



Production of Extracellular Adenosine by CD73⁺ Dendritic Cells Is Crucial for Induction of Tolerance in Contact Hypersensitivity Reactions

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Dendritic cells (DCs) express the ecto-5'-nucleotidase CD73 that generates immunosuppressive adenosine (Ado) by dephosphorylation of extracellular Ado monophosphate and diphosphate. To investigate whether CD73-derived Ado has immune-suppressive activity, 2,4-dinitrothiocyanobenzene (DNTB) was applied to skin of wild-type (WT) or CD73-deficient (CD73^{-/-}) mice, followed by sensitization and challenge with 2,4-dinitrofluorobenzene. In this model, we show the induction of tolerance by DNTB against 2,4-dinitrofluorobenzene only in WT but not in CD73^{-/-} mice. Analysis of skin DCs showed increased expression of CD73 after application of DNTB in WT mice. That was accompanied by elevated concentrations of extracellular Ado in the lymph node. Moreover, T cells expressed markers for anergy, namely EGR2 and NDRG1 in DNTB-treated WT mice and they exhibited impaired proliferation upon ex vivo re-stimulation. Similarly, in vitro we observed that Ado-producing WT DCs, but not CD73^{-/-} DCs, rendered transgenic T cells from OTII mice (OTII T cells) hyporeactive, decreased their T-cell costimulatory signaling, and induced up-regulation of EGR2 and NDRG1. Thus, these data show that expression of CD73 by DCs, which triggers elevated levels of extracellular Ado, is a crucial mechanism for the induction of anergic T cells and tolerance.

Journal of Investigative Dermatology (2019) 139, 541–551; doi:10.1016/j.jid.2018.10.016

INTRODUCTION

Adenosine (Ado) has potent anti-inflammatory properties because it blocks activation and secretion of T-cell cytokines and maturation of dendritic cells (DCs) (Ben Addi et al., 2008; Challier et al., 2013; Linden and Cekic, 2012). In skin, leukocytes are able to modulate intercellular concentrations of Ado by enzymatic dephosphorylation of extracellular adenosine triphosphate (ATP) (Burnstock et al., 2012). They express the ATP-degrading ectoenzymes CD39 and CD73, whereby CD39

catalyzes the degradation of ATP to adenosine diphosphate and then adenosine monophosphate (AMP), and CD73 converts these substrates to Ado.

Extracellular ATP is a well-characterized “danger” molecule in skin (Onami et al., 2014; Ring et al., 2010; Schenk et al., 2008; Woehrle et al., 2010) that activates immune cells by engaging purinergic receptors (Cekic and Linden, 2016). Accordingly, in healthy tissues the extracellular concentration of ATP has to be kept low, and many cells therefore express the ATP-degrading ectonucleotidases CD39 and CD73 (Romio et al., 2011).

Defined lymphocyte populations use the production of extracellular Ado to convey immunosuppressive effects. For instance, regulatory T cells (Tregs) produce extracellular Ado upon activation (Deaglio et al., 2007; Ring et al., 2010). Ado thereby exerts suppressive actions in contact hypersensitivity reactions and during tumor growth. Moreover, Treg-derived Ado acts as a “find me” signal for DCs, guiding the interaction between Tregs and DCs and modulating induction of tolerance (Ring et al., 2015; Saez et al., 2017).

T cells predominantly express A2a and A2b Ado receptors, and pharmacological engagement of these receptors during cognate TCR-major histocompatibility complex II interaction prevents their activation (Linnemann et al., 2009; Zarek et al., 2008). This led us to hypothesize that the production of Ado by means of degradation of extracellular ATP by DCs during antigen presentation may affect the outcome of an immune response.

In bone marrow-derived DCs (BMDCs), we found higher expression of CD73 and increased Ado production in

