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 (Dougan et al., 2007). In cells, CD1 proteins bind and display hundreds of molecular species of self-sphingolipids, phospholipids, and

© 2015 Bourgeois et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike license for the first six months after the publication date (see http://www.rupress.org/terms). After six months it is available under a Creative Commons License Attribution–Noncommercial–Share Alike 3.0 Unported license, as described at http://creativecommons.org/licenses/by-nc-sa/3.0/.

The Journal of Experimental Medicine
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, CD1d 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 αβ 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 during tuberculosis (Moody et al., 2000) and seasonal allergy (Agea et al., 2005), and as in other pathogens that infect humans

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 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), 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.

Aphis 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 (Égner 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. *Vespula* 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 *Vespula* 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 *Vespula* spp and *Apis mellifera* venoms. CD1a-restricted, venom-specific responses were measured in 21 donors for *Vespula* spp venom (C) and *Apis mellifera* venom (D). mDC or in vitro LC-like cells derived from CD14+ cells were pulsed with 1 µg/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 ± 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 act on the APC in some way or if lipid autoantigens are presented 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; 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 dideoxymycobacterin 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; Caux et al., 1997; Geissmann et al., 1998) 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* species with crude venom and then fractionated the venom into subunits by ion exchange and gel chromatography. As a result, we identified a tripeptide fragment of protein A (from *Vespula* species) that mimics the pattern of patient responses seen ex vivo (Fig. 1). This tripeptide fragment is composed of cysteine and histidine residues, which are highly conserved in natural venoms and are recognized by T cells. Thus, this tripeptide fragment could be used for the subsequent assays needed to isolate and identify stimulatory molecules in venom.
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 lipid 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 of activating T cells.
Bee venom generates CD1a ligands | Bourgeois et al.

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. 4 C), 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. 4 D) and cord blood CD34-derived LC-like cells (Fig. 4 E) act as APCs 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 phosphatidylcholine comprised of singly unsaturated C18 fatty acyl chains (PC 18:1/18:1) and phosphatidylcholine comprised of singly unsaturated C18 fatty acyl chains (PC 18:1/18:1) and phosphatidylcholine comprised of singly unsaturated C18 fatty acyl chains (PC 18:1/18:1) and phosphatidylcholine comprised of singly unsaturated C18 fatty acyl chains (PC 18:1/18:1) and phosphatidylcholine comprised of singly unsaturated C18 fatty acyl chains (PC 18:1/18:1) and phosphatidylcholine comprised of singly unsaturated C18 fatty acyl chains (PC 18:1/18:1).
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

---

**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 µg/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.
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 using suction cup blisters (Salimi et al., 2013). This method uses low pressure, sustained (~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 µg 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)
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 manoalide 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 PLA2-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 CD1d dependence of lipid-specific T cell responses is observed in polyclonal T cells and among many unrelated human donors, these data suggest
that phospholipases are important in CD1a biology, providing a potential molecular pathway underlying previous observations of T cell responses to fatty acids, which may be important for skin barrier sensing and inflammation (de Jong et al., 2014).

Recent studies of CD1a function have found that CD1a autoreactive T cells and CD1a proteins are both abundantly present within adjacent compartments of the skin (de Jong et al., 2010, 2014). In general, there exists a physical separation of CD1a proteins, which are mainly expressed on LC in the epidermis, and natural autoantigens such as fatty acids, wax esters, and squalene, which are concentrated more superficially in the sebum and cornified epithelium. The observations suggested a near neighbor model in which intact skin prevents direct contact of autoantigens from CD1a, but skin breach through infection or injury deposits antigenic substances more deeply within the skin so that antigens come into contact with CD1a expressing epidermal LC (Colonna, 2010; de Jong et al., 2010; Kronenberg and Havran, 2014). Considering bee or wasp sting as a variant of this model, phospholipases are normally injected up to 2 mm deep into 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.

Venoms injure and thereby neutralize diverse predators and prey through transfer of toxic substances that act on many types of animals or insects encountered in the wild. Thus, venoms are typically comprised of many toxic substances
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

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 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 reaction 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 ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
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 isofoms 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 mM 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/beem 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 1 µg/ml Ionomycin was included as a positive control, and T cells alone were set up in duplicate or triplicate. 10 ng/ml phorbol myristate acetate and 1 µg/ml Ionomycin plus 10% human serum (R10*) were added in 200 µl/well 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-β1 (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 µl/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 µl/ml; Sanofi US Services, Inc.) and TGF-β1 (1 ng/ml; R&D Systems) to increase CD1a expression. LCs 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-CD1a antibodies were added on streptavind-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 venom, their lipidic extracts, deoxyxymyocobactin (DDM), arachidonic acid, phosphatidylcholine 18:1, phosphatic acid 16:0, lysophosphatidylcholine 18:1, lysophosphatic acid 16:0, and anti–HLA-ABC and anti–HLA-DR blocking antibodies (W6/32 and L243, respectively) 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 venom, 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 IgGl isotype control (P3), or 100 µM manoxide, 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 (Vespula 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 intradermally, a dose which is at the low end of venom normally administered in a sting (ALK). Suction was applied to the skin to 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 confinned 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′-Azino-bis[3-ethylbenzothiazoline-6-sulfonic acid]-di-ammonium 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 venom or 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′-Azino-bis[3-ethylbenzothiazoline-6-sulfonic acid]-di-ammonium salt (ABTS).

**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.

We thank John Altman and the NIH tetramer facility for providing recombinant CD1 proteins.

This work was funded by the MRC and NIHR Biomedical Research Centre, the National Institutes of Health (NIH; N1AMS R01 048632), and the Burroughs Wellcome Fund Program in Translational Medicine. GO also acknowledges the support of the National Institute for Health Research Clinical Research Network.

**REFERENCES**


dos Santos, L.D., A.R. da Silva Menegasso, J.R. dos Santos Pinto, K.S. Santos, F.M. Castro, J.E. Kahil, and M.S. Palma. 2011. Proteomic characterization of the multiple forms of the PLAs from the venom of the...


