Danger Signaling through the Inflammasome Acts as a Master Switch between Tolerance and Sensitization

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Efficient priming of adaptive immunity depends on danger signals provided by innate immune pathways. As an example, inflammasome-mediated activation of caspase-1 and IL-1β is crucial for the development of reactive T cells targeting sensitizers like dinitrofluorobenzene (DNFB). Surprisingly, DNFB and dinitrothiocyanobenzene provide cross-reactive Ags yet drive opposing, sensitizing vs tolerizing, T cell responses. In this study, we show that, in mice, inflammasome-signaling levels can be modulated to turn dinitrothiocyanobenzene into a sensitizer and DNFB into a tolerizer, and that it correlates with the IL-6 and IL-12 secretion levels, affecting Th1, Th17, and regulatory T cell development. Hence, our data provide the first evidence that the inflammasome can define the type of adaptive immune response elicited by an Ag, and hint at new strategies to modulate T cell responses in vivo. 

Materials and Methods

Cell culture and stimulation

Primary keratinocytes (human epidermal keratinocytes (Biocoba), were cultured in Epilife medium supplemented with Human Keratinocyte Growth Supplement (Biocoba) and 60 μM of Ca²⁺. HaCaT cells were cultured in DMEM F-12 medium 1/1 (Invitrogen) supplemented with 5 μg/ml human insulin, 10 ng/ml choleratoxin, 0.4 μg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, and 10% FCS. Cell cultures were maintained at 37°C in humidified incubators with 5% CO₂. Primary keratinocytes were pre-treated with 0.1 ng/ml hTNF (Alexis) for 6 h, and stimulated with TNCB (0.5–10 μg/ml) (Fluka), DNCB (0.5–10 μg/ml), DNFB (0.5–10 μg/ml) (Fluka), DNCB (0.5–10 μg/ml), DNFB (0.5–10 μg/ml) (Fluka), DTNB (Lancaster Synthesis) (0.5–50 μg/ml) (a gift from Ian Kimber, Faculty of Life Sciences, The University of Manchester, Manchester, U.K.), urushiol, 1–5 μg/ml and SDS (15 μg/ml), for 24 h with or without Z-Val-Ala-Asp-fluoromethylketone (Alexis) at 50 μM for 24 h before IL-1β secretion was determined by ELISA.

Analysis of contact hypersensitivity and tolerance in mice

Mice were handled according to institutional and Swiss Federal Veterinary Office guidelines. IL-1R and ASC-deficient mice were obtained from M. Kopf (Molecular Biomedicine ETHZ, Zürich-Schlieren, Switzerland) and V.M. Dixit (Genentech, San Francisco). For classical CHS studies: 6–7-wk-old mice were sensitized by topical (external) applications of 20 μl of 0.5% TNCB, DNCB, DNFB, oxazolone, or 1% DTNB in acetone-olive oil (A0O) or A0O alone to the skin of the right ear at days 0 and 1. Elicitation was done at day 5 by topical application of 20 μl of 0.3% DNCB, DNFB, oxazolone, or 1% DTNB in A0O to the contralateral ear. Ear thicknesses were measured with a digital thickness gauge (Mitsutoyo) before and 24 h after hapten challenge. For tolerance induction, mice were shaved on the belly on day −1, then treated with 20 μl of 0.5% DTNB or DNFB on day 0 and 1, then sensitized on the right ear and challenged on the left ear as described above. For adoptive transfer, mice were shaved on the belly on day −1, then treated with 20 μl of 0.5% DTNB or DNFB on day 0 and 1, sacrificed on day 5. Single cell suspension from inguinal lymph nodes and spleen were washed in DMEM medium, counted, concentrated, and injected in the tail vein of strain-matched naive mice. Alternatively, single cell suspension obtained from the lymph node and spleen of DTNB-treated mice were depleted of CD4⁺/CD25⁺ cells using a two-step magnetic sorting (AutoMACS and Treg depletion kit, Miltenyi Biotech) according to the manufacturer’s protocol. Recipient mice were sensitized on the right ear with 20 μl of 0.5% DNFB on day 1 and 2 after adoptive transfer, then challenged on the left ear on day 6, as described above.