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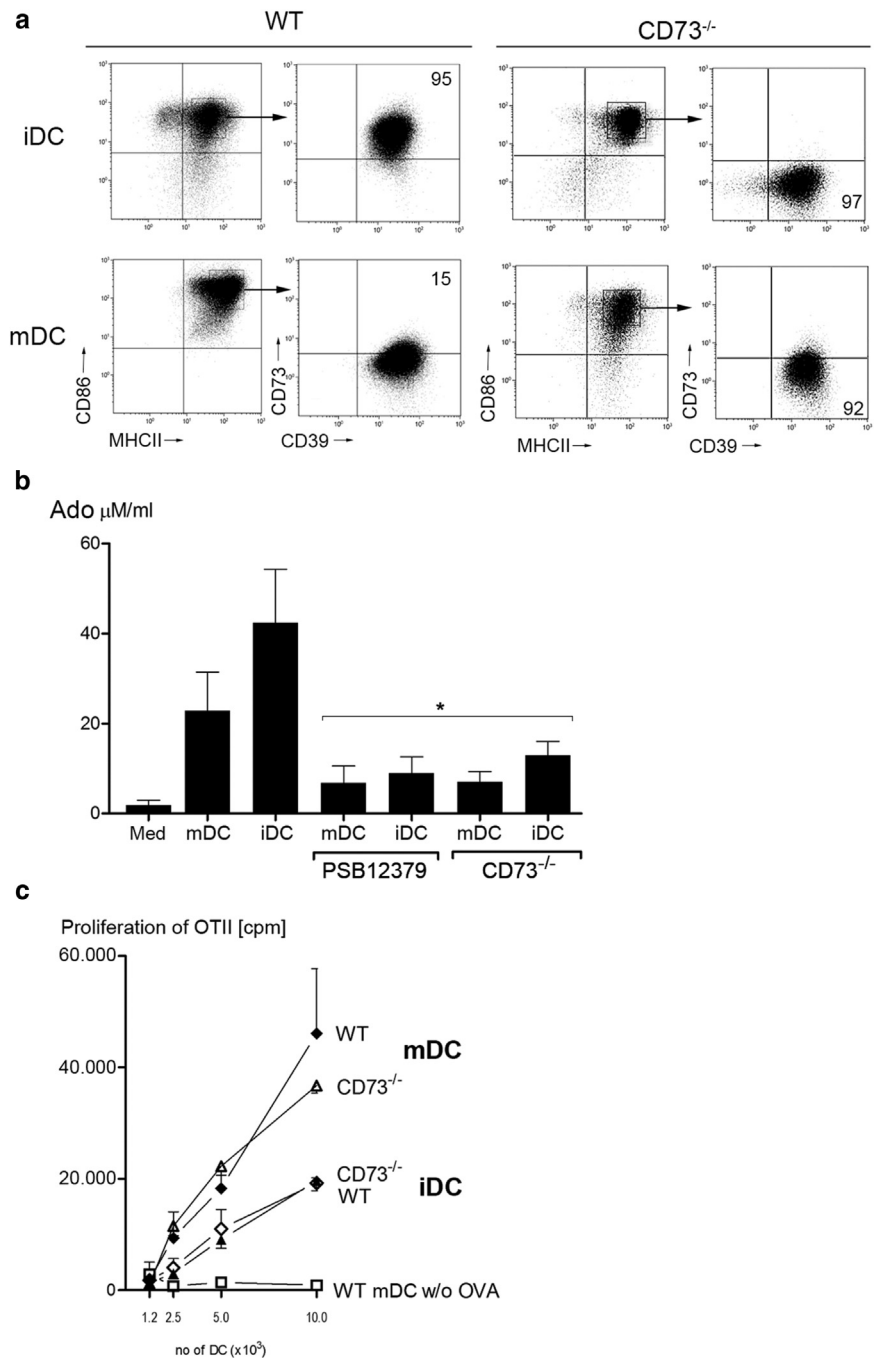
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Abbreviations: Ado, adenosine; AMP, adenosine monophosphate; ATP, adenosine triphosphate; BMDC, bone marrow-derived dendritic cell; cAMP, cyclic adenosine monophosphate; DC, dendritic cell; DNFB, 2,4-dinitrofluorobenzene; DNTB, 2,4-dinitrothiocyanobenzene; iDC, immature bone marrow derived dendritic cell; LC, Langerhans cell; mDC, mature bone marrow derived dendritic cell; LN, lymph node; OTII, ovalbumin specific transgenic T cell receptor expressing T cells from OTII mice; Ova, ovalbumin peptide, amino acid 323–339; smDC, skin-migrating dendritic cell; Treg, regulatory T cell; WT, wild type

Received 28 June 2018; revised 2 October 2018; accepted 15 October 2018; accepted manuscript published online 28 October 2018; corrected proof published online 12 January 2019

Figure 1. WT iDCs produce Ado. (a)

For FACS analysis, WT and CD73^{-/-} iDCs were harvested after 5 days of culture. In parallel, mDCs were iDCs treated with lipopolysaccharide for the last 24 hours of culture. Numbers in quadrants indicate percentage of positive cells. **(b)** iDCs and mDCs were cultivated for 6 hours in ATP-containing medium, followed by analysis of Ado in tissue culture supernatants. Samples contained CD73^{-/-} DCs, or were treated with the CD73 inhibitor PSB12379, or contained medium only (as indicated). Means $\pm$ standard error of the mean are shown. * $P < 0.05$ compared with iDCs, t test; $n = 4$. **(c)** Graded numbers of Ova-pulsed WT or CD73^{-/-} mDCs and iDCs, respectively, were co-cultivated with 5×10^5 OTII T cells. Proliferation was determined by ³H-thymidine incorporation. Means $\pm$ standard error of the mean are shown of triplicates from one out of four similar experiments. Ado, adenosine; ATP, adenosine triphosphate; DC, dendritic cell; iDC, immature dendritic cell; mDC, mature dendritic cell; Med, medium; Ova, ovalbumin peptide; PBS, phosphate buffered saline; WT, wild type.



immature BMDCs (iDCs) than in mature BMDCs (mDCs). Only CD73⁺ and Ado-producing iDCs were able to render T cells refractory to restimulation and triggered up-regulation of anergic markers such as EGR2 and NDRG1. In contrast, CD73^{-/-} iDCs that are unable to produce sizable amounts of extracellular Ado failed to induce T-cell anergy. Moreover, we showed that CD73^{-/-} animals were resistant to tolerance induction in a 2,4-dinitrothiocyanobenzene (DNTB)/2,4-dinitrofluorobenzene (DNFB) contact hypersensitivity model. Thus, these data indicate that the regulated expression of CD73 by iDCs and the concomitant production of Ado represent a crucial mechanism by which DCs induce anergic T cells and prevent immune reactions.

