptide/DR1 DAP cell lines were obtained, we transiently transfected vSAG7 and monitored proliferation of mouse vSAG7–responsive T cell hybridomas (44). We used two different hybridomas bearing Vβ6 regions cognate for vSAG7. Because vSAG7 expression is low, we coexpressed a reporter GFP to monitor
vSAG presentation is dictated by the MHCII-associated peptide. (A) vSAG presentation by DM⁺ and DM⁻ B cell lines following vSAG transfer from the vSAG7⁺ DAP cell line (10). Presentation is monitored by the stimulation of Vβ6⁺ T cell hybridomas Kmls 13.11 and KR3⁺. BJAB (DR3) is used as a positive B cell control and vSAG7 to monitor the transfection efficiency. L243/XD5 MFI ratio of the two cell lines is shown. Both cell lines expressed L243 and SEB binding but does not affect vSAG presentation. However, it is hard to pinpoint whether it is one of these effects, or a combination of both, that accounts for the reduction in stimulatory capacity of vSAG7. On the one hand, it was reported that the L243- and SEB-binding sites are already occupied by the vSAG7 peptide, even before the MHCII pocket is saturated. On the other hand, superimposition of the crystal structures of DR1 bound to either CLIP81–86 or CLIP90–101 showed conformational alteration in the loop connecting the third and fourth strands of the MHCII β-sheet platform that includes lysine α39 (50). Mutation of K39 abolishes L243 and SEB binding but does not affect vSAG presentation (51). Therefore, although there is a known conformational change between CLIP-MHCII variants, it is unlikely to affect vSAG presentation due to steric hindrance between the CLIP81–86 extension and L243. This result suggests that vSAG presentation is sensitive to variations in the peptide, we used different mAbs: L243, which binds a conformational epitope on the DRα-chain, and XD5.117, which binds a linear epitope on the DRβ-chain (28, 46). Fig. 1C shows for each cell line the MHCII cell surface expression and the MFI of the reporter GFP (lower panels). Both cell lines expressed similar amount of MHCII and were similarly receptive to DNA upon the subsequent transient transfection of vSAG7 and GFP.

After vSAG7 transfection, cells were incubated with the Vβ6⁺ T cell hybridomas Kmls 13.11 or 12.6. As shown in Fig. 1D, vSAG7 presentation by ICLIP-DR was inefficient compared with cCLIP-DR, the latter giving rise to strong T cell response. Interestingly, we also observed a decrease in L243 mAb reactivity with the ICLIP-DR1 transfectant (Fig. 1C), better portrayed by the iGraph in Fig. 1E showing normalized MFI for L243 over XD5. The reduced L243 reactivity for ICLIP/MHCII complexes can either stem from an MHCII conformation alteration or steric hindrance between the CLIP81–86 extension and L243. This result suggests that vSAG presentation is sensitive to variations in the peptide, we used different mAbs: L243, which binds a conformational epitope on the DRα-chain, and XD5.117, which binds a linear epitope on the DRβ-chain (28, 46). Fig. 1C shows for each cell line the MHCII cell surface expression and the MFI of the reporter GFP (lower panels). Both cell lines expressed similar amount of MHCII and were similarly receptive to DNA upon the subsequent transient transfection of vSAG7 and GFP.

Transfected cell lines from (C), (F), and (H) were used as APCs to stimulate Kmls 13.11, Kmls 12.6 hybridomas, or Jurkat T cells (D, G, I, J). All data are representative of at least three independent experiments.
as Jurkat) (33, 52). Our rationale was to be able to distinguish both the conventional and vSAG presentation in the same setting, using different T cells. Additionally, to minimize adverse effects linked to the bound peptide’s primary sequence, we performed the same assays using another peptide supporting vSAG presentation, the tetanus toxin peptide (TT829–842). Similar to HA-DR1, TT-DR1 has great kinetic stability and also fills the P1 pocket with a tyrosine, thus providing a system to test the exclusive contribution of peptide-protruding side chains (Fig. 1B) (53, 54). The TCR contacting side chains of these two peptides were interchanged, resulting in the backbone (b) HAα and TTβ variants, and involved a charge modification at P-1 (K-Q), a change of a small residue (V) to a bulkier one (I) at P2, and finally a change from a positively charged residue to a highly hydrophobic one (K-F) at P8. When any of the peptide-protruding side chains were implicated in specific vSAG7 contacts, one should expect to see differential stimulation between these peptides, independently of the T cell Vβ specificity. The stably transfected HAβ and TTβ constructs (Fig. 1F, 1H) were then tested for their capacity to stimulate T cells (Fig. 1G, 1I). Every variant successfully presented vSAG7, suggesting that a peptide’s protruding side chains are not critical for vSAG7-mediated response (Fig. 1G, 1I). Conversely, presentation of HAα failed to stimulate Jurkat T cells (Fig. 1J), as specific TCR contact residues have been modified (52). Many covalently-linked peptides supported strong vSAG7 presentation, ruling out a role for the peptide’s C-terminal linkers and thus PFR at this end. Altogether, these results suggest a model in which vSAG7 presentation is tolerant of the conformation adopted by the N-terminal extension of some peptides.