SDS, IL-1β, zYVAD, and IL-1R agonist (IL-1RA) administration

Twenty microliters of either SDS in dimethylformamide (1% w/v) or dimethylformamide alone was applied 30 min before each sensitization with DNFB. Ten microliters of either recombinant mIL-1β (BD Biosciences) in PBS (200 ng/ml) or PBS alone was injected s.c. in the ear 30 min before each sensitization with DNFB. Twenty microliters of either acyloxy-Z-Val-Ala-Asp-chloromethylketone (Alexis) in DMSO (0.2 mM) or DMSO alone was applied on the right ear 30 min before sensitization with DNFB on days 0, 1, and 2. One hundred microliters of IL-1R antagonist (Amgen) (150 μg/ml) or PBS was injected i.p. twice daily for three consecutive days, starting 12 h before the first sensitization with DNFB.

Cytokine detection by ELISA

Supernatants from primary keratinocyte cultures were analyzed by ELISA for IL-1β (BD Biosciences) according to the manufacturer’s instructions. ASC or wild-type littermates were treated with 20 μl of 0.3% DNFB on day 0 and 1 were sacrificed on day 2. Single cell suspension of draining neck lymph nodes were obtained and cultivated in DMEM medium for 5 days. Supernatants were then analyzed by ELISA IL-6 (R&D Systems), IL-10 (R&D Systems), and IL-12 (mouse IL-12/IL-23 p40 allele-specific DuoSetDY499, BD Biosciences) according to the manufacturer’s instructions.

Statistical analysis

Groups were compared using one-tailed Student’s or Aspin-Welch’s t tests.

Results

DNFB and DTNB provide similar epitopes yet differ in their capacity to activate the inflammasome and induce contact hypersensitivity

DNFB, DNCB, TNCB, and DTNB have been used as model hapten to study contact hypersensitivity and T cell reactivity in mice (11–14). As we had previously observed that TNCB activates the inflammasome in vitro and in vivo, we decided to study the effect of DNCB, DNFB, and DTNB on this signaling pathway. We found that primary keratinocytes exposed to DNFB, DNCB, and TNCB, but not DTNB, secrete significant amounts of active IL-1β, in a dose dependent manner, while the viability of the cells was not affected at these concentrations (Fig. 1A). This effect was inhibited by the caspase-1 inhibitor zYVAD, strongly suggesting an inflammasome dependent mechanism. Interestingly, the active component of poison ivy, urushiol, also potently activates IL-1β secretion, as previously reported (15). Because detergents with irritant properties potentiate allergic responses (4–6) and have been reported to promote IL-1β secretion by keratinocytes (15), we included SDS in our assays and found that it could also trigger IL-1β
activation. Our results hinted that DNTB might fail to trigger danger signals necessary to induce CHS in vivo. Indeed, DNFB, DNCB, and TNCB induce strong sensitization (Fig. 1B), while DNTB is a weak sensitizer at best (11–14). The difference in the sensitizing potency of these molecules may be due to the antigenicity of these molecules, but our results rather suggest that it is linked to their capacity to provide danger signals through the activation of the inflammasome.