RESULTS**CD73-derived Ado is necessary to induce T-cell hyporesponsiveness**

When investigating BMDCs, increased expression of CD73 by iDCs as compared with mDCs was recorded. However, expression of CD39 was similar (Figure 1a). In CD73^{-/-} DCs we observed a similar maturation level compared with wild-type (WT) DCs, and expression of CD39 was also not altered. We detected increased amounts of Ado in tissue culture supernatants of iDCs, and the production of Ado ceased after cultures were treated with the CD73 antagonist PSB12379 or when iDCs from CD73^{-/-} animals were tested in these assays (Figure 1b).

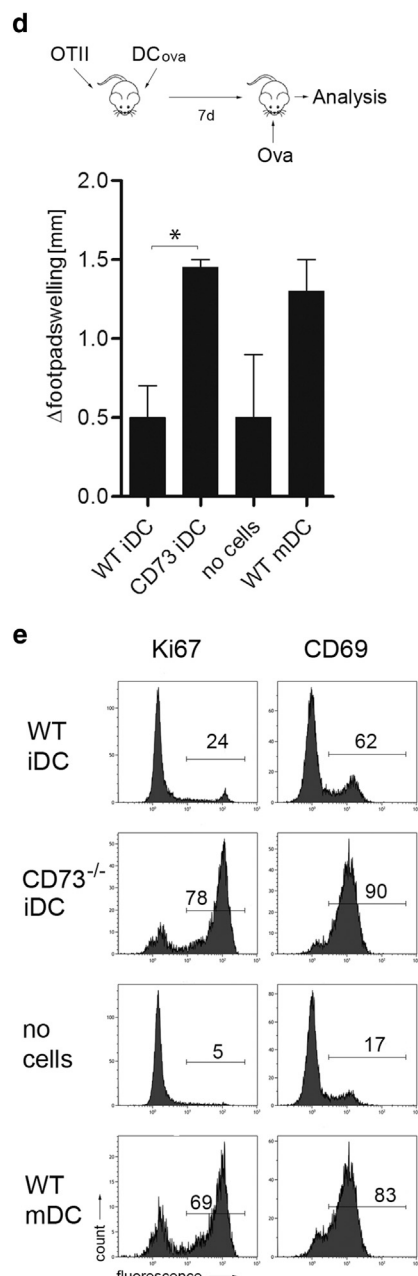
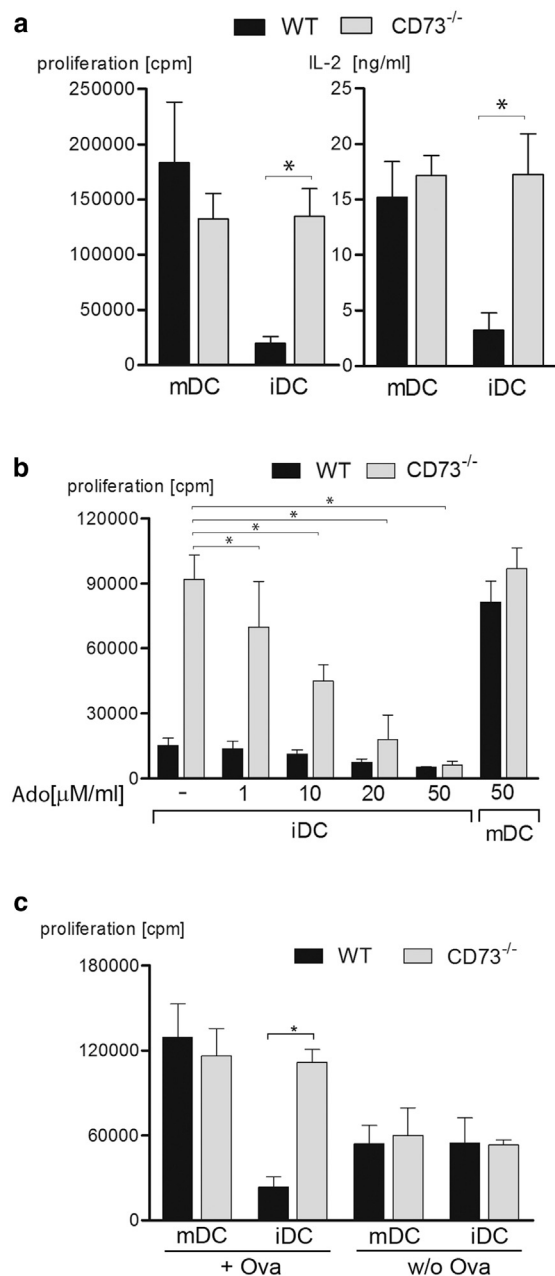


