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*J Immunol* 2008; 180:5826-5832; doi: 10.4049/jimmunol.180.9.5826

http://www.jimmunol.org/content/180/9/5826

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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. The Journal of Immunology, 2008, 180: 5826–5832.

The Journal of Immunology

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www.jimmunol.org

Received for publication December 20, 2007. Accepted for publication February 20, 2008.

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1 This work was supported by grants from the Swiss National Science Foundation. H.W. was supported by Mahuco Co. Ltd., Japan.
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4 Abbreviations used in this paper: NLR, nod-like receptor; CHS, contact hypersensitivity; DNFB, dinitrofluorobenzene; ASC, apoptosis-associated speck-like protein containing a CARD domain; DNFB, dinitrothiocyanobenzene; TNF, TNF-related apoptosis-inducing ligand; IL-1Ra, IL-1 receptor antagonist; AOO, acetone olive oil; DNTB, dinitrothiocyanobenzene; Treg, regulatory T cell.

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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 CaCl₂. HaCaT cells were cultured in DMEM: F-12 medium (1/1) (Invitrogen) supplemented with 5 µg/ml human insulin, 10 ng/ml cholera toxin, 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 preincubated with 0.1 ng/ml IL-1β (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), DNFB (0.5–10 µg/ml) (Fluka), NTBC (Alexis) (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) (a gift from Ian Kimber, Faculty of Life Sciences, The University of Manchester, Manchester, U.K.), urushiol, (1–5 µg/ml) (Fluka), DNCB (0.5–10 µg/ml), DNFB (0.5–10 µg/ml) (Fluka), DNCB (0.5–10 µg/ml) (Fluka), DNFB (0.5–10 µg/ml) (Fluka), and 10% FCS. Cell cultures were cultured in DMEM: F-12 medium (1/1) (Invitrogen) supplemented with 5 µg/ml human insulin, 10 ng/ml cholera toxin, 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 preincubated with 0.1 ng/ml IL-1β (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) (Fluka), DNFB (0.5–10 µg/ml) (Fluka), NTBC (Alexis) (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 30 µM for 24 h before IL-1β secretion was determined by ELISA.

Analysis of contact hypersensitivity and tolerance in mice

Mice were housed 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% DNTB in acetone-olive oil (AOO) or AOO 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% DNFB on day 0 and 1 and 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 DuoSet DY499, BD Biosciences) according to the manufacturer’s instructions.

Statistical analysis

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

Results

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

DNFB, DNCB, TNCB, and DNTB have been used as model reporters 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 DNTB on this signaling pathway. We found that primary keratinocytes exposed to DNFB, DNCB, TNCB, and DNTB 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 assay and found that it could also trigger IL-1β secretion.

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 acryloxy-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. Groups were compared using one-tailed Student’s or Aspin-Welch’s tests.

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 and 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 DuoSet DY499, BD Biosciences) according to the manufacturer’s instructions.

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FIGURE 1. DNFB and DNTB provide similar epitopes, yet differ in their capacity to activate the inflammasome and induce contact hypersensitivity. A, Primary keratinocytes exposed in vitro to contact sensitizers such as TNCB (10 µg/ml), DNCB (10 µg/ml), DNFB (0.5–10 µg/ml), and urushiol (5 µg/ml) secrete IL-1β, whereas DNTB (0.5–10 µg/ml), which induces tolerance, does not. The skin-irritant SDS (15 µg/ml) also induced significant IL-1β release, but not benzalkonium (BZK). IL-1β activation is blocked by the specific caspase-1 inhibitor zYVAD. B, Mice sensitized at day 0 and 1 on the left ear, then challenged at day 5 on the right ear develop contact hypersensitivity to TNCB, DNCB, and DNFB, but not to DNTB, as shown by the ear swelling 24 h after challenge. Interestingly, mice sensitized to DNFB and challenged with DNTB develop CHS, whereas DNTB-sensitized mice challenged with DNFB do not. Taken together, these findings suggest that both molecules provide cross-reactive epitope(s), but that only DNFB provides danger signals necessary to mount a CHS. (Cells: n = 4 independent duplicates, mice = 5–6/group.)
activation. Our results hinted that DNFB might fail to trigger danger signals necessary to induce CHS in vivo. Indeed, DNFB, DNBCB, and TNCB induce strong sensitization (Fig. 1B), while DNFB 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), this suggests that the level of inflammasome signaling induced by a given molecule may better predict its sensitizing potential than the antigenic properties itself.

**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 DNFB failed to mount an immune response when challenged with DNTB in ASC-/- mice (Fig. 2A), although a direct irritant effect of DNFB could be readily observed. However, when SDS was applied topically during DNTB 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.

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 weekly 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.

**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 elicte 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 littersmates. 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 targets, is significantly increased after the injection of femto-moles of IL-1β, hence making IL-6 a surrogate marker for active IL-1β 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 DNFB 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 DNFB 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 DNFB. 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 littersmates. 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
IL-12/23

0 - 4 mice/group). ND: Nondetectable.

Treg population variation is too small to affect the total number of regulatory T cells present in the lymph node (Fig. 5A), as previously reported (14). We reasoned that if Treg do play a role in tolerance induction, adoptive transfer of Treg depleted T cells would not protect recipient mice from DNFB. We therefore extracted lymph node and spleen cells from DNTB-exposed donor mice and used magnetic bead-associated cell sorting to remove CD4+CD25+ double positive cells. We found that after adoptive transfer of these cells, sensitivity to DNFB was partially restored (Fig. 5B). We cannot fully rule out that this type of depletion could have a less specific general effect on Tregs numbers, affecting general suppressor cytokine level, but this is unlikely because the overall number of Treg is not affected by ASC deficiency (Fig. 5A).

Interestingly, when DNFB-treated animals were used as donors, depletion of Treg did not induce further increase in the response to DNFB, suggesting that there are no or very few DNFB-specific Treg cells in naive mice (Fig. 5B). Taken together with previous reports (11, 12, 14), our results suggest that molecules which are present on the skin but do not activate the inflammasome are nevertheless presented to T cells, resulting the development of a small population of Tregs with suppressive capacity. This is consistent with the current belief that, in noninflammatory conditions, LC travel to the lymph nodes (although at low level) and are tolerogenic (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 signaling is provided. More importantly, blocking inflammasome signaling 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, IL-18R, and IL-12R may all favor the development of CHS to DNTB or other Ags, as reported for IL-12 (14). Consequently, 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).
important implications in the fields of virology and oncology. As an example, human papillomaviruses elicit little response from the immune system, although they can provide highly antigenic epitopes (32). Danger signaling mediated by imiquimod-induced TLR7-triggering results in a rapid destruction of the lesions by T cells (33), suggesting that the virus “invisibility” depends on the absence of inflammation. Cancer development and immune-escape may rely on similar mechanisms, as most tumors develop without eliciting inflammation. Interestingly, activation of the innate immune system through TLRs (33) or NLRs (31) may also result in activating their signaling pathways or downstream targets are already available. For example, down-modulation of the inflammasome may rely on similar mechanisms, as most tumors develop without eliciting inflammation. Interestingly, we have conducted experiments that indicate that the presence of an intact inflammasome is essential for DNFB-induced 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 DNFB- 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-κB 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 autoinflammatory 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.

Acknowledgments
We thank Maria Olleros for help with ELISAs.

Disclosures
The authors have no financial conflict of interest.

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