We found that DNFB-sensitized mice display a strong reaction to DNTB (Fig. 1B). Our data are consistent with previously published observations and tend to confirm that DNFB and DNTB provide epitopes that can be recognized by the same T cells, yet differ in their capacity to prime the adaptive immunity (11, 12, 16). Although the value of DNTB as a tolerogen is still debated (17, 18), some find it to behave as a weak sensitizer (17, 18), whereas others find it to behave as a weak sensitizer (17, 18). Moreover, concomitant injection of IL-1β, but not PBS alone, favored the development of CHS to DNTB (Fig. 2A). Conversely, the sensitizing effect of DNFB was abrogated when inflammasome signaling was inhibited, either by the local application of a caspase-1 inhibitor (zYVAD), as previously reported (20, 21). Likewise, the sensitizing capacity of DNFB was abrogated by the systemic injection of an IL-1 receptor antagonist (Anakinra) (Fig. 2B), which is consistent with a report showing local intradermal injection of IL-1ra 6–24 h before sensitization (22). These results are concordant with the response of mice deficient for ASC and IL-1R.

Although our in vitro data strongly suggested that SDS induces IL-1β activation through an inflammasome-mediated process, we had no proof that it was the case for its in vivo adjuvant effect. We therefore assessed the effect of SDS in ASC-deficient mice. SDS potently and significantly increased the sensitizing capacity of DNTB in ASC heterozygote mice but only very weakly in their littermate ASC-deficient mice (Fig. 2C). Hence, the adjuvant effect we observed for SDS is mediated primarily by the inflammasome. Taken together, these data strongly suggest that the inflammasome is a key mediator of danger signals that prime the adaptive immune response directed against potent contact sensitizers.

**Figure 2.** Concomitant danger signaling allows sensitization to DNTB, while blocking danger signaling prevents DNFB sensitization. A, DNFB-sensitized mice (days 0 and 1 on the left ear) challenged with DNFB (day 5 on the right ear) do not develop CHS, except if danger signals, such as i.v. IL-1β or topical SDS, are provided at the time of sensitization. Application of either SDS or IL-1β alone, in the absence of any hapten (AOO), had no effect, while neither could further increase the sensitization capacity of DNFB. B, Blocking of danger signals provided by DNFB during sensitization, using i.v. IL-1Ra (Anakinra) or topical zYVAD prevents the development of CHS. Similar results are obtained when the sensitization is performed in ASC or IL-1R-deficient mice. C, SDS increases the sensitizing capacity of DNTB in ASC+/− but not ASC−/− littermates. (n = 5 mice/group, unless indicated in the figure). The irritant effect of DNFB alone is limited, as observed in mice treated with AOO during the sensitization phase later challenged with a single application of DNFB (A and C).

**Danger signaling mediated by the inflammasome allows sensitization to DNTB, while blocking danger signaling prevents DNFB sensitization**

IL-1β had been previously reported to play a key role for the CHS response to small irritants like DNFB, acting as an adjuvant promoting effector T cell response (19). We reasoned that whether the inflammasome is responsible for the activation of IL-1β, it should be possible to turn weak sensitizers into strong sensitizer by providing danger signals. Mice exposed twice on the right ear to DNTB failed to mount an immune response when challenged with DNFB on the contralateral ear (Fig. 2A), although a direct irritant effect of DNFB could be readily observed. However, when SDS was applied topically during DNFB sensitization, DNFB elicited a strong immune reaction (Fig. 2A). Danger signaling by these two adjuvants failed to trigger CHS in the absence of hapten copresentation (AOO), ruling out a nonspecific effect on T cell development during the challenge phase. It did not either further enhance the response to DNFB, which can activate the inflammasome very efficiently on its own (Fig. 2A). These data are in accord with previous reports showing an adjuvant effect of a similar detergent (sodium lauryl sulfate) in men (4, 5). Moreover, concomitant injection of IL-1β, but not PBS alone, favored the development of CHS to DNTB (Fig. 2A). Conversely, the sensitizing effect of DNFB was abrogated when inflammasome signaling was inhibited, either by the local application of a caspase-1 inhibitor (zYVAD), as previously reported (20, 21). Likewise, the sensitizing capacity of DNFB was abrogated by the systemic injection of an IL-1 receptor antagonist (Anakinra) (Fig. 2B), which is consistent with a report showing local intradermal injection of IL-1ra 6–24 h before sensitization (22). These results are concordant with the response of mice deficient for ASC and IL-1R.