Figure 2. Ado production by CD73^{-/-} iDCs induces T-cell hypo-responsiveness. (a) OTII cells were co-cultured for 7 days with iDCs or mDCs from WT or CD73^{-/-} mice. After re-isolation, 5×10^5 T cells were stimulated with anti-CD3/anti-CD28 antibodies (1 μ g/ml). Proliferation was determined by ³H-thymidine. IL-2 was determined by ELISA. Means $\pm$ standard error of the mean are shown. * $P < 0.05$, t test; $n = 4$. (b) Cocultures as in a, except Ado was added as indicated. Mean proliferation $\pm$ standard error of the mean upon restimulation, * $P < 0.05$, t test; $n = 3$. (c) Co-cultures as in a, except, co-cultures were with (+ Ova) or without Ova-pulsed DCs (w/o Ova). Means $\pm$ standard error of the mean are shown. * $P < 0.05$, t test; $n = 3$. (d) First, 4×10^6 OTII cells were injected, followed by 2×10^6 DCs 24 hours later and then challenge by Ova injection into footpad at day 7. Mean swellings $\pm$ standard error of the mean are shown 48 hours later. * $P < 0.05$, t test; $n = 3$. (e) Procedure as in d. Expression of Ki67 and CD69 by OTII cells (V β 5⁺/V α 1⁺) was measured in knee-draining LNs. Numbers indicate percentage of positive cells. Ado, adenosine; cpm, counts per minute; d, day; DC, dendritic cell; iDC, immature dendritic cell; M, mol/L; mDC, mature dendritic cell; Med, medium; LN, lymph node; Ova, ovalbumin peptide; w/o, without; WT, wild type.

For functional analysis we set up antigen-presentation assays with ovalbumin peptide (Ova)-pulsed iDCs and mDCs derived from WT or CD73^{-/-} mice. No difference in their T-cell stimulatory capacity was apparent (Figure 1c). However, iDCs were generally less effective in stimulating proliferation of OTII T cells than mDCs. Next, we prepared DC/OTII T-cell cultures for 7 days under conditions that normally induce energy (Pletinckx et al., 2015). After restimulation with anti-CD3/anti-CD28, we observed reduced proliferation of T cells derived from cocultures with WT iDCs (Figure 2a). In contrast, T cells cocultured with CD73^{-/-} iDCs were not hyporeactive and proliferated strongly. Moreover, we recorded lower amounts of IL-2 in tissue culture supernatants of WT iDC-derived T cells than in their CD73^{-/-} iDC-stimulated counterparts (Figure 2a).

To prove that not just the mere absence of CD73 from the surface of iDCs is responsible for the hypoactivation of T cells, we supplemented CD73^{-/-} iDC/OTII T cell cultures with Ado before analyzing T-cell proliferation 7 days later. Here, Ado reestablished the hypoactivating effect of iDC on OTII T cells (Figure 2b). Finally, we tested the requirement of TCR engagement during iDC–T-cell interaction and found that T-cell hyporeactivity was not induced in the absence of Ova (Figure 2c).

Because iDCs can induce T-cell hyporesponsiveness in transplantation models in vivo (Lutz et al., 2000), we first injected OTII responder T cells into WT mice, followed by injection of Ova-pulsed WT or CD73^{-/-} iDCs (Figure 2d). To challenge the T cells, Ova was injected into the hind footpad 7 days later, and swelling and the activation status of OTII