N-vSAG7 has an overlapping binding site with SEA on the DRβ-chain

The above-described data support the notion that C-vSAG7 binds to the MHCII α-chain. However, it has been proposed that the vSAG7 polypeptide has two binding sites on MHCII, one of which shares the same interface as SEA on the β-chain or binds the peptide-binding groove (18, 21, 22). Maturing vSAGs are composed of two domains that remain noncovalently associated upon processing. C-vSAG participates in MHCII/TCR bridging but the role of N-vSAG is less clear. To gain insight on the role of the latter, we designed two SC polypeptides in which the luminal domain of either DR chain is linked to that of vSAG7. These straightforward SCs were inspired by a similar fusion between MHCII and Ii (55). Such SCs were made possible because HLA-DR and vSAG7 are type I and type II proteins, respectively, allowing fusion of the C terminus of DR chains to the N terminus of vSAG7. The resulting DRα- and DRβ-based fusion molecules were named oSCD and βSCD, respectively, and are depicted in Fig. 2A. For brevity, when an SC is mentioned in an experimental setting, it is always cotransfected with its matching complementary wt DR chain partner; for example, DRα plus βSCD is referred to as βSCD.

First, we characterized the interaction between the SCs and their complementary MHCII chain partner. For these experiments,
we used HEK 293T cells given their high transfection efficiency. First, the α-SCD apparently failed to form a functional complex with DRβ, as judged by the lack of L243 surface staining and the fact that DRβ remained entirely EndoH-sensitive (Fig. 2B, Supplemental Fig. 2, left panel). Also, the α-SCD migrates as two major bands, which are smaller than βSCD and appear to be devoid of sugars (Supplemental Fig. 2, middle and right panels). In contrast, the βSCD properly associated into heterodimers with DRα, trafficked to the plasma membrane, and was recognized by a conformational mAb (Fig. 2B, left panel). Using the anti–C-vSAG7 mAb VS7, we also observed MHCII-bound vSAGs at the cell surface (Fig. 2B, right panel). Altogether, these results indicate that only the βSCD can generate ordered complexes and allow vSAG surface expression.

Next, to confirm that vSAG7 behaved similar to the wt vSAG when part of the βSCD, we verified whether it still competed the binding of SEA. We transfected HEK 293T cells with either wt DR or βSCD and performed SEA binding assays monitored by flow cytometry. Fig. 2C shows the DR (left panel) and SEA (right panel) staining profiles, which reveal weak SEA binding to cells expressing βSCD. Once normalized for MHCII surface expression, we noted a marked reduction in the binding of SEA for βSCD compared with the wt or a control βα-SCD controls (Fig. 2A, 2D). These results indicated that the vSAG moiety on DRβ interferes with SEA binding. However, because SEA has a minor low-affinity binding site on the MHCII α-chain, the possibility remained that the observed competition was due to the presence of vSAG7 on DRα (56, 57). To test this, we performed the same experiment using a DRα mutant that is unable to bind SEA (DRαK39A) (56). As shown in Fig. 2E, binding of SEA is the same whether DRβ is associated with wt MHCII α-chain or K39A mutant. Moreover, truncating C-vSAG from βSCD, generating the βSCDΔCt, still reduced SEA binding, confirming that N-vSAG7 and SEA have an overlapping binding interface (Fig. 2D). The high SEA concentration and affinity for βHis81 are factors likely to favor displacement of some of the vSAG7 molecules and could explain why competition was not total. Likewise, processing of the vSAG7 moiety is observed and could favor its dissociation from the β-chain (Supplemental Fig. 2). These data confirmed that N-vSAG has an overlapping binding site with SEA on the MHCII β-chain.

