

Bee venom processes human skin lipids for presentation by CD1a

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Venoms frequently co-opt host immune responses, so study of their mode of action can provide insight into novel inflammatory pathways. Using bee and wasp venom responses as a model system, we investigated whether venoms contain CD1-presented antigens. Here, we show that venoms activate human T cells via CD1a proteins. Whereas CD1 proteins typically present lipids, chromatographic separation of venoms unexpectedly showed that stimulatory factors partition into protein-containing fractions. This finding was explained by demonstrating that bee venom-derived phospholipase A2 (PLA2) activates T cells through generation of small neoantigens, such as free fatty acids and lysophospholipids, from common phosphodiacylglycerides. Patient studies showed that injected PLA2 generates lysophospholipids within human skin *in vivo*, and polyclonal T cell responses are dependent on CD1a protein and PLA2. These findings support a previously unknown skin immune response based on T cell recognition of CD1a proteins and lipid neoantigen generated *in vivo* by phospholipases. The findings have implications for skin barrier sensing by T cells and mechanisms underlying phospholipase-dependent inflammatory skin disease.

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Abbreviations used: APC, anti-gen presenting cells; DDM, dideoxymycobactin; LC, Langerhans cells; mDC, monocyte-derived DC; PC, phosphatidylcholin; PLA2, phospholipase A2.

Extensive evidence for the important role of peptide-MHC complexes in T cell activation evolved into a widespread belief that peptides are the only common and natural target of human T cell responses. Therefore, until recently, nearly all human clinical studies of T cell action in autoimmune, allergic, and infectious diseases were targeted at peptide antigens. For example, most candidate antigens for human T cell-mediated autoimmune diseases are proteins (Klein et al., 2014). Subunit vaccines (Tameris et al., 2013) and diagnostic tests (Lalvani and Pareek, 2010) rely on defined peptide motifs, and mouse models

of autoimmunity start with protein and peptide antigen vaccination. However, the discovery of the function of CD1a, CD1b, CD1c, and CD1d proteins (McMichael et al., 1979; Calabi and Milstein, 1986) as antigen-presenting molecules expands the biochemical spectrum of natural antigens for T cells to include many types of lipids (Porcelli et al., 1989, 1992; Kronenberg and Kinjo, 2005).

CD1 proteins are conserved among mammals (Kasmar et al., 2009) and are expressed at high density on thymocytes and professional APCs in the periphery, including Langerhans cells (LCs), B cells, macrophages and myeloid DCs (Dogan et al., 2007). In cells, CD1 proteins bind and display hundreds of molecular species of self-sphingolipids, phospholipids, and

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acylglycerides (Huang et al., 2011), and >20 types of stimulatory lipid antigens for T cells are now known (Young and Moody, 2006). The molecular bases by which lipids are recognized by T cells are well established through crystal structures of CD1, CD1 bound to lipid, and CD1-lipid bound to a TCR (Zeng et al., 1997; Gadola et al., 2002; Borg et al., 2007). The alkyl chains of lipids are sequestered within the grooves of CD1 proteins, allowing the carbohydrate, sulfate, phosphate, and other polar elements to protrude and interact with TCRs.

Despite the wealth of molecular and cell biological data showing that mammalian $\alpha\beta$ T cells can recognize lipids, translational research to determine the roles of CD1-restricted lipid antigens in vivo or during diseases that are commonly seen by physicians is limited. Most reported studies have focused on CD1d and CD1d-restricted T cells, known as NKT cells, because CD1d proteins are the only CD1 isoform expressed in commonly used mouse models (Godfrey and Rossjohn, 2011). Yet CD1a, CD1b, and CD1c differ from CD1d proteins, and from one another, in their trafficking and tissue distribution, suggesting that they exert different physiological roles (Kasmar et al., 2009). Notably, CD1a, unlike CD1b and CD1c proteins, has been known for decades as a phenotype-specific marker of human epidermal LCs (Dougan et al., 2007). In addition to studies of guinea pigs (Hiromatsu et al., 2002a,b) and transgenic mice (Felio et al., 2009), the functions of CD1a, CD1b, and CD1c proteins have been studied in humans during tuberculosis (Moody et al., 2000) and seasonal allergy (Agea et al., 2005), and as in other pathogens that infect humans (Zeissig et al., 2010). However, human studies rely mostly on T cell clones whose functions change over time during in vitro culture and may not represent the natural populations of T cells in vivo.

To study polyclonal autoreactive human T cells ex vivo, APCs that lack detectable surface expression of MHC proteins (K562 cells) were engineered to express CD1a, CD1b, CD1c, or CD1d proteins at high density (K562-CD1). K562-CD1 cells provide lipid autoantigens, and such MHC^{low} CD1^{high} APCs largely avoid MHC alloreactivity (de Jong et al., 2010). Therefore, T cells from groups of randomly chosen or unrelated donors can be tested for antigen responses, allowing cohort studies and quantitation of CD1 autoreactivity in healthy subjects. Three recent studies show that CD1a autoreactive T cells are present in the peripheral blood of nearly all humans tested, defining CD1a autoreactive T cells as a distinct component of the human immune system (de Jong et al., 2010, 2014; de Lalla et al., 2011). These studies also found that the rates of T cell autoreactivity are higher to CD1a, as compared with CD1b, CD1c and CD1d. In addition to studies of peripheral blood, recent studies implicate CD1a autoreactive T cells as functioning in organ-specific immunity in the skin. CD1a is the major human CD1 protein expressed at high density on LCs (Dougan et al., 2007), and CD1a autoreactive T cells express skin-specific homing receptors (cutaneous lymphocyte antigen, CCR4, CCR6, and CCR10) and home to the skin in large numbers (de Jong et al., 2010).

In addition, skin-specific oils, including squalene, function as autoantigens presented by CD1a proteins (de Jong et al., 2014).

Venoms act within the skin to cause pain or death by transfer to highly unrelated organisms; they involve multiple active substances that typically function by coopting host responses and thus have a long history as useful probes of natural host inflammatory responses. In particular, venom-induced immune responses have been relevant beyond venom reactivity and have provided broader immunological insights in to novel pathways of inflammation and tolerance (Bilò and Bonifazi, 2008; Aslam et al., 2010; Gutierrez and Rodewald, 2013). Because bee and wasp venom responses are localized initially in skin, where CD1a is so abundantly expressed, and because the lipidic content of the bee and wasp venoms was unexplored, we sought to use bee and wasp venom as a model system to investigate novel pathways of inflammation in the skin. After initial studies documented a CD1a-mediated response in individual T cell clones and among cohorts of patients, mechanistic studies tracked the antigenic substance to venom proteins. By investigating venom-derived proteins, we discovered an unexpected mechanism by which venom phospholipases create lipid neoantigens in vivo providing a new view of venom responses and insight into the role of phospholipases in CD1a biology.

RESULTS

Responses of T cells to CD1 molecules and venom

Stinging insects in the genera *vespula* (wasp) and *apis* (bee) generate a local intradermal inflammatory response. To investigate a possible role of CD1, we obtained CD3⁺ T cells from healthy adult individuals and mixed them with wasp venom-treated and irradiated K562-CD1 APCs, which were mock transfected or transfected with either CD1a, CD1b, CD1c, or CD1d. As expected based on prior work (de Jong et al., 2014), we observed low but detectable alloreactive background responses of ~ 1 in 2,000 T cells after 12–14 d in culture to mock-transfected target cells in donor C1175 (Fig. 1 A, left), which is representative of three donors. In the absence of venom, T cells showed responses to K562-CD1a cells at rates higher than to mock transfected cells or cells expressing other CD1 isoforms, confirming the presence and relatively high rates of CD1a autoreactive T cells seen previously (de Jong et al., 2010, 2014; de Lalla et al., 2011). Further, polyclonal responses to wasp venom above background levels were detected, but were seen only when using CD1a-expressing cells as targets ($P < 0.05$, Fig. 1 A, left, representative of three donors). These responses demonstrate that a T cell response to wasp venom is restricted by CD1a proteins.