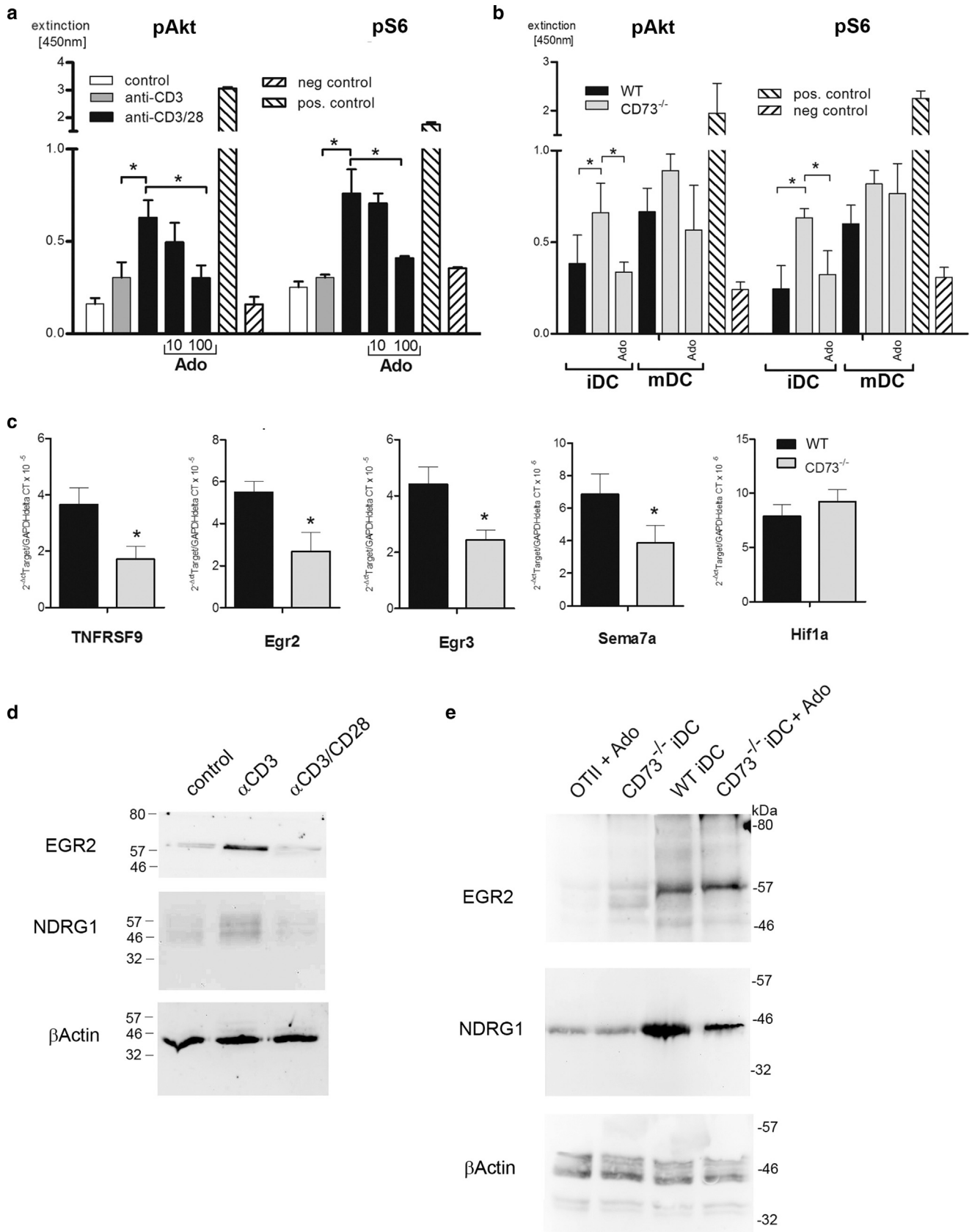


Figure 3. CD73⁺ iDCs induce anergy markers in T cells. (a) Untreated (control) or anti-CD3/anti-CD28-stimulated OTII cells, with or without Ado (as indicated) were lysed and pAkt and pS6 contents were determined by ELISA. (b) OTII cells co-cultivated with iDCs or mDCs were re-isolated, and pAkt and pS6 contents were determined as in a. In a and b, the means ± standard error of the mean are shown of triplicates of a typical experiment. **P* < 0.05, *t* test; *n* = 4. Positive and negative controls were provided by the supplier. (c) Co-cultures as in b for 24 hours. mRNA from T cells was quantified using qRT-PCR. Means ±