**Exposure to topical sensitizers in the absence of inflammasome-mediated danger signals leads to tolerance**

The tolerogenic effect of DNTB is a matter of debate. Indeed, some find it to behave as a weak sensitizer (17, 18), whereas others find it induces tolerance (11, 13, 14). There is, on the contrary, no doubt on the fact that DNFB is a strong sensitizer. In our experimental setting, DNTB not only failed to elicit CHS, but also induced tolerance, as mice painted on two consecutive days on their shaved belly could not be sensitized any longer with DNFB, whereas the response to Oxazolone was normal (Fig. 3A). As DNTB and DNFB provide cross-reactive epitopes, which are different from those provided by oxazolone (16), our findings suggest that the
Cytokine profile in draining lymph nodes of DNFB treated ears from ASC deficient mice and their control littermates. Mice sensitized with DNFB on day 1 and 2 were sacrificed at day 3 and the draining lymph nodes were dissociated and cultivated for 5 days. Under these conditions, we could not detect significant amounts of secreted IL-1β (data not shown). It is important to note here that IL-1β is secreted in minute amounts and that IL-6, one of its bona fide downstream target, is significantly increased after the injection of femto-moles of IL-1β, hence making IL-6 a surrogate marker for active IL1β secretion (25). Interestingly, we found that wild-type lymph nodes secreted significantly higher amounts of IL-6 and IL-12/23 than those from ASC-deficient mice, suggesting a cytokine environment likely to favor a Th1 and Th17 response and hamper regulatory T cell (Treg) development (26, 27) (Fig. 4 and see below). These results are consistent with a previous report showing that IL-12 can turn DN TB into a strong sensitizer (14). IL-10 secretion, which antagonizes LC migration and IL-12 priming of T cells (28), was slightly higher in ASC deficient mice, but the difference was not statistically significant and the very low levels observed may undermine the relevance of our finding. Hence, our results suggest but do not prove that the inflammasome may modulate the secretion levels of IL-10, which may in turn regulate T cell fate.

Mechanisms of tolerance transfer

The mechanism of DN TB induced tolerance is still controversial. However, early experiments, including adoptive transfer studies, have suggested that regulatory T cells (previously called suppressive T cells) may be implicated (11, 12, 14). We therefore studied the impact of regulatory cells on tolerance to DN FB. We first analyzed the repartition of CD4+ CD25+ FoxP3+ T cells in lymph nodes draining the skin of DNFB treated ASC-deficient mice and their control littermates. We found that the overall number of CD4+ CD25+ FoxP3+ cells was the same, suggesting either that Treg are not implicated in tolerance or that the DNFB-specific
Mechanisms of tolerance induction, adoptive transfer of Treg depleted T cells (14). We reasoned that if Treg do play a role in T cell dependency.

Mechanisms of tolerogenesis (29). Moreover, this is also consistent with the observation that the vast majority of people exposed to nickel, a weak hapten that does not activate the inflammasome (H. Watanabe, manuscript in preparation), exhibit nickel-specific Tregs with suppressive activity, but nickel-allergic patients do not (30). Hence, the Treg pathways may represent a default pathway, which is negatively regulated by danger signals, including the IL-1β activation mediated by the inflammasome.