**vSAG7 does not bind the MHCII peptide groove**

Our results described so far define the binding site of C-vSAG7 and N-vSAG to the MHCII α-chain and β-chain, respectively. Thus, it was difficult to envision a model where vSAG7 interacted with both DR chains at once. Huber and colleagues (22) proposed that a segment of MMTV SAGs (called the MHCII peptide-binding motif) interacts with the MHCII peptide-binding groove upon synthesis in the endoplasmic reticulum. The MHCII peptide-binding motif is part of N-vSAG and could bridge both vSAG segments through the MHCII clefT. To verify whether vSAG7 occupies the MHCII groove, we designed a new construct, HASCT, in which the HA peptide was appended to the βSCD N terminus (Fig. 2A). Based on the Hsu et al. (22) premise, our rationale was that N-vSAG7 would prevent HA binding to the groove. Fig. 3A shows the surface DR expression for both βSCD and HASCT in comparison with wt DR and HADR. Interestingly, DR surface staining was increased in both transfectants expressing the linked HA peptide. These data corroborate well with the increased stability of MHCIIIs when optimal peptide occupies the groove (25). Soluble versions of DRβ and HAβ (sβ and sHAβ) chains were used as controls and ruled out transmembrane domain–mediated bias between the transmembrane-devoid βSCD and controls. L243 MFI ratios of sβ and βSCD against sHAβ and HASCT, respectively, showed a 4-fold increase in expression when HA was fused to DRβ (Fig. 3B). That the tethered HA peptide increased expression of DR is incompatible with a model in which vSAG would occupy the binding groove.

To confirm that the HA peptide was lodged inside the groove and not displaced by the immature vSAG7 polypeptide, we tested the complex for SDS sensitivity. Indeed, HA/DR1 complexes resist SDS denaturation and migrate as compact peptide-loaded heterodimers...
under nonboiled conditions (58). Cell lysates obtained from the transfectants presented in Fig. 3A as well as a pHSCD control were analyzed by SDS-PAGE. XD5 mAb was used to probe the immunoblots. As expected, in absence of HLA-DM, neither Ii nor vSAG7 SCDs formed compact complexes with DRα (Fig. 3C, lanes 4 and 6). In contrast, when HA was linked to either DRβ or βSCD (lanes 2 and 8), compact, SDS-resistant heterodimers were observed, confirming that the HA peptide was present in the groove.

To unequivocally prove that vSAG7 did not bind to the MHCII cleft, we tested whether the HASCT could induce both Jurkat and vSAG-specific T cell responses. To this end, we moved from the HEK 293T to HeLa cells, the former being unable to present vSAG (20). Of note, although the molecular basis of this defect remains obscure, HeLa cells are unable to present vSAGs unless transfected with CIITA or treated with IFN-γ (20). This provided a useful system to evaluate the presentation of HA independently of vSAG in the absence of CIITA and of both HA and vSAG7 in the presence of CIITA. We transiently transfected our various β-chains into either HeLa or HeLa CIITA+ cells and assessed the response of Jurkat or Kmls 13.11 cells. As expected, vSAG7 presentation only occurred in the presence of CIITA, whether from the SCD or SCT (Fig. 3E). Surprisingly, HeLa cells transfected with HASCT failed to stimulate Jurkat cells in contrast to the control sHA (Fig. 3D).