Apis mellifera (honeybee or bee) venom also causes skin immune responses and shares certain mechanisms of immune action with wasp venom (Müller et al., 2009). For example, patients that develop hypersensitivity toward wasp venom can also show reactivity to bee venom (Egner et al., 1998). Therefore, we next investigated the CD1-restricted T cell response to bee venom and found a pattern of response that was similar to that of wasp venom, including augmentation of the baseline

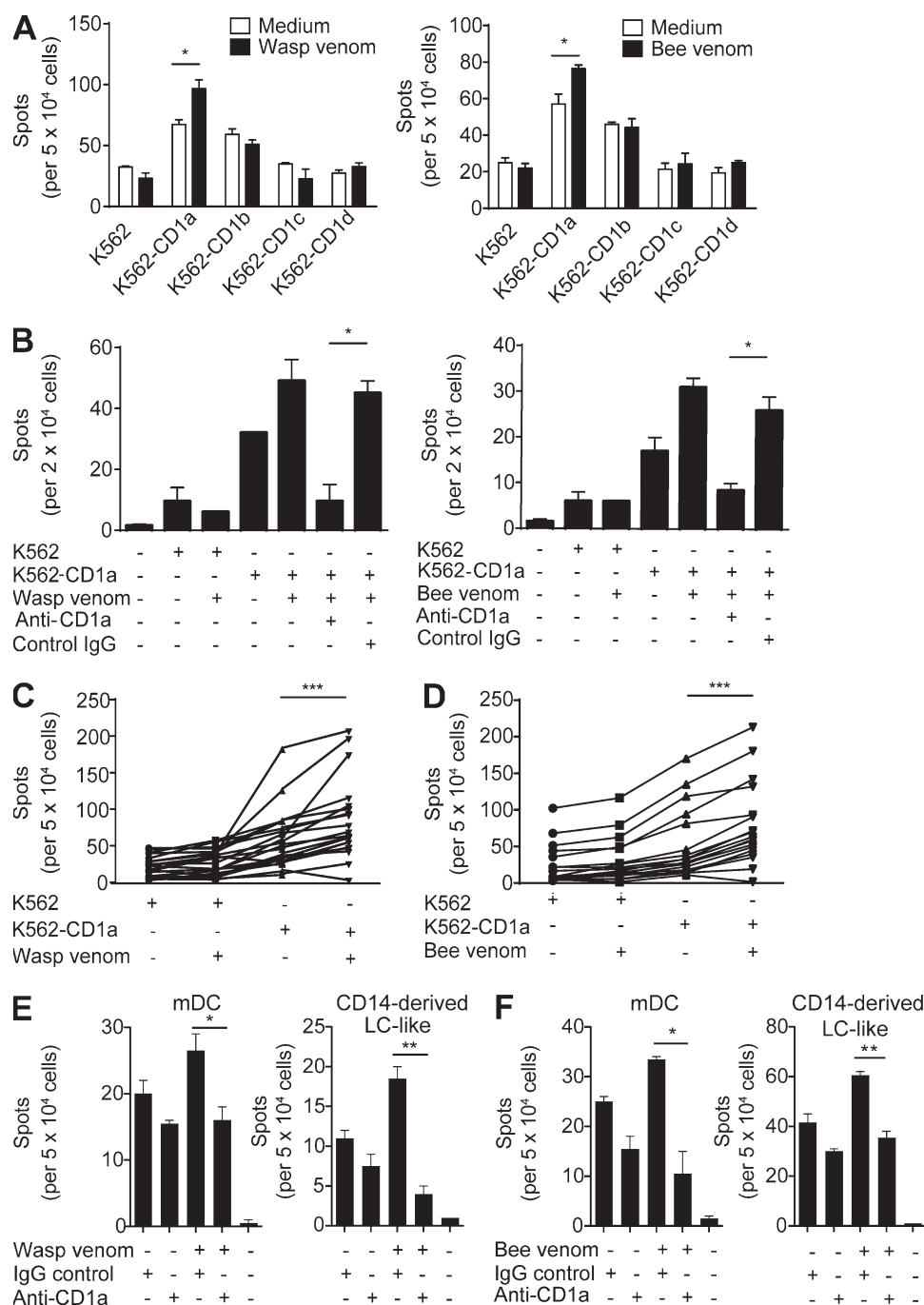


Figure 1. *Vespa* spp and *Apis mellifera* venoms induce a preferential activation of CD1a-restricted T cells among the group1-CD1 reactive cells. (A) T cells were isolated by CD3 MACS beads from healthy donor PBMCs and cultured for 12–14 d with IL-2 and irradiated K562 cells transfected with CD1a (K562-CD1a), CD1b (K562-CD1b), CD1c (K562-CD1c), CD1d (K562-CD1d), or an empty vector (K562) in the presence of venom. CD1 reactivity was then examined by IFN- γ ELISpot with transfected or untransfected K562 cells either in the presence or absence of *Vespa* spp or *Apis mellifera* venoms. Representative data for one donor (C1175) of three are shown. (B) The CD1a-restricted T cell response in the presence or absence of anti-CD1a (donor C1098) and *Vespa* spp and *Apis mellifera* venoms. CD1a-restricted, venom-specific responses were measured in 21 donors for *Vespa* spp venom (C) and *Apis mellifera* venom (D). mDC or in vitro LC-like cells derived from CD14⁺ cells were pulsed with 1 μ g/ml wasp venom (E) or bee venom (F) and incubated with the T cells in the presence or absence of anti-CD1a antibody or isotype control. IFN- γ production was measured by IFN- γ ELISpot. Representative data for one donor (C556 [E] and C560 [F]) of three are shown. Data were mean of triplicate measurements \pm SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

autoreactive response by venom in the presence of CD1a (Fig. 1 A, right). Thus, this pilot study provided proof of principle for CD1a-mediated human cellular responses to wasp and bee venom among human polyclonal T cells.

The total IFN- γ response of fresh polyclonal T cells consisted of three identifiable components: a response to K562, an increment in response to CD1a and a further increment to added venom, consistent with CD1a presentation of venom antigens. Expansion of T cells with antigen ex vivo offered the opportunity to more definitively establish the molecular mechanisms involved. We cultured T cells in IL-2 for 2 wk in the presence of wasp venom-treated K562-CD1a cells to permit the outgrowth of responder cells. ELISpot studies clearly confirmed reactivity to CD1a alone, as well as to CD1a plus wasp venom, but no response to venom was seen in the absence of CD1a (Fig. 1 B, left, donor C1098). Further, anti-CD1a mAb blocked the response to background levels ($P < 0.05$), proving that CD1a is required. A similar pattern of response was seen to T cells treated with K562-CD1a and bee venom (Fig. 1 B, right).

CD1a-restricted venom responses in a healthy cohort

Next, we sought to test this pattern of wasp venom responses in a larger cohort of 21 healthy individuals. Measuring mean IFN- γ spots for all donors, we observed higher ($P < 0.0001$) response to CD1a transfectants, as compared with untransfected target cells (Fig. 1 C). Similar to the pilot studies, we detected higher spot numbers when wasp venom was added, but only when CD1a was present ($P < 0.001$). We observed a pattern of IFN- γ production to bee venom that was dependent on CD1a expression ($P < 0.001$) and similar in magnitude to that seen with wasp venom (Fig. 1 D).

K562 cells are transformed cells that represent useful tools which bypass MHC alloreactivity. The two types of primary human skin APCs that express CD1a proteins are myeloid DCs and LCs. To investigate whether these cell types mediate the venom response similarly to K562 cells, we differentiated monocytes with GM-CSF and IL-4 to produce monocyte-derived DCs (mDCs), which mimic key features of myeloid DCs. In addition we activated cells in vitro with cytokines to mimic LCs (in vitro LCs), including high CD1a expression (Porcelli et al., 1992; Sallusto and Lanzavecchia, 1994; Caux et al., 1997; Geissmann et al., 1998). Both types of human primary APCs were able to mediate wasp venom (Fig. 1 E) and bee venom (Fig. 1 F) responses, which were abrogated in the presence of anti-CD1a. Overall, this study showed that CD1a autoreactivity and CD1a-dependent venom reactivity are common in humans. This pattern of combined CD1a autoreactivity and antigen-dependent reactivity is reminiscent of invariant NKT cells, which are activated weakly by CD1d and strongly by CD1d with lipid antigen (Kawano et al., 1997).