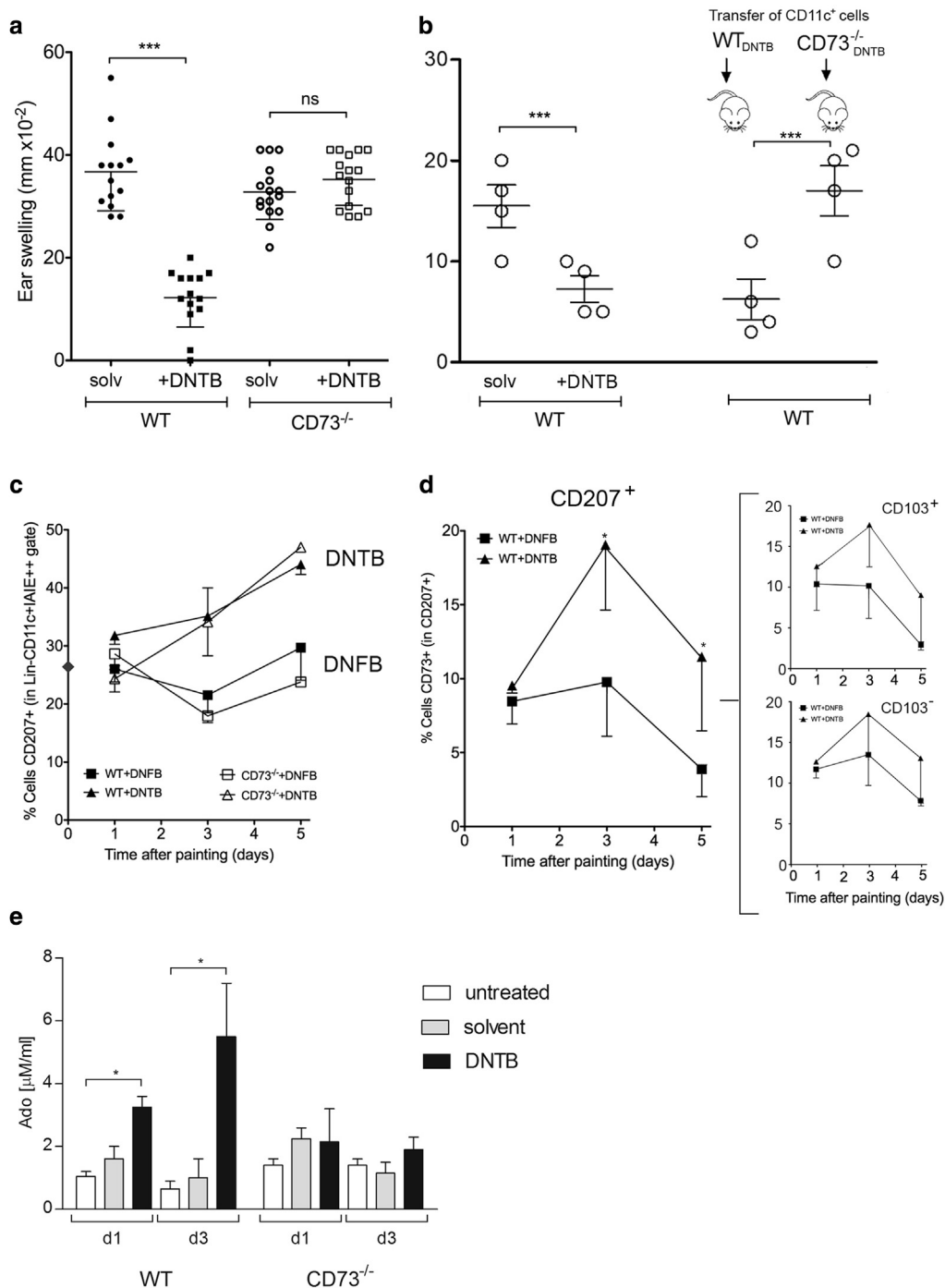


Figure 4. CD73^{-/-} mice are refractory to tolerance induction. (a) WT and CD73^{-/-} mice were treated with DNTB or solvent, followed by sensitization and challenge. Change in ear swelling after 24 hours is shown. Mean $\pm$ standard error of the mean is shown. * $P < 0.05$ indicates significance, t test; $n = 12$). (b) WT mice were either treated as in a or received CD11c⁺ DCs from DNTB-treated WT or CD73^{-/-} animals. After sensitization and challenge, ear swelling was determined as in a. * $P < 0.01$, t test; $n = 4$. (c) Frequency of CD207⁺ smDCs in draining LNs of WT and CD73^{-/-} mice treated with DNTB or DNFB. Means $\pm$ standard error of the mean are shown, $n = 4$. (d) As in c, except CD207⁺ smDCs were dissected into CD103⁺/CD103⁻ subsets and analyzed for CD73 expression. Means $\pm$ standard error of the mean are shown. * $P < 0.05$, t test; $n = 4$. (e) Mice were treated as indicated. At 1 day and 3 days thereafter, LNs were removed and cultured in ATP-containing medium, and tissue culture supernatants were analyzed for Ado. Means $\pm$ standard error of the mean are shown. * $P < 0.05$, t test; $n = 4$. Ado, adenosine; ATP, adenosine triphosphate; d, day; DC, dendritic cell; DNFB, 2,4-dinitrofluorobenzene; DNTB, 2,4-dinitrothiocyanobenzene; LN, lymph node; ns, not significant; smDC, skin-migrating dendritic cell; solv, solvent; WT, wild type.

cells were analyzed. A reduced footpad swelling was recorded in WT iDC-injected mice. Moreover, OTII cells exhibited reduced activation (CD69 expression) and proliferation (Ki67 expression) after WT iDC injection. Thus, these data indicate that CD73-mediated generation of Ado is necessary to induce T-cell hyporesponsiveness during cognate iDC–T-cell interactions.

iDCs reduce co-stimulatory signals and induce anergic markers in T cells by Ado

Ado dampens CD28-mediated intracellular signaling (Linnemann et al., 2009). Therefore, the production of Ado by iDCs may decrease T-cell activation, leading to anergy. We next investigated the phosphorylation of Akt and S6 kinases (Kane and Weiss, 2003) in OTII cells under various

standard error of the mean are shown. * $P < 0.05$, t test; $n = 4$. (d) Cell cultures as in a. OTII cells were re-isolated (48 hours) and analyzed by Western blot for EGR2, NDRG1, and β -actin expression. (e) OTII T cells were cultivated alone (OTII) or with DCs as indicated (Ado = 100 μ M/L). Thereafter (24 hours), analysis was performed by Western blot as in d. Ado, adenosine; DC, dendritic cell; iDC, immature dendritic cell; mDC, mature dendritic cell; neg, negative; p, phosphorylated; pos., positive; qRT-PCR, quantitative reverse transcriptase–PCR; WT, wild type.