Discussion

The central role of innate immunity in the activation of the adaptive immune response is no longer a matter of debate, in particular regarding TLR signaling. We herein provide evidence that danger signaling through the inflammasome can also affect the fate of the adaptive immune response. Others and we had already shown that efficient sensitization does not occur in the absence of the inflammasome and IL-1β (9, 10, 22), yet these studies did not evaluate the impact of this signaling pathway, or its absence, on the type of adaptive response elicited. We now provide evidence that the inflammasome may act as a major switch between tolerance and sensitization, laying emphasis on the sequential role of innate immunity and adaptive immunity in CHS (Fig. 6). Indeed, we found that DNTB fails to activate the inflammasome in vitro, unlike other members of this family. Furthermore, DNTB becomes a sensitizer if concomitant inflammasome signalling is provided. More importantly, blocking inflammasome signalling turns a bona fide sensitizer, DNFB, into a tolerizer. Taken together, these findings suggest that the inflammasome controls the development of tolerance or sensitization to (irritant) chemicals. Hence, NLR and TLR not only share structural resemblance, but also a similar function. Because both signaling pathways ultimately result in NF-κB activation, it is reasonable to think that a certain amount of redundancy is present. We therefore propose that stimulation of TLR, IL-1R, NLR activation may be an interesting target as an adjuvant for vaccines and tumor vaccination, and it should be noted here that DNCB has been used to promote inflammation and favor immune-rejection in metastatic melanoma patients (31).

Our data further suggest that the Treg-mediated development of tolerance may represent a default pathway in noninflammatory conditions, as we observed that ASC deficiency favors tolerance to hapten present in the skin. The potential physiologic benefit to this may be to avoid unnecessary/harmful T cell responses against Ags that are not accompanied by significant cell stress or damage, and therefore are unlikely to originate from a pathogen (introducing the notion of an environmental self). This hypothesis may have

FIGURE 4. Cytokine profile in tolerant vs sensitized mouse lymph nodes. Lymph node cells from DNFB treated ASC−/− mice (days −5 and −4 before lymph nodes extraction) secrete significant amounts of IL-6 and IL-12, as determined by ELISA. In contrast, cells from their ASC+/− littermates secreted only small amounts of these cytokines. IL-10 levels were slightly increased in ASC−/− cells, yet statistical significance was not reached. (n = 4 mice/group). ND: Nondetectable.

FIGURE 5. Mechanisms of tolerance transfer and inflammasome dependency. A. The repartition of CD4+ CD25+ FoxP3+ Tregs is the same in lymph node cells extracted from DNFB treated ASC heterozygote and deficient mice, as demonstrated by FACS staining. B. Adoptive transfer of DNTB exposed donor mice fails to induce tolerance to DNFB in the recipient mouse, if CD4+CD25+ cells are removed by magnetic associated cell sorting, before their transfer. (n = 4 mice/group).
proliferation by draining lymph node cells such as DNCB, but fails to induce LC migration (36), suggesting the possible recruitment of dermal DC by DTNB (37). We propose that DTNB fails to activate the inflammasome and IL-1β activation, which is crucial for LC migration, but does activate other molecular pathways that may, depending on the context, be sufficient to trigger an efficient immune response and result in sensitization. We are currently generating LC-specific IL-1β and ASC-deficient mice to study this hypothesis. Interestingly, we have conducted experiments that indicate that the presence of an intact inflammasome is essential for DTNB and DNCB-induced LC migration (data not shown). Additional experiments are necessary, including a characterization of the role of the inflammasome on dermal DC, as the exact role of both cell types in CHS remains controversial (38–41). Finally, the CHS outcome may also depend on the viral and bacterial environment of the host, as TLR and NLR activate similar targets such as NF-kB and IL-12.

Although NLR discovery is recent, several therapeutic tools targeting their signaling pathways or downstream targets are already available. For example, down-modulation of the inflammasome has been achieved successfully in patients suffering from autoimmune inflammatory disorders, either by blocking caspase-1 or IL-1β signaling (42–44). Additional tools will soon be added to this list, including anti-IL-1β, anti-IL-6, and anti-IL12/23 Abs. As for NLR activation, Freund’s adjuvant has been used extensively in vaccination studies (3, 8). We believe that gathering further insights into the role of each member of this large family will increase the number of patients benefiting from this therapeutic advance and will lead to the development of even more potent and specific drugs.

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Disclosures
The authors have no financial conflict of interest.

References