Isolation of stimulatory factors from venom

To neutralize diverse predators or prey, natural venoms are usually high potency toxins that have multiple mechanisms of action (King et al., 2003). Repeated exposure of wasp and

bee venoms to lymphocytes resulted in apparent toxicity and cell death based on monitoring of T cells by Trypan blue exclusion. We could not generate long-term T cell lines by direct and repeated exposure to venom in vitro. Instead, we screened existing panels of CD1a-reactive T cell clones for responses to *Vespula* species (wasp) and *Apis mellifera* (bee) venoms. To minimize toxicity and interrogate the mechanisms of venom action, we switched from K562-CD1a activation assays (cellular assays) and took advantage of assays in which biotinylated CD1a proteins are coated onto streptavidin plates (plate assays). In the plate assay, CD1a proteins are pulsed with venom-derived products, washed and then exposed to T cells. This method diminishes venom exposure to APCs and responding cells, thereby minimizing toxicity. Also, the plate method might favor detection of stimulants that act by forming complexes with CD1 proteins in preference to indirect effects of venom on APCs or T cells that do not involve binding to CD1 proteins. The T cell clone BC2 showed a dose-dependent increase in IFN- γ secretion when cultured in the presence of CD1a proteins pretreated with wasp venom (Fig. 2 A). The response was likely to be specific antigen recognition rather than a general mitogenic activation, because no response was seen when plates were coated with CD1b, CD1c (Fig. 2 A), or CD1d (not depicted), or when venom-treated CD1 proteins were exposed to another T cell line, CD8-2, which normally recognizes dideoxymycobactin lipopeptides presented by CD1a (Moody et al., 2004; Fig. 2 B). Furthermore, we observed a similar dose-dependent recognition by T cell clone BC2 of bee venom in the presence of CD1a proteins (Fig. 2 C).

Using cellular K562 assays, we again observed a CD1-dependent and dose-dependent response to bee venom, but this time with significant CD1a autoreactivity. The higher responses seen in cellular assays might be explained if venom acts on the APC in some way or if lipid autoantigens are provided by K562 cells (Fig. 2 D). In addition to using monocyte-derived DCs, we also aimed to use an alternative system for the generation of LC-like cells and so sorted CD34⁺ progenitors and activated them with relevant cytokines, which are well established to serve as a model of LC function (Hunger et al., 2004). The cells express CD1a at moderate to high levels (unpublished data; Porcelli et al., 1992; Sallusto and Lanzavecchia, 1994; Yakimchuk et al., 2011) and, when added to cultures, mediated CD1a- and venom-dependent responses of BC2 at a ratio of 1 APC to 25 T cells (Fig. 2, E and F). Thus, both transformed and primary CD1a⁺ cells were used to formally show that T cells can be activated by bee and wasp venom, and the primary cells validated BC2 as a reagent that mimics the pattern of patient responses seen ex vivo (Fig. 1). Thus, this T cell clone could be used for the subsequent assays needed to isolate and identify stimulatory molecules in venom.

Fractionation of venom to discover antigens

To fractionate natural venoms and discover the stimulatory substance, we treated *Apis mellifera* (bee) and a mix of *Vespula*

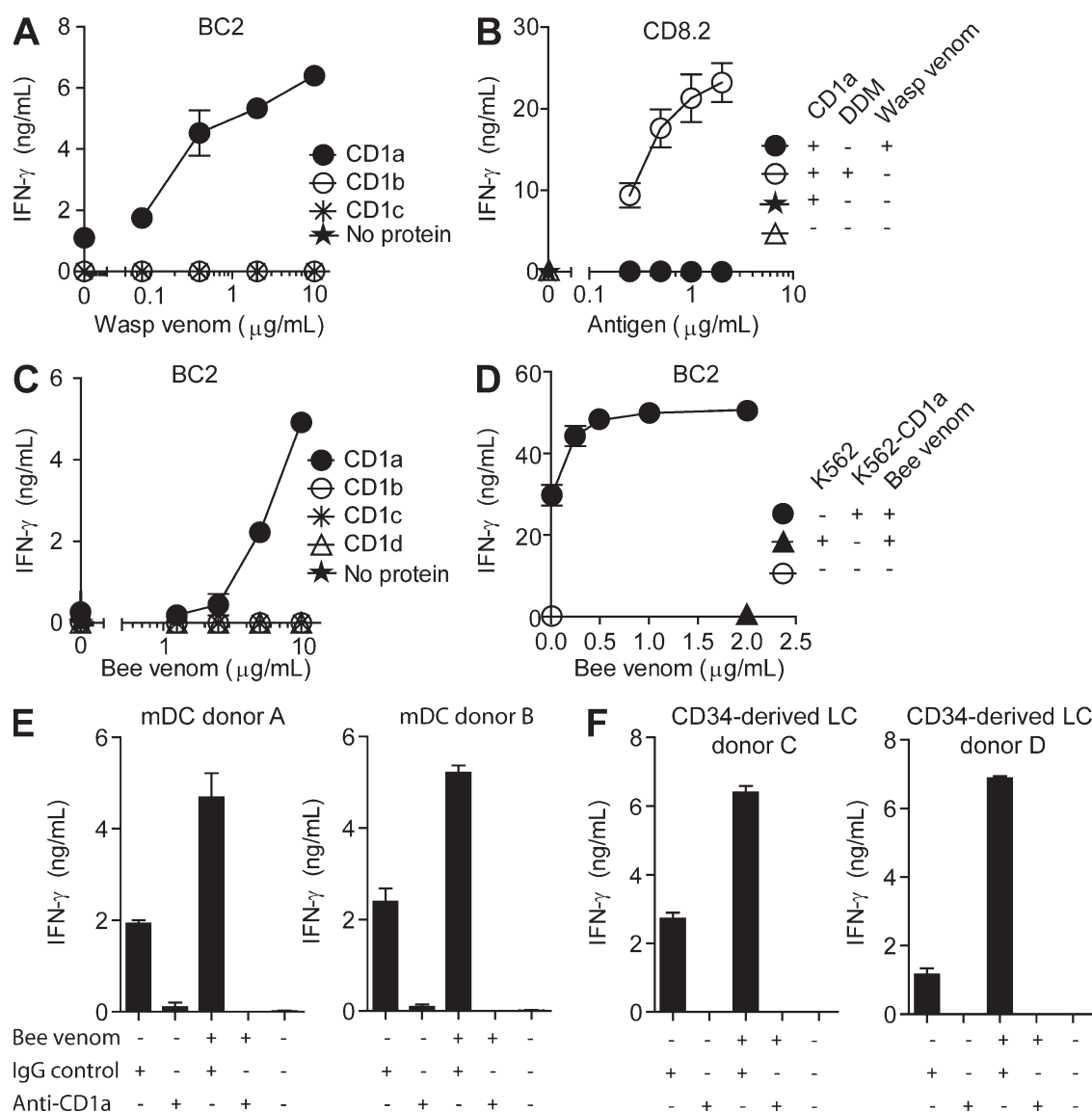


Figure 2. The autoreactive CD1a-restricted T cell clone BC2 is activated by wasp and bee venoms. Biotinylated CD1a, CD1b, and CD1c and CD1d proteins were coated on streptavidin plates. After washing, wasp (A and B) and bee (C) venoms and dideoxymycobactin (DDM; B) were added to the wells for 24–48 h. After washing, BC2 cells (A and C) or CD8.2 cells (B) were added, and the supernatants were collected 24 h later and IFN- γ quantified. (D) BC2 cells were co-cultured for 24 h with universal target cells (K562) as APCs, transfected with CD1a (K562-CD1a) or empty vector (K562) and bee venom. IFN- γ in supernatants was measured by ELISA. Data are representative of three separate experiments. (E) mDCs and (F) CD34-derived LC-like cells were incubated with IgG control or anti-CD1a antibodies for 1 h at 37°C. Bee venom was then added at 2 μ g/ml for 1 h. BC2 cells were then added at a 1:25 APC/T cell ratio. Two representative donors out of three are shown.

species (wasp) venoms with chloroform and methanol to create a two-phase mixture. Lipids partition into the organic solvents (extract), whereas proteins, nucleotides and other polar molecules are excluded (precipitate; Fig. 3 A). Nearly all CD1-presented antigens are lipids, so it was surprising that the lipidic extract failed to activate BC2 cells (Fig. 3 B). Likewise, the protein-enriched precipitate failed to activate T cells. However, T cell activation was detectable when the extract and precipitate were recombined. This result excluded the possibility that chloroform and methanol somehow destroyed the capacity of the whole venom to activate

BC2, and it suggested that multiple compounds within the venom acted in concert to generate T cell response. An analogous experiment performed using the K562-CD1a cellular assay showed different results, which provided possible explanations for the unexpected pattern. In contrast to the plate assay, venom precipitate applied to intact K562-CD1a APCs was sufficient to activate T cells. Again, extracted lipids alone failed to activate BC2 (Fig. 3 C). These seemingly contradictory results might be explained if venom does not provide an activated lipid antigen, but instead provides a protein that precipitates in organic solvents and then is capable