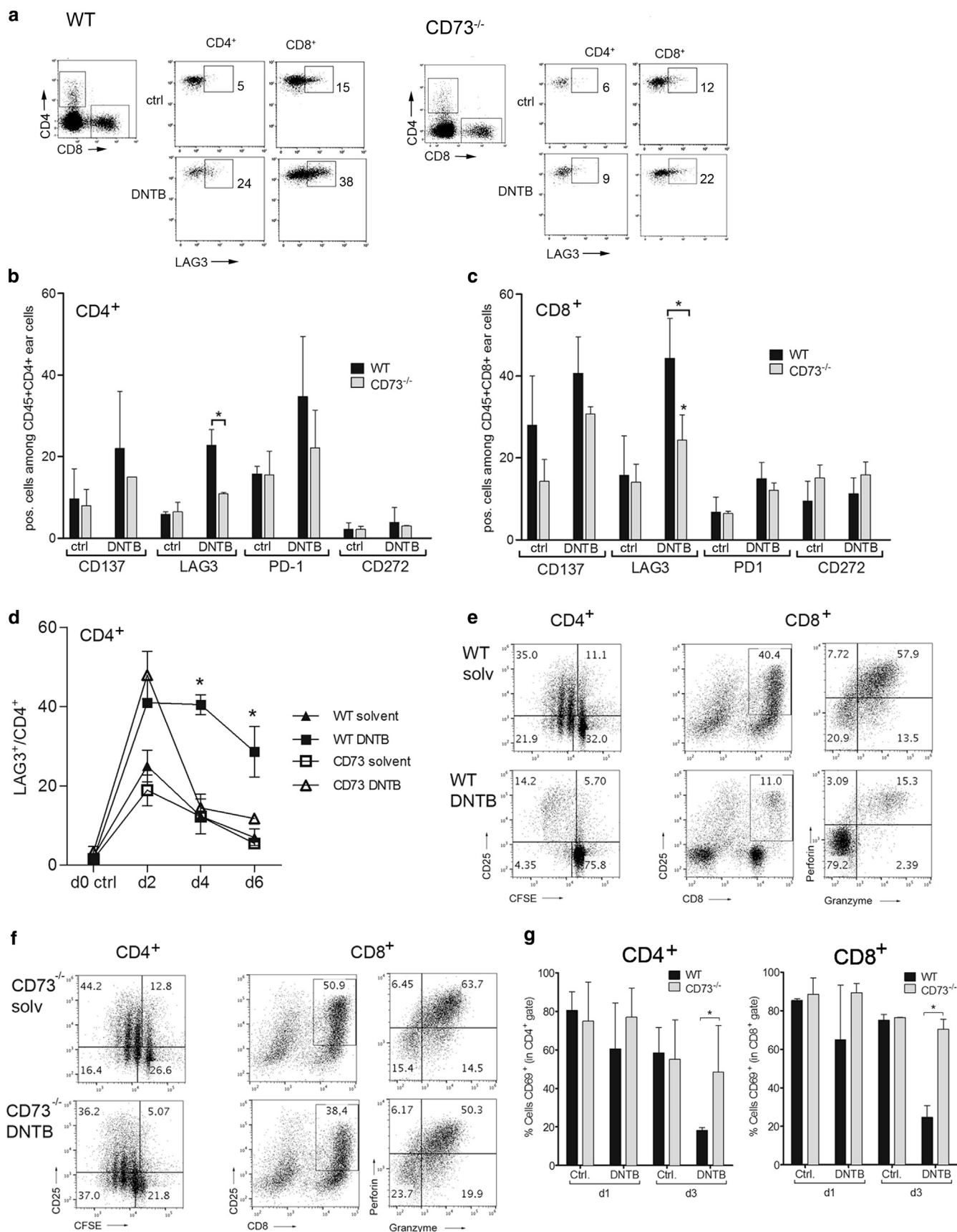


Figure 5. DNTB induces hyporeactive T cells in WT but not in CD73^{-/-} mice. (a–c) CD4⁺ and CD8⁺ skin T cells (gated on CD45⁺ cells) were analyzed (a) for expression of LAG3 and (b, c) for other surface markers 3 days after solvent (ctrl) or DNTB application. Means $\pm$ standard deviation of four experiments are shown. (d) Shows a time course study as in a. * $P < 0.05$, t test; $n = 3$. (d) CFSE-labeled T cells from solvent- or DNTB-treated WT animals were re-stimulated with

conditions. As expected, stimulation of T cells with anti-CD3/anti-CD28 induced stronger phosphorylation of Akt and S6 than anti-CD3 alone (Figure 3a). In the latter setting, addition of Ado was able to reduce phosphorylation of Akt and S6 kinases to control levels. Likewise, when using DCs to stimulate OTII T cells, CD73^{-/-} iDCs were potent inducers of Akt and S6 phosphorylation as opposed to WT iDCs (Figure 3b), and addition of Ado to the CD73^{-/-} iDC/OTII cocultures reduced Akt and S6 phosphorylation to levels obtained with WT iDCs. This indicates inferior activation of signaling pathways in T cells by Ado-producing iDCs.