The lack of HA presentation can be attributed to either an MHCII conformation flaw or to the vSAG moiety of the HASCT hampering proper TCR docking. We argue for the latter alternative because the tested mAbs did not distinguished the HASCT from the sHA control (Fig. 3A), and in the presence of CIITA, HA presentation was restored (Fig. 3D). However, that Jurkat failed to respond to the HASCT in CIITA+ cells was puzzling. This is not due to degradation of the SCT-RA peptide and its subsequent association to endogenously expressed MHCII, as the DRβ0102 allele in the homozygous HeLa CIITA+ cells does not present HA307–318 to Jurkat or Kmls 13.11 cells (59). As expected, vSAG7 presentation only occurred in the presence of CIITA, whether from the SCD or SCT (Fig. 3E).

vSAG7 presentation relies on MHCIIa/TCR interactions

To better pinpoint the C-vSAG7 binding site on DRα and investigate the TCR binding topology, we generated an array of mutants from DQ2 and I-Aq, which present vSAG poorly and span aa 63–68, the interface associated with SEB binding (12). Fig. 4A shows the point mutants on the HA-DR1 model (Protein Data Bank no. 1FYT) as well as DRβ residues implicated in the cross-linking of MHCII molecules. Binding of C-vSAG to the MHCII α-chain is conformation-dependent and abrogated by a peptide N-terminal extension. The TCRβ CDR3 binds the MHCII α-chain specifically instead of the peptide, whereas vSAG binds the FR3 and HV4 region of the Vβ. The exact nature of Vα binding to the MHCII β remains to be fully characterized (60).
DR expression in DAP cells is given in Fig. 4B as assessed by L243 and XD5 staining. As above, stable cell lines were transiently cotransfected with vSAG7 and the reporter GFP. Then, cells were cocultured with Kmls 13.11 and Kmls 12.6 T cells and IL-2 production was measured. In an effort to minimize the effect of the cells’ intrinsic associated peptides, we coexpressed each DRα variant with the HADRβ-chain. Additionally, this allowed us to monitor the stimulation of HA-specific Jurkat cells, which could be impeded by the α-chain mutations (Fig. 4D). As predicted from the HA/DR1/HA1.T CCR cocystal (52), only mutation of TCR contact residues α64, α65, and α67 abrogated presentation of HA to Jurkat cells.

Of note, the reactivity of L243 was strongly decreased toward DR mutants α63 and α67 (Fig. 4B). This is best portrayed by the graph in Fig. 4C showing L243 MFI over the MFI of the expression-insensitive XD5 mAb. Surprisingly, those same DR mutants had a dramatically reduced ability to present vSAG7 (Fig. 4E, 4F), suggesting again overlapping binding regions between L243 and vSAG. Because the side-chain of α63 points laterally, the weakened stimulation observed with both hybridomas by the α163E MHCIIs mutant cell line suggests that C-vSAG7 binding is somehow affected by this mutation. Indeed, a less obstructive mutation, α63A, restored vSAG7 stimulation (Supplemental Fig. 3A, 3B).

Interestingly, whereas DRαA64R allowed a strong Kmls 13.11 stimulation, Kmls 12.6 failed to respond (Fig. 4E, 4F). This result indicates a TCR recognition effect unrelated to vSAG7 binding and highlights the importance of specific DRα/TCR contacts for efficient T cell stimulation. In line with this, it became difficult to evaluate whether the effect of DRα K67A on vSAG presentation is actually linked to a weakened association between vSAG and DRα or to a TCR recognition defect. Indeed, DRα K67 is characterized by a lack of both L243 reactivity and inefficient presentation of HA peptide to Jurkat cells, making both outcomes plausible (Fig. 2C, 2D). Although weak, the stimulatory response observed exclusively with the Kmls 13.11 hybridoma suggests that TCR recognition is a factor.

The solvent-exposed DRα α-helix is used by SEB to bridge TCRs (61). The fact that vSAG7 presentation implicates DRα/TCR interactions demonstrates that the topology of the ternary complex is different from the one involving SEB. Interestingly, only the DRα mutant A64R cell line was unable to support SEB presentation (Supplemental Fig. 3C). The highly conserved A64 is buried inside the SEB/DRα interface and the Arg substitute is likely to sterically hinder SEB association, probably reflecting the effect of I63E on vSAG7 presentation. Overall, it is apparent that the mechanism leading to TCR recognition of the vSAG/MHCIIs complexes differs from SEB and canonical peptide/MHCIIs recognition.