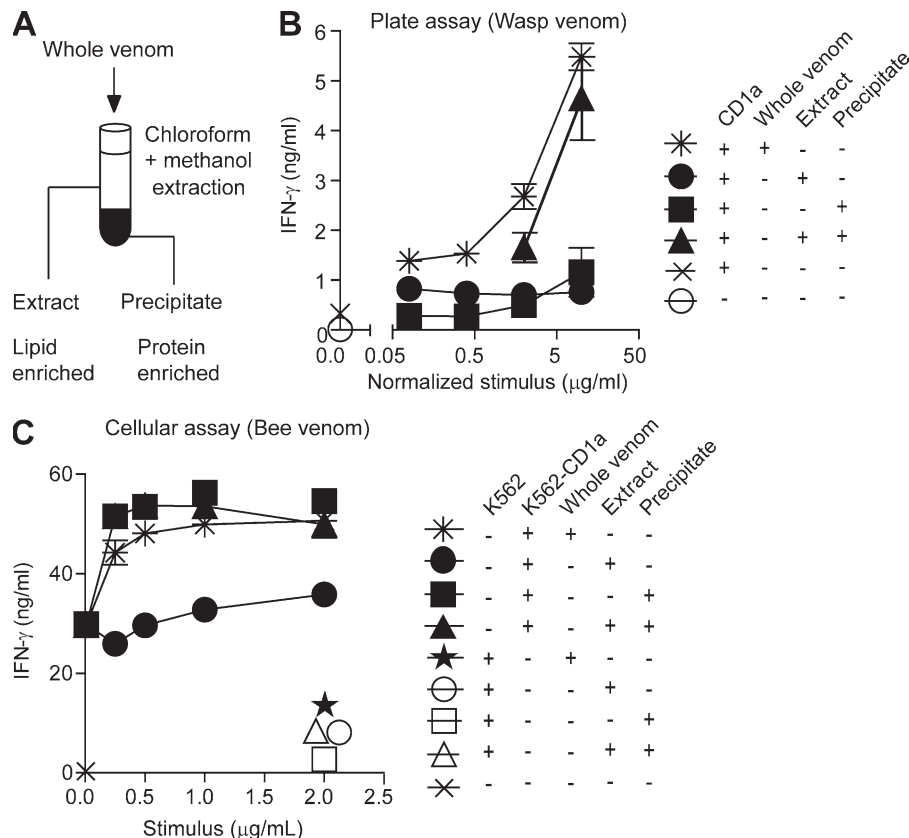


Figure 3. The co-incubation of both proteins and lipids from wasp and bee venoms triggers BC2 cells activation when presented by CD1a protein. (A) Protein and lipid fractions of bee and wasp venoms were separated by organic solvent extraction using chloroform and methanol. (B) CD1a proteins were coated on streptavidin plates. After washing, wasp or bee venoms and their extracts were added to the wells for 24 to 48 h. After washing, BC2 cells were added, supernatants were collected after 24 h and IFN- γ quantified. (C) BC2 cells were co-cultured with universal target cells transfected with CD1a (K562-CD1a) or empty vector (K562) and bee venom or their extracts. Supernatants were collected after 24 h. IFN- γ in supernatants was measured by ELISA. Data are representative of three separate experiments.

of producing antigenic substances when combined with venom-derived or K562-derived lipids.

Phospholipase A2 activates T cells

In considering protein factors that might generate antigens for CD1a proteins, it is well established that wasp and bee venoms contain phospholipase A1 (PLA1) and A2 (PLA2). Phospholipases are major components of venom that have been extensively studied in regard to their antigenicity toward MHC-restricted T cells and B cells (Aslam et al., 2006; Sin et al., 2011). Viral phospholipases can promote activation of NKT cells by CD1d (Zeissig et al., 2012). Based on our preliminary experiments with recombinant protein and lipid fractions, we hypothesized that phospholipases act on venom-derived lipids (Fig. 2) or the K562 membrane phosphodiacylglycerols (Fig. 3) cleaving ester bonds and generating free fatty acids, and lysophospholipids. Purified venom PLA2 from *Apis mellifera* strongly activated BC2 T cells (Fig. 4A). No T cell activation was seen when PLA2 was exposed to T cells with K562 cells lacking CD1a (Fig. 4A). Therefore, the mechanism of PLA2 on T cells is indirect and requires CD1a proteins.

Mechanism of PLA2 action

Another well-known polypeptide present in bee venom that binds lipids and enhances PLA2 activity is mastoparan (Argiolas and Pisano, 1983; Cabrera et al., 2011). However, mastoparan acting alone (Fig. 4B) did not activate BC2.

PLA2 releases large amounts of lipid mediators of inflammation through downstream action of cyclooxygenases, which create arachidonic acid metabolites. Therefore, testing another candidate mechanism of action relating to PLA2, we hypothesized that free arachidonic acid might bind to CD1a proteins as an antigen or act indirectly as cyclooxygenase substrate to stimulate BC2 via receptors other than the TCR. However, we observed no arachidonic acid-mediated stimulation and saw dose-dependent inhibition of BC2 activation by arachidonic acid in K562 cellular and plate assays when added at high absolute dose (Fig. 4C), ruling out both hypotheses. Although arachidonic acid could inhibit responses at pharmacological doses, PLA2 had a reproducible and strong stimulatory effect, suggesting that the smaller amounts of arachidonic acid liberated by this enzyme do not have a dominant negative effect. Finally, mDC (Fig. 4D) and cord blood CD34-derived LC-like cells (Fig. 4E) act as APCs and substrates of the PLA2 to activate BC2 cells in a CD1a-dependent manner, suggesting that neo-antigens can be generated in primary APCs and that neither mDCs nor LCs express inhibitory factors.

To directly test the hypothesis that bee venom PLA2 acts by cleaving intact cellular phospholipids to create neoantigens, we preincubated the enzyme with two synthetic substrates for bee venom PLA2, phosphatidylcholine comprised of singly unsaturated C18 fatty acyl chains (PC 18:1/18:1) and phosphatidic acid with C16 fatty acyl chain in *sn*-1 and