Several anergy marker genes have been described recently (Oh et al., 2015), and PCR analysis showed up-regulation of *TNSFR9*, *EGR2*, *EGR3*, *SEMA7a*, and *Hif1α* in OTII T cells after co-culture with WT but not with CD73^{-/-} iDCs (Figure 3c). This gene expression was complemented by protein data obtained with EGR2 and NDRG1, which are essential for a hyporesponsive phenotype of T cells. Both proteins were up-regulated after stimulation of T cells with anti-CD3, that is, when signal 2 is missing (Figure 3d). A similar up-regulation was detected in T cells after co-culture with WT but not with CD73^{-/-} iDCs (Figure 3e). Addition of Ado re-established the up-regulation of EGR2 and NDRG1 in T cells in CD73^{-/-} iDC/OTII co-cultures. In conclusion, these data indicate that iDCs induce T-cell hyporesponsiveness by means of CD73-mediated Ado production.

CD73 is up-regulated in skin-migrating DCs after tolerance induction by DNTB

To investigate the effects of CD73 ablation on induction of tolerance in vivo, we used a contact hypersensitivity model in which pretreatment of mice with DNTB induces tolerance against the related hapten DNFB (Iijima and Katz, 1983). Although application of DNTB renders WT mice tolerant against DNFB, CD73^{-/-} animals showed no reduction of ear swelling after DNTB pretreatment (Figure 4a). Transfer of CD11c⁺ cells from DNTB-treated WT but not from CD73^{-/-} mice was able to transfer tolerance (Figure 4b), indicating a crucial role of CD73⁺ skin-migrating DC (smDCs) during tolerance induction. The subsequent analysis of different smDC subsets in draining lymph nodes (LNs) showed similar frequencies of all defined smDCs in the steady state in WT and CD73^{-/-} mice (see Supplementary Figure S1 online). Moreover, comparable migration patterns for DC subpopulations, shown as an example for CD207⁺CD11c⁺ major histocompatibility complex II^{high} cells, including Langerhans cells (LCs) and langerin⁺ dermal DCs, were recorded after application of DNTB (Figure 4c). However, the migration of LCs in response to DNTB is still a matter of debate. Long-standing reports showed no effect of DNTB on LC localization in the epidermis (Weinlich et al., 1998), whereas more recent transgenic approaches gave indications that LC migration is indeed induced by DNTB (Gomez de

Aguero et al., 2012). Nevertheless, although our results cannot finally discriminate between LC and CD207⁺ dermal DCs, we observed up-regulation of CD73 expression in CD207⁺ smDCs 3–5 days after DNTB application, whereas the hapten DNFB failed to induce CD73 expression (Figure 4d). Further dissection of CD207⁺ cells into CD103⁺ and CD103⁻ subsets did not show any difference in CD73 expression. Moreover, up-regulation of CD73 by epidermal CD207⁺ LCs was already recorded 6 hours after DNTB treatment (see Supplementary Figure S2 online), whereas CD11b⁺ and CD11b⁻ dermal DCs did not up-regulate expression of CD73 after migration into the LNs (see Supplementary Figure S3 online).

To support our conclusion that smDCs and not LN-resident DCs are responsible for increased expression of CD73 after DNTB application, we applied FITC together with DNTB to the skin, allowing direct analysis of smDCs by gating on FITC⁺ cells in FACS. Up to 40% of all FITC⁺CCR7⁺ smDCs were CD73⁺ (see Supplementary Figure S4 online), whereas resident DCs were not FITC⁺, nor did they express substantial amounts of CD73. Moreover, when comparing the mean fluorescence of CD73⁺ smDCs in LNs after DNTB or solvent treatment, only minor changes (mean fluorescence intensity: control = 1,560 vs. DNTB = 1,920) became apparent (see Supplementary Figure S4). To test for antigen presentation function, smDCs and resident DCs from LNs were sorted 3 days after DNTB painting and were co-cultured with DNFB-specific T cells. DNTB and DNFB share similar dinitrophenyl-protein antigenic motifs (Gomez de Aguero et al., 2012); therefore, T cells primed with DNFB can be reactivated when restimulated by DC-carrying dinitrophenyl-protein-derived antigens. In these experiments, only smDCs induced robust T-cell proliferation compared with resident DCs (see Supplementary Figure S4), indicating that, in particular, CD73⁺ smDCs engage with antigen-specific T cells in vivo. The expression of CD73 remained unaltered in CD4⁺ and CD8⁺ T cells (20%–30% CD73⁺) (see Supplementary Figure S5 online), with mean fluorescence levels comparable to those in DCs. Finally, we investigated the effects of CD73 expression by smDCs on the production of Ado in LNs. Here, increased amounts of Ado in the tissue culture supernatants of cultivated LNs of WT mice 3 days after treatment with DNTB were detected (Figure 4e), indicating increased degradation of extracellular ATP to Ado by LN cells. From these results, we conclude that DNTB treatment leads to increased extracellular levels of Ado in LNs, which is driven by elevated CD73 expression in CD207⁺ smDCs.

DNTB up-regulates LAG3 on T cells and renders them refractory to stimulation with DNFB

When analyzing the T