vSAG7 mediates TCR activation through a conserved TGXY motif

The specific interactions between the TCR and the MHCIIs α-chain suggest the existence of conserved vSAG/TCR contacts. Given the numerous responsive Vβs for all vSAGs, it is unlikely that the TCR interaction is solely based on the polymorphic vSAG C terminus, as it would severely impede the likelihood of a cognate partnership. Interestingly, an alignment of MMTV SAGs and MMTV SAG-related domain from other viruses (e.g., herpesvirus) revealed a conserved TGXY motif (Fig. 5A) located at residues 226–229 of C-vSAG. We mutated the Tyr229 to a Phe and assessed the ability of vSAG7-YF to stimulate different T cell hybridomas. Fig. 5B indicates that the Y229F mutation introduced either in full length or SC vSAG7 abrogated the activity. The TGXY motif could impact vSAG activity in many ways. On the one hand, it could perturb C-vSAG binding to MHCIIs or its overall structural integrity. Both of these hypotheses are refuted by the fact that the mutation did not affect surface expression of MHCIIs or vSAG7 (Fig. 5C, 5D). On the other hand, the Tyr229 could be implicated in direct TCR contact. Supporting this mechanism is the presence of a similar motif, LGNY, on a SEB contacting loop that interacts with the HV4 region of the TCRβ. Accordingly, mutation of the Tyr on SEB prevents its presentation (63, 64). Furthermore, Digglemann and colleagues (65) described the region of C-vSAG bearing the TGXY motif as part of the TCR-interacting domain. These results suggest that in addition to highly polymorphic regions responsible for Vβ specificities, vSAG binding also relies on conserved vSAG-specific TCR interactions.

Discussion

The data presented in this study define how vSAG7 bridges MHCIIs to the TCR. C-vSAG binds the MHCIIs α-chain in a conformation-sensitive manner at the interface formed between the α-helix and the β-sheet platform. We demonstrated that, when coerced by vSAG, the TCR recognizes the MHCIIs in a near canonical manner, which constitutes a unique topology among those previously described for SAGs. Next, our results suggest that maturing N- and C-vSAG7 bind the MHCIIs β- and α-chains, respectively, on distinct MHCIIs. Finally, we identified a conserved motif in MMTV SAGs responsible for TCR binding independently of the TCR Vβ specificity. Based on our results and those of others, we propose a model in which the N- and C-terminal domains of a single vSAG crosslink two MHCIIs and bridge only specific TCR-bearing Vβ elements that can be skewed to recognize the MHCIIs α-chain instead of the associated peptide (Fig. 4G).
C-vSAG7 binding to the MHCIId α-chain is influenced by the associated peptide

Presentation of vSAG7 requires that MHCIId be filled with a diverse peptide repertoire (11, 20, 38). In the present study, we demonstrated that a single peptide supports vSAG7 presentation. However, the MHCIId associated peptide must meet certain criteria to be part of a permissive pMHCIId. By comparing cell lines expressing either cCLIP-DR1 ΔCLIP, ICCLIP-DR1ΔCLIP, and ICCLIP-HADR1ΔCLIP, we showed that the CLIP ΔPFRs, PFRs inhibit vSAG7 presentation (Fig. 1D, Supplementary Fig. 1). Interestingly, it was previously described that CLIP N-terminal extensions also interfered with SEB binding to MHCIId (49). The crystal structures of CLIP-DR3 and CLIP-ΔI63A show that the CLIP ΔPFRs N-terminal extension is disordered, suggesting an idiosyncratic effect of this CLIP region on vSAG function (66, 67). Future studies will address this issue by characterizing the impact of a larger panel of N-terminal extensions linked to various core peptides. Nevertheless, knowing the profound effect of PFRs on canonical and toxic shock syndrome toxin-1 T cell responses (39, 68, 69), and that DM favors the binding of peptides that tightly fit the groove, our findings point to the presence of N-terminal peptide trimming as a potentially important determinant for vSAG7 presentation (40, 70). These results explain why DM-deficient cells, either murine or human, are unable to present vSAG7, as they are predominantly charged with CLIP peptide bearing a 4- to 6-aa extension (34, 45, 71). Hsu et al. (22) reported that a DM+ B cell line was able to present vSAG7. However, as opposed to T2 cells expressing a single MHCIId (DR3) or our DAP cell lines (DR1), the proportion of the various alleles of DR, DQ, or DP that are bound to CLIP at steady-state, or which form of CLIP is bound, was not determined in this DM− model.