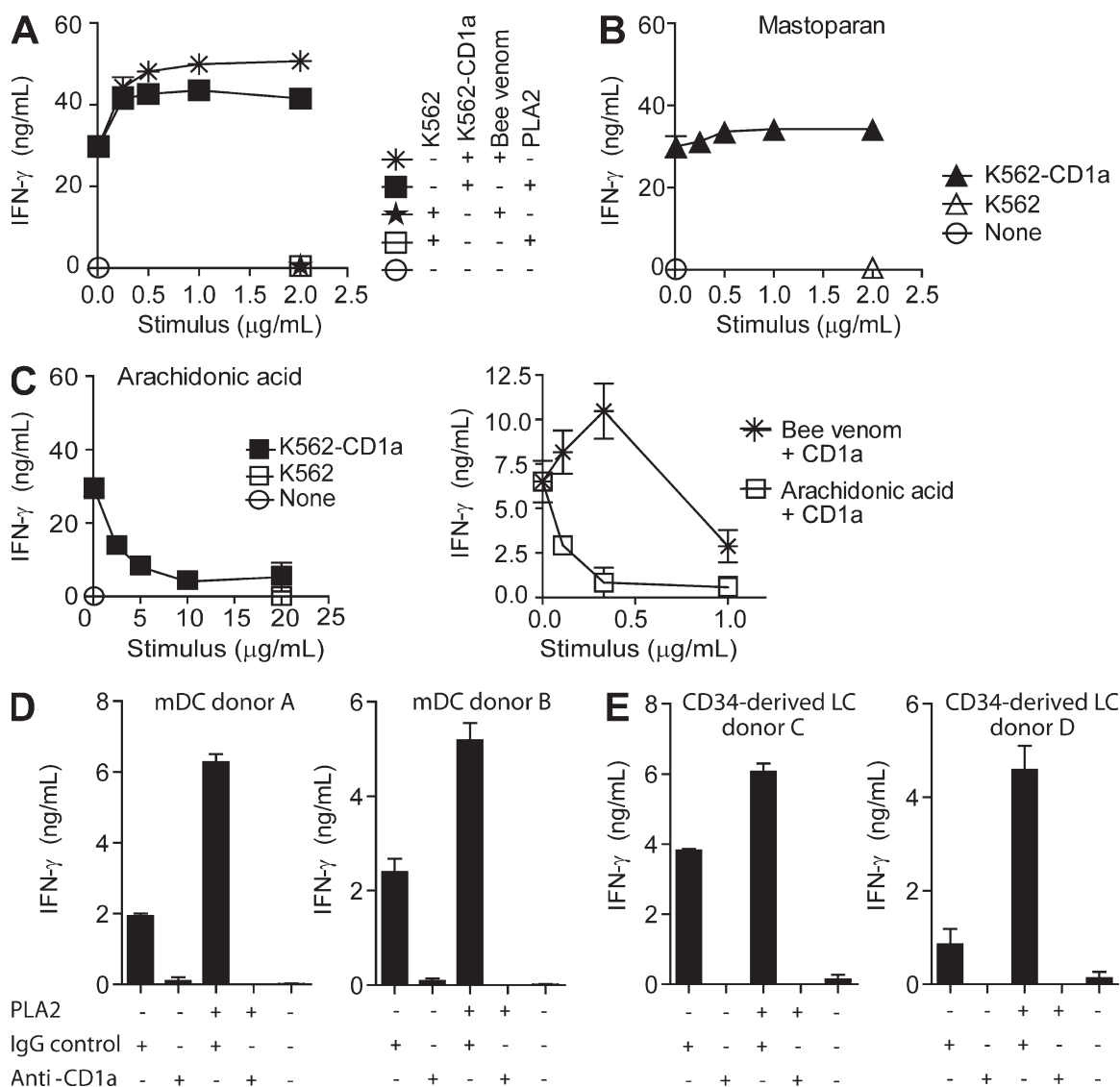


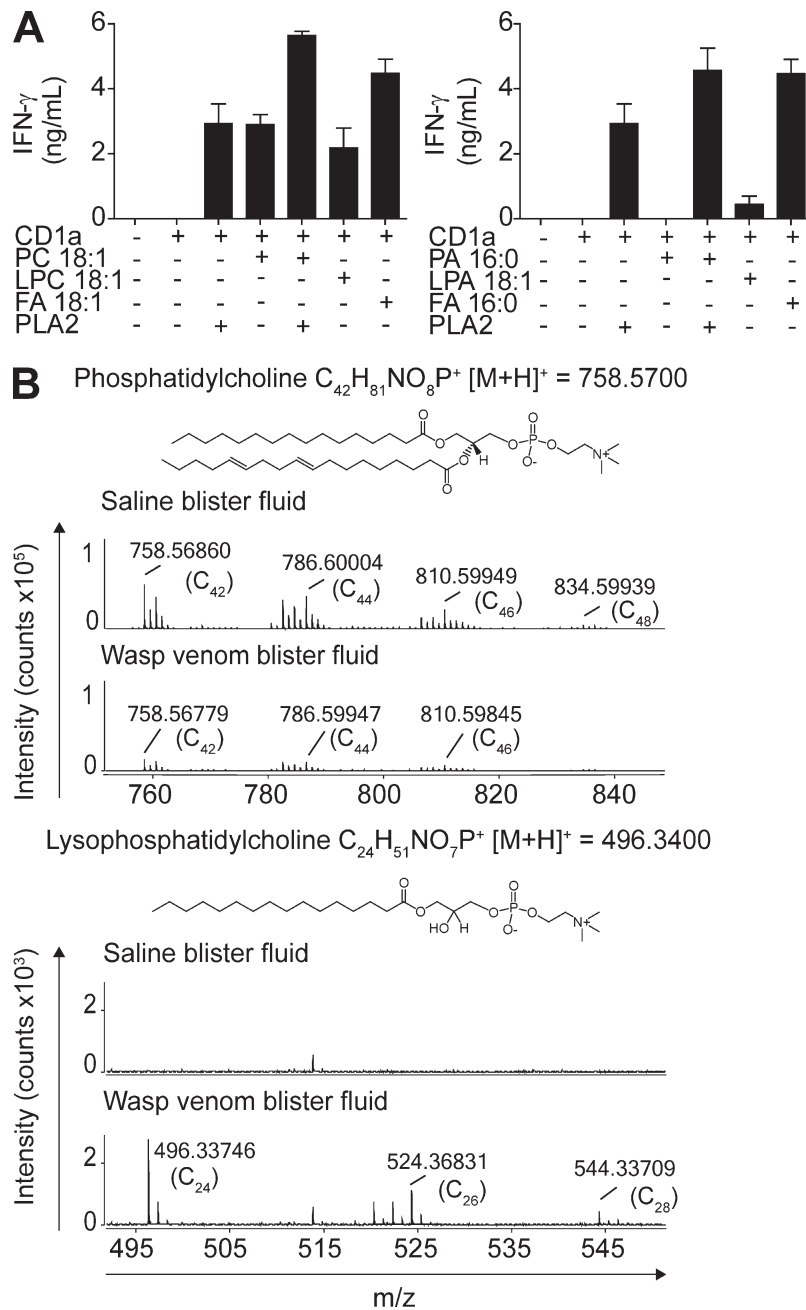
Figure 4. Bee venom PLA2 alone induces a CD1a-restricted T cell response by releasing free fatty acids. BC2 cells were co-cultured for 24 h with universal target cells (K562) transfected with CD1a (K562 CD1a) or empty vector (K562) and bee venom or bee venom PLA2 (A), mastoparan (B), or arachidonic acid (C). IFN- γ in supernatants was measured by ELISA. In (C) right panel, biotinylated CD1a proteins were coated on streptavidin plates. After washing, bee venom or arachidonic acid were added to the wells for 24 to 48 h. After washing, BC2 cells were added for 24 h, and supernatant was sampled for IFN- γ quantification by ELISA. Data are representative of three separate experiments. (D) mDC and (E) CD34-derived LC-like cells were incubated with IgG control or anti-CD1a antibodies for 1 h at 37°C. PLA2 was then added at 2 μ g/ml for 1 h. BC2 cells were then added at a 1:25 APC:T cell ratio. Two representative donors out of three are shown.

unsaturated C18 fatty acyl chain in *sn*-2 (PA 16:0/18:1). We also tested for a T cell response to products of cleavage by PLA2 and control lipids (Fig. 5 A), including purified free fatty acids and lysophospholipids: lysophosphatidylcholine (LPC 18:1), oleic acid (FA 18:1), lysophosphatidic acid (LPA 18:1), and palmitic acid (FA 16:0). Higher production of IFN- γ was obtained in response to phospholipids when preincubated with PLA2. BC2 T cells responded to fatty acids to a greater extent than intact phospholipids or lysophospholipids. This result suggests that PLA2 activates CD1a-restricted T cells by cleaving nonantigenic phospholipids into lysophospholipids

and antigenic fatty acid, in agreement with a recent study identifying free fatty acids as CD1-presented antigens (de Jong et al., 2014).

Venom generates lysophospholipids in vivo

Membrane phospholipids and free fatty acids are both abundant in blood and tissue. However phospholipids largely self-assemble into bilayer membrane structures, and free fatty acids exist mainly as cytosolic metabolites or lipoprotein structures generated during gastrointestinal absorption. Thus, lysophospholipids and free fatty acids are normally



stored in macromolecular clusters and do not reach high concentration as free molecules in the extracellular space, where CD1a lipid loading is thought to occur (Manolova et al., 2003). Furthermore, although extracellular lipids, including fatty acids, accumulate in the stratum corneum and sebaceous glands, they are not detectable in normal dermis. Accordingly, most models of self- or altered self-lipid display emphasize release of free fatty acids and other antigens into the extracellular space in the proximity of CD1⁺ epidermal LC (Colonna, 2010; de Jong et al., 2010, 2014). Injection of bee and wasp venoms into skin might locally generate such cleavage of intact phosphodiacylglycerides to lysolipids and free fatty acids, so we tested this in a human model for skin immune responses

Figure 5. Wasp venom phospholipase is active in vivo in the skin. (A) Biotinylated CD1a proteins were coated on streptavidin plates. After washing, lipids were added at 10 μ g/ml for 24–48 h. Lipids tested were as follows: phosphatidylcholine 18:1 (PC18:1), lysophosphatidylcholine 18:1 (LPC18:1), and oleic acid 18:1 (FA 18:1; left) or phosphatidic acid 16:0 (PA16:0), lysophosphatidic acid 18:1 (LPA18:1), and palmitic acid 16:0 (FA16:0; right). Phosphatidylcholine and lysophosphatidic acid were preincubated or not with PLA2 at 50 μ g/ml for 1 h at 37°C. 24 h later, wells were washed and BC2 cells were added. Supernatants were collected after 24 h. IFN- γ in supernatants was measured by ELISA. Data are representative of three separate experiments. (B) Healthy donor epidermis was injected with 10 μ g of wasp venom or saline. Skin blisters were raised and, 24 h later, the blister fluid was sampled. Lipids were extracted with chloroform, methanol, and water using the Bligh-Dyer method and analyzed on a quadrupole time of flight mass spectrometer. Positive mode EIC-MS detected ions at m/z 758.5686, 786.6000, 810.5994, and 834.5993, corresponding to the homologous series of phosphatidylcholine $C_{42}H_{81}NO_8P^+$ (16:0/18:2), $C_{44}H_{85}NO_8P^+$ (18:0/18:2), $C_{46}H_{89}NO_8P^+$ (18:2/20:2), and $C_{48}H_{93}NO_8P^+$ (20:4/20:2). Positive mode EIC-MS detected ions at m/z 496.3374, 524.3683, and 544.3370, corresponding to the homologous series of lysophosphatidylcholine $C_{24}H_{51}NO_7P^+$ (16:0), $C_{26}H_{55}NO_7P^+$ (18:0), and $C_{28}H_{59}NO_7P^+$ (20:4). Data from one representative donor out of two are shown.

using suction cup blisters (Salimi et al., 2013). This method uses low pressure, sustained (\sim 60 min) suction to produce extracellular blister fluids that are captured for immunological and biochemical analysis before or after antigen challenge (Salimi et al., 2013).

After injection of 10 μ g of venom, which mimics the approximate volume and dose of a wasp sting, or saline vehicle, 2 mm into the skin at two sites in the arm of one patient, we obtained two blister fluid samples. After extracting the lipids from blister fluids using a mixture of chloroform, methanol and water, we assessed for the presence of substrates and products of the wasp venom PLA by mass spectrometry using sensitive quadrupole time of flight (QToF)

methods (Fig. 5 B). Focusing on the main natural substrate for PLA2, we monitored the phosphatidylcholine series for key ions corresponding to lipids with differing fatty acid content: $C_{42}H_{81}NO_8P^+$ (16:0/18:2), $C_{44}H_{85}NO_8P^+$ (18:0/18:2), $C_{46}H_{85}NO_8P^+$ (18:2/20:2), and $C_{48}H_{85}NO_8P^+$ (20:4/20:2; Fig. 5 B). The masses of detected ions (m/z 758.5686, 786.6000, 810.5994, and 834.5993) matched these PC variants within the mass accuracy of the detector. Comparing saline- and venom-injected sites, we found a twofold lower intensity for signals corresponding with all molecular variants of PC in the PLA2-injected skin samples, indicating that the enzyme substantially consumed local PC (Fig. 5 B). In a second patient, signals were reduced after venom injection for all PC species except PC18:1 18:1 (unpublished data). Conversely, ions matching the lysophosphatidylcholine series, $C_{24}H_{51}NO_7P^+$ (16:0), $C_{26}H_{55}NO_7P^+$ (18:0), and $C_{28}H_{51}NO_7P^+$ (20:4), were readily detected in wasp venom blister fluid with high signal but were not detected in the blister fluid from saline injection sites. Thus, lysolipids derive from PLA itself and not some other aspect of the suction blister model. We measured signals corresponding to the expected masses of free fatty acids in the positive and negative mode in these samples, but could not detect them. Mass spectrometry detection of fatty acids is less sensitive than detection of anionic phospholipids, so we cannot derive any direct conclusions about fatty acid concentrations in these in vivo experiments. Nevertheless, these results demonstrate that the injection of venom alters the local lipid content in ways that are predicted from the specificity of PLA2 and that the cleavage reactions needed to generate fatty acid and lysophospholipid antigens do occur.

Direct measure of wasp and bee venom PLA bioactivity

In individual donors (Fig. 1, A and B) and cohorts (Fig. 1, C and D) and in vitro studies with clones (Figs. 2–4), we noted strikingly similar responses to bee and wasp venom, which are currently thought to differ in their PLA subtypes, as described above. However if fatty acids are the CD1a-lipid ligand, similar immunological responses are expected, as fatty acids will be generated whether PLA1 or PLA2 cleave acyl chains from phospholipids at *sn*-1 or *sn*-2, respectively. Interestingly, PLA2 has been found in venom of the neotropical social wasps *Polybia paulista* (de Oliveira and Palma, 1998; dos Santos et al., 2011) and *Agelaia pallipes pallipes* (Costa and Palma, 2000). This led us to investigate whether PLA2 activity is also exerted by *Vespula* species of wasp venom, and furthermore whether there were shared specificities between bee and wasp venom phospholipase. Using *sn*-2 thiol-labeled PLA2 substrates (arachidonyl thio-PC, heptanoyl thio-PC, diheptanoyl thio-PC, and palmitoyl thio-PC), we observed PLA2 activity with both bee and wasp venom indicating that wasp venom contains PLA2 activity in addition to its known PLA1 activity, and that specificities are partially shared with bee venom PLA2 (Fig. 6 A). We confirmed the PLA2 activity of wasp venom could be inhibited by manolide, a previously known PLA2 inhibitor (Fig. 6 B). Collectively, these

data suggest that both wasp and bee venom phospholipases can generate common CD1a fatty acid ligands.

PLA2 induced polyclonal responses ex vivo

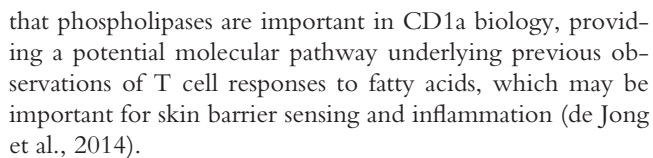
Having identified PLA2 as sufficient to generate CD1a-presented antigens in vitro using T cell clones, we next sought to determine if bee venom PLA2 is sufficient to activate polyclonal T cells ex vivo in cellular assays (Fig. 7). As with whole venoms (Fig. 1), we detected higher IFN- γ -producing cells with the addition of 100 ng/ml bee venom PLA2 but only in the presence of CD1a and not other CD1 isoforms (Fig. 7 A, left). We observed significantly ($P < 0.001$) higher CD1a-restricted responses in the presence of PLA2 in a cohort of 18 donors (Fig. 7 A, right). More detailed testing of an individual responder showed that anti-CD1a antibodies prevented the response to CD1a and PLA2 ($P < 0.05$), confirming the essential role of CD1a (Fig. 7 B, left).

PLA2 is the essential component of venom for CD1a ligand generation

Collectively, these studies showed that PLA2 was sufficient to recapitulate the response to venom, so we next sought to determine if PLA2 was necessary, or instead whether the many known active compounds in venom could generate a response. We observed an inhibition of IFN- γ production in response to the venoms treated with neutralizing antibodies that bind PLA2 ($P < 0.05$; Fig. 7 B, right). Further, using manolide we noted a blockade of response to wasp venom and bee venom PLA2 to background levels seen with CD1a alone ($P < 0.05$; Fig. 7 C) and not to lower levels, which might be expected in the event of nonspecific toxicity to cells. Lastly, we showed that monocyte-derived DC and LC-like cells derived in vitro, as well as CD1a⁺ cells isolated directly ex vivo from skin could induce a response by PLA2-specific polyclonal T cells in a CD1a- and PLA-dependent manner (Fig. 7 D). We conclude that PLA2 is a necessary factor involved in venom-dependent activation of CD1a-reactive T cells.

DISCUSSION

Using venoms as a model system, our data identify a new mechanism of in vivo antigen processing by which phospholipases cause local alterations in lipid content and conversion of nonantigenic substances to smaller lipids, which have CD1-mediated T cell antigenicity. Specifically, we identify wasp and bee venom phospholipases, which act as the key proteins that are necessary and sufficient to cleave common cell membrane phosphodiacylglycerides, to release free fatty acid and lysophospholipids (de Jong et al., 2014). Using the particular clone BC2, we ruled in free fatty acids as neoantigens. However, polyclonal T cells may be responding to other lipids as well, and it is notable that CD1d presents lysophospholipids to NKT cells (Gumperz et al., 2000; Fox et al., 2009; Zeissig et al., 2012). Because the CD1a dependence of lipid-specific T cell responses is observed in polyclonal T cells and among many unrelated human donors, these data suggest



the skin in a process that can be meaningfully mimicked by needle injection (Cortellini et al., 2012). Several aspects of our data fulfill predictions that phospholipase injection might lead to antigen generation beneath cornified epithelia in proximity to CD1a proteins. Responses to venom and phospholipase are detected most strongly in response to the particular CD1 isoform (CD1a) that is most extensively expressed in the skin. PLA substrates can derive from both venom and cellular sources, and PLA2 would presumably have access to venom or cell derived phospholipids during a sting. Also, although free fatty acids could not be detected *ex vivo* by mass spectrometry, phospholipid and lysophospholipid concentrations are demonstrated to change in suction blisters in a process that is dependent on PLA itself. Thus, bee or wasp stings might represent a means to locally remodel lipids and generate neo-antigens in proximity to CD1a proteins, mimicking a natural system of barrier sensing.