It is unclear whether the previously reported need for peptide diversity reflects a direct contribution of the peptide’s sequence to part of the vSAG binding site or is an indirect negative effect echoed in the MHCIId conformation. The findings presented in this study support the latter hypothesis. It is unlikely that vSAG7 interacts with the MHCIId-bound peptide’s protruding side chains because severe changes in charge or size between those of HA, TT, and CLIP did not affect their ability to activate Vβ6- and Vβ8.1-bearing T cells (Fig. 1, Supplemental Fig. 3D). Although we did not conduct a thorough saturation mutagenesis of the peptides, our observation of DRαa/TCR contacts portrays a setting in which vSAG7/pseudo interactions are improbable (see below).

Our results suggest that C-vSAG7 binds to the lateral interface formed by the solvent-exposed DRαa helix/β-sheet junction (Fig. 4A), an area highly susceptible to peptide-induced conformational changes (72). That HA-DR1ΔI63A, but not HA-DR1ΔI63A, reduced both L243 mAb reactivity and vSAG7 presentation capabilities points to an MHCIId conformation defect (Fig. 4C, 4D, Supplemental Fig. 3A, 3B). It was previously reported that mice expressing Ea-binding I-Aβ as the sole pMHCIId were unable to mount vSAG7-specific responses, supporting the conclusion that a diverse MHCIId peptide repertoire was required (38). In light of our results, we can speculate as to why this pMHCIId failed to present vSAG. The Ea(52-68) peptide bears no N-terminal PFRs, and the C-terminal linker between the peptide and the MHCIId β-chain does not influence vSAG presentation. Thus, as previously presented by the authors, the inability of Ea-I-Aβ to successfully present vSAG must be due to its intrinsic conformation (38). Indeed, whereas the 25-9-17 mAb binds a panel of peptide-bound I-Aβ, Ea-I-Aβ was not recognized (73, 74). One possibility is that vSAG7 binding is affected by the αF24 residue, reported to be pushed outward by the bulky pl-filling Phe of Ea at the C-vSAG7 binding site (75). Although we did not observe an effect of a Y-A substitution at the p1 HA residue (Supplemental Fig. 3E), it would be interesting to determine whether a bulkier amino acid (e.g., Trp) substitution would reduce vSAG7 presentation accordingly. Collectively, these results indicate that the interface formed on the DRαa helix/β-sheet junction is paramount for vSAG7 binding and that its presentation is vulnerable to both peptide-mediated conformational changes and N-terminal PFRs.

TCR engagement by vSAG7 is unique among SAGs

Most of the SAG-mediated T cell signaling complexes outline a typical interaction between the TCR ββ and the SAG, where the Vβ sometimes participates in MHCIId β-chain binding (4, 76, 77). Consequently, Vβ/MHCIId α-chain contacts are sterically precluded by the bound bacterial SAGs. Given the lack of an MMTV SAG crystal structure, it is challenging to predict how vSAGs force MHCIId/TCR association. Previous analyses of the response of mature peripheral T cells against SAGs have clearly established that non-Vβ components of the TCR are involved in the pMHCIId/vSAG complex recognition (78–81). The Vβ-chain was shown to be an important part of the complex, and a skewed repertoire in responding cells has been identified (82, 83). Within a given Vβ family, reactivity was seen with only certain Vβ subfamily members (83, 84). Thus, the variations in the CDR regions, especially CDR3, are likely to affect the capacity of these TCRs to bind the MHCIId and might be at the origin of many of the reported cases of MHCIId allelic/isotype restriction (32, 47, 85).

Based on these facts and our results demonstrating that DRαa A64R and K67A mutant cell lines differentially stimulated distinct hybridomas bearing the same Vβ, it is clear that the TCR/MHCIId contacts are imperative to vSAG7 signal transduction (Fig. 4E, 4F). Moreover, these findings highlight direct MHCIId α-chain/TCRβ contacts and would imply that vSAG7-mediated TCR engagement resembles that of conventional peptides, as hypothesized by the Marrack and Kappler group following their discovery that the TCR βT24Y mutant abrogated both conventional Ag and vSAG presentation (13, 86). Along the same lines, our MHCIId α64 and α67 mutants were unable to trigger HA-specific activation of Jurkat cells (Fig. 4D). Based on the crystal structure of HA/DR1/HAI.7 TCR, DRαa A64 and K67 are bound to the CDR2β D51 (52). However, one must be cautious in unifying these results. Indeed, as we have shown with our many different peptide/MHCIId combinations, the peptide’s protruding side chain did not affect vSAG response by the tested hybridomas, as would be expected in a traditional pMHCIId-TCR setting (Fig. 1). Furthermore, mutations at residues α65 and α68, which are also implicated in TCR binding (52), had no effect on the ability of MHCIId to present vSAG7 (Fig. 4D, 4E). Thus, it is fitting to picture a near canonical docking between MHCIId and TCR that is coerced by vSAG. Such architecture allows eschewing of traditional CDR1/3 binding with the associated peptide in favor of MHCIId chain binding as proposed by Nguyen et al. (87). These Vα/MHCIIdβ and Vβ/MHCIIdα contacts remain to be verified biochemically (88).

A large body of literature suggests that the β chain junctional region could influence TCR recognition of SAGs (79, 89–91). Especially relevant was the demonstration by many groups that Vβ6 thymic deletion is incomplete in vSAG7 mice and that Vβ junctional diversity regulates vSAG reactivity (91, 92). At first glance, the need for a diverse peptide repertoire and the importance of Vβ junctional diversity may seem paradoxical. Because many peptides support vSAG binding, we do not expect skewing in CDR3. However, our results point to the existence of nonpermissive peptides regulating vSAG binding and to unconventional...
interactions between MHCII and TCR. In order words, there may be extra pressure on CDR3s to have more intimate contacts with the MHCII chains than during canonical peptide recognition. Indeed, thymic positive selection does not directly influence the fine specificity of T cells toward MHCII, and it has been suggested that TCR/MHCII interactions taking place during vSAG presentation are unconventional and linked to TCR recognition of haplotype-specific MHCII residues (79, 87). This is in line with the recent demonstration by Kilgannon et al. (92) that three of eight Vβ6+ T cells, although all specific for the same pMHCII (K5-I-Aβ), did not respond to vSAG7.

A recent report by Stadinski et al. (93) stressed the fact that TCR specificity for pMHC ligands is not driven by germline-encoded pairwise interactions. Notably, they described a single TCR Vβ using alternate strategies to bind pMHC when paired with different Vα. The authors argued that because CDR1 and CDR2 loops have the flexibility to bind their ligands in many ways, this could not fit a pairwise coevolution model. Accordingly, it demonstrated that the CDR3 loops can markedly alter those evolutionarily selected contacts (94). Canonical CDR/pMHCII contacts can differ under different pressures, supporting a model in which vSAG enforces such unconventional docking.

vSAG7 moieties bind distinct MHCII

Given that vSAG7 N- and C-terminal moieties bind respectively the MHCII β1 and α1 domains, one must ask how such binding is possible while remaining noncovalently associated. We propose a model where C-vSAG binds the α-chain of an adjacent MHCII, while still bound to N-vSAG, with the latter attached to the β-chain. In other words, unable to reach the α-chain of its MHCII partner in cis, C-vSAG interacts with another MHCII in trans. This model highly resembles the manner in which SEA crosslinks MHCII molecules via a low-affinity binding site to the α-chain and the Zn-dependent binding site on the β-chain (95, 96). In the context of the SCT, our results showing that HA presentation to Jurkat cells is null in endogenous MHCII context of the SCT, our results showing that HA presentation to specific TCR Vα and the Zn-dependent binding site on the cis pair were implicated in the recognition of the vSAG/peptide/MHCII by the TCR suggests that T cell activation is influenced by the polymorphic CDR3β region, linked to the recognition of the MHCII α-chain. To our knowledge, this represents a novel SAG-mediated MHCII/TCR architecture. According to a recent study by Nur-ur Rahman et al. suggesting that the TCR CDR2β is the critical determinant for the functional recognition of bacterial SAGs, it is clear that the topology of vSAG differs from those previously described (97). Because both the MHCII allele and associated peptide influence the potential interaction between MHCII and TCR, a given TCR could only recognize a fraction of the vSAG/pMHCII complexes (79). MMTV SAGs are expressed at very low density on the cell surface and still remain highly potent T cell activators. This expression pattern is of great importance, as it mimics the low density of conventional antigenic peptide/MHCII complexes, critical for T cell activation (98, 99).

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Disclosures

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