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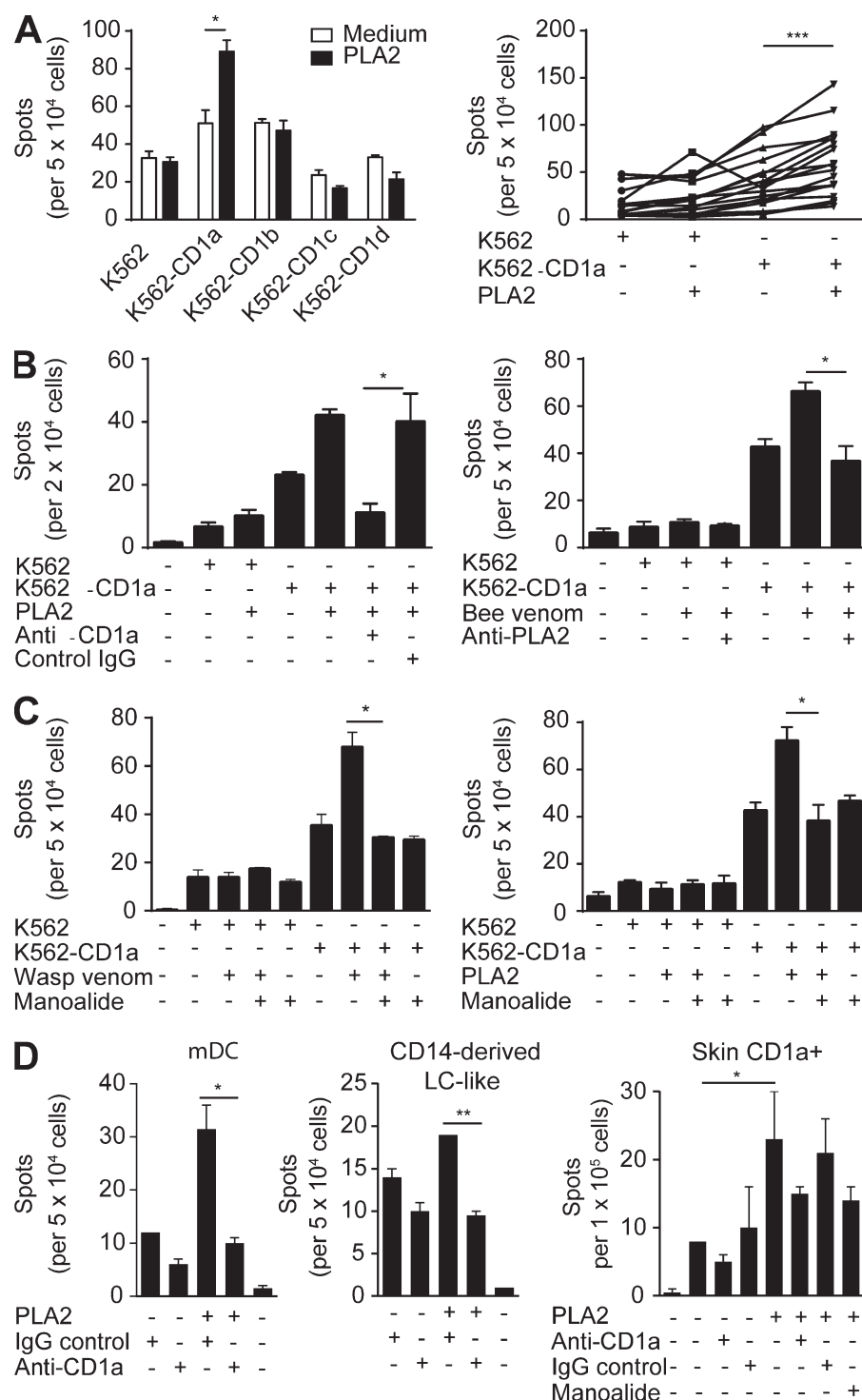


Figure 7. CD1a-restricted reactivity to *Apis mellifera* venom PLA2 is found among polyclonal T cells from blood of healthy donors. CD3⁺ cells were isolated by magnetic beads from healthy donor PBMC and cultured for 12–14 d with IL-2 and irradiated K562 cells transfected with CD1a (K562 CD1a) or CD1b (K562 CD1b) or CD1c (K562 CD1c) or CD1d (K562 CD1d) or an empty vector (K562) in the presence of *Apis mellifera* venom PLA2. CD1 reactivity was then examined by IFN- γ ELISpot with transfected or untransfected K562 cells either in the presence or absence of *Apis mellifera* venom 100 ng/ml PLA2 (A, left). Data from one representative donor of three are shown. Venom PLA2 specific CD1a-restricted T cell responses were measured in 18 donors (A, right). CD1a-restricted responses were examined in the presence or absence of anti-CD1a (B, left) for donor C1098, and PLA2-neutralizing antibodies (7B, right), and manoalide (specific PLA2 inhibitor) for wasp venom in donor C559 (C, left) and *Apis mellifera* venom PLA2 in donor C334 (C, right). Representative results are shown from three independent experiments. (D) IFN- γ production was measured by co-incubations of PLA2-specific T cells with mDCs (left) or CD14-derived LC-like DCs (middle) in the presence or absence of anti-CD1a for donor C558. IFN- γ production was measured from co-incubations of PLA2-specific T cells and freshly isolated skin CD1a⁺ cells in the presence or absence of anti-CD1a or manoalide (right). Representative data from 3 (left and middle) or 2 (right) separate donors are shown. Data are mean of triplicate measurements \pm SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

with parallel actions, and act by coopting conserved neurological or immunological mechanisms in the stung animal. We speculate that injection of venom PLA represents a means to coopt, through overstimulation, the natural effects of endogenous mammalian PLAs in barrier sensing and immune response. Mammalian PLA2 enzymes exist as lysosomal, cytosolic, and secreted (sPLA2) forms. The sPLA2 superfamily is

divided into 6 groups (I, II, III, V, X, and XII) based on disulfide bridge structure (Starkl et al., 2013), with bee venom PLA having the closest homology to type III human sPLA (Scott et al., 1990; Valentin et al., 2000). sPLA2 enzymes act in lipid digestion, host defense, and inflammation (Murakami and Lambeau, 2013). Other evidence to suggest that mammalian PLAs might have CD1 or lipid-mediated effects on

T cells includes a recent study of mammalian PLAs from hepatitis virus that activate NKT cells (Fox et al., 2009; Zeissig et al., 2012) and the role of thymic PLA in controlling NKT cell selection (Paduraru et al., 2013). In these studies of blood-derived T cells, CD1a autoreactivity predominates, but in the gastrointestinal tract CD1d is highly expressed, and NKT cells home to the intestine and liver. Thus, both studies emphasize a role of phospholipases, but the differing effects of CD1a and CD1d isoforms may relate to the organ-specific patterns of expression and T cell response. Free fatty acids were identified as natural skin antigens for CD1a earlier this year (de Jong et al., 2014). Collectively these studies provide multiple lines of evidence that PLAs control various aspects of T cell response in different organs. The clinical study of bee and wasp sting responses represents a model system to study the basic role of PLAs because it is characterized by single, discreet exposures and there exist practical means to mimic a stinging exposure and study the response in vivo and ex vivo. Having established a role for CD1a and PLA in the normal response to venom, future studies will likely examine their roles in hypersensitivity and immunotherapeutic systems and take advantage of information about lysophospholipids and free fatty acids as natural pathways that could be considered for diagnosis through skin testing and antigen-based immunotherapy.

MATERIALS AND METHODS

Isolation of human PBMC, lines, and clones. PBMCs were isolated from healthy adult donors with a previous history of bee or wasp sting under local ethics approval (NRES Committee South Central, Oxford C, 09/H0606/71). T cells were purified from ficollized PBMCs using CD3 MACs beads (Miltenyi Biotec) and cultured for 10–14 d with pulsed irradiated K562-CD1a cells in RPMI supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 5% human serum (Sigma-Aldrich), nonessential amino acids, Hepes, sodium pyruvate, and 2-mercaptoethanol (Life Technologies) in the presence of 1 nM IL-2 (PeproTech; 5,000,000 T cells and 200,000 K562-CD1a per well of 24-well plate). CD1a reactivity was then assessed by IFN- γ ELISPOT (Mabtech). ELISPOT plates (Millipore) were coated with anti-IFN- γ antibody overnight (Mabtech). K562 cells were pulsed with 1 µg/ml wasp/bee venom (Sigma-Aldrich) or 100 ng/ml bee venom PLA2 (Sigma-Aldrich) overnight, and were then washed and resuspended in R5* (RPMI supplemented with 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin plus 5% human serum). The plates were washed six times with RPMI and blocked for 1 h with RPMI supplemented with 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin plus 10% human serum (R10*). 20,000–50,000 T cells were added per well to which 10,000–25,000 K562 or primary cells were added. Wells were set up in duplicate or triplicate. 10 ng/ml phorbol myristate acetate and 500 ng/ml Ionomycin was included as a positive control, and T cells alone in the absence of K562 was included as a negative control. After overnight incubation at 37°C and 5% CO₂, plates were washed 6 times in PBS-Tween 0.05% and incubated with 1 µg/ml of biotin-linked anti-IFN- γ monoclonal antibody (Mabtech) for 2 h. After washing 6 times in PBS-Tween 0.05%, the plates were incubated for an additional 1 h with streptavidin-alkaline phosphatase (Mabtech). Spots were visualized using an alkaline phosphatase conjugate substrate kit (Bio-Rad Laboratories) and enumerated using an automated ELISPOT reader (Autimmun Diagnostika GmbH). The T cell lines, BC2 and CD8-2, as well as K562-CD1 cells have been described previously (Partners Health Care Institutional Review Board; Rosat et al., 1999; de Jong et al., 2010, 2014).

Isolation and generation of human APCs. PBMCs were incubated with GM-CSF (300 U/ml) for 30 min. After removing the nonadherent cells, medium with GM-CSF (300 U/ml) and IL-4 was added (200 U/ml) for 3–4 d. Adherent mDCs were collected by addition of PBS/0.5 mM of EDTA for 20 min, and then irradiated by γ -irradiation (5,000 rad). High CD1a expression was verified by flow cytometry. We generated LC-like cells in vitro using two published protocols. Starting with CD14⁺ monocytes from human buffy coats from the NHS Blood Bank (John Radcliffe Hospital, Oxford, UK), CD14⁺ human cells were isolated using MACS cell separation (Miltenyi Biotec) according to the supplier's instructions. In brief, such in vitro LC-like cells were prepared as previously described (Caux et al., 1997; Geissmann et al., 1998) from CD14⁺ cells, which were cultured in 6-well plates in complete medium in the presence of IL-4 (250 ng/ml; PeproTech), GM-CSF (100 ng/ml; PeproTech), and TGF- β ₁ (10 ng/ml; PeproTech). At days 2 and 4, cultures were replated in the presence of the aforementioned cytokines to generate cells which were 10–15% CD1a⁺ CD207⁺. The in vitro LC-like cells were incubated with 10 µg/ml anti-HLA-ABC and anti-HLA-DR blocking antibodies (W6/32 and L243, respectively) for one hour before co-culture with T cells, to minimize HLA-restricted responses. Alternatively, samples of human cord blood were obtained from CFAR Virology Core Laboratory at UCLA (Los Angeles, CA). CD34⁺ human progenitor cells were isolated using MACS cell separation (Miltenyi Biotec) according to the suppliers' instructions. LCs were then prepared as previously described (Hunger et al., 2004) but with some modifications. Specifically, cultures of CD34⁺ cells were established in the presence of stem cell factor (25 ng/ml; R&D Systems), GM-CSF (200 U/ml; Sanofi US Services, Inc.), and TNF (2.5 ng/ml; Thermo Fisher Scientific). At day 5, cultures were replated in the presence of GM-CSF (200 U/ml; Sanofi US Services, Inc.) and TGF- β ₁ (1 ng/ml; R&D Systems) to increase CD1a expression. LC were harvested at day 12. The LC quality of the cells was checked by flow cytometry, and found to consist of 30–40% CD1a⁺CD207⁺ cells.

Isolation of CD1a⁺ cells from skin. After removing subcutaneous fat, skin sections were cut into ~5-mm-wide pieces and cultured in 2 mg/ml dispase solution at 4°C overnight. The epidermis was then separated from the dermis and cultured in complete media for 5 d. Migratory adherent cells were harvested and enriched by density gradient centrifugation, and CD1a⁺ cells were isolated using MACs cell separation (Miltenyi Biotec). The CD1a⁺ cells were incubated with 10 µg/ml anti-HLA-ABC and anti-HLA-DR blocking antibodies (W6/32 and L243, respectively) for 1 h before co-culture with T cells, to minimize HLA-restricted responses.

CD1-plate assays. Biotinylated CD1a, CD1b, CD1c, and CD1d proteins (National Institutes of Health Tetramer Core Facility, Emory University, Atlanta, GA) and anti-CD11a antibodies were added on streptavidin-coated plates (Thermo Fisher Scientific) at 10 µg/ml and 2.5 µg/ml, respectively, for 24 h at room temperature as detailed previously (de Jong et al., 2014). Wasp and bee venoms, their lipidic extracts, dideoxymycobactin (DDM), arachidonic acid, phosphatidylcholine 18:1, phosphatic acid 16:0, lysophosphatidylcholine 18:1, lysophosphatidic acid 16:0 or fatty acids 18:1, and 16:0 (Avanti) were loaded at 10 µg/ml in PBS for 24–48 h at room temperature. BC2 cells and CD8-2 cells were then added to the well at 100,000 cells/well.

Cellular assays. 150,000 BC2 cells were co-cultured with 20,000 K562-CD1 cells for 24 h with wasp and bee venoms, chloroform and methanol extracts, chloroform and methanol precipitates, PLA2 (Sigma-Aldrich), mastoparan (Sigma-Aldrich) or arachidonic acid (Sigma-Aldrich). In some experiments 10 µg/ml anti-CD1a blocking antibody (OKT-6), or 10 µg/ml IgG1 isotype control (P3), or 100 µM manoalide, an inhibitor of PLA2 that acts by covalently modifying lysine residues (Enzo Life Sciences), were added to K562 or primary cells before addition of T cells.

Venom extraction. 120 µg of lyophilized wasp (*Vespa species*; ALK) and bee venom were resuspended in a solution of (1:1) methanol/chloroform.

After 40 min of rotation and a centrifugation at 300 g for 5 min, supernatant was collected. Lipid enriched-supernatant and protein-enriched precipitates were collected and resuspended in 1.2-ml of sterile PBS and normalized to the input mass, or when possible, weighed to determine the exact mass.

Blister fluids. Healthy donors were injected with 10 µg of wasp venom or saline intraepidermally, a dose which is at the low end of venom normally administered in a sting (ALK). Suction was applied to the skin at 200 mmHg for 1 h, which induced a split between the epidermis and dermis (Salimi et al., 2013). The blister fluids were collected by aspiration at 24 h and immediately stored at −20°C. Lipids were extracted with chloroform, methanol, and water using the Bligh-Dyer method (Bligh and Dyer, 1959).

HPLC-(electrospray ionization)-quadrupole time of flight mass spectrometry. The analyses were performed using an Agilent Technologies 6520 ESI-QToF coupled with HPLC (Agilent Technologies 1200). Lipids were separated using a 3 µm × 150 mm × 2 mm diol column (Varian) as previously described (Layre et al., 2011). Spectra were collected in positive mode from 100 to 3,000 *m/z* at 1 spectrum/s. Internal calibrations were performed using calibrants at 121.050873 *m/z* and 922.009798 *m/z* that were coinfused into the spray chamber and used to monitor the stability of ion signal. Data were analyzed using MassHunter Workstation Qualitative Analysis Software (Agilent Technologies).

ELISA. Culture supernatants were harvested and tested for IFN-γ production by ELISA using the clone 2G1 (Thermo Fisher Scientific) for coating at 2 µg/ml and the biotin-labeled clone B133.5 (Thermo Fisher Scientific) at 0.1 µg/ml. Streptavidin-HRP (BD) was incubated for 30 min and the substrate was revealed by 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS).

PLA2 biochemical activity experiments. PLA2 activity in wasp and bee venom were detected using site-specific substrate kit (Cayman Chemicals), according to manufacturer's instructions. In a flat-bottom 96-well plate, 50 ng wasp or bee venom or 10 ng PLA2 (Sigma-Aldrich), plus 5 µl assay buffer, were incubated for 1 h at RT with a substrate solution. Substrates used have a thiol group attached at the *sn*-2 position of the phospholipid, and included arachidonoyl thio-PC, heptanoyl thio-PC, diheptanoyl thio-PC, and palmitoyl thio-PC. In the presence of PLA2, cleavage of the substrate at the *sn*-2 position resulted in release of the thiol group. After 1 h incubation, 10 µl DNTB/EGTA was added, which reacts with free thiol groups producing a colored precipitate, which was measured at 415 nm wavelength (iMark Microplate Reader; Bio-Rad Laboratories).

Statistics. Cohort of healthy donors investigated for CD1a-restricted wasp venom, bee venom, and PLA2-specific responses were analyzed using one-tailed Wilcoxon matched-pairs signed rank test. All other polyclonal T cells responses were analyzed using one-tailed Mann-Whitney tests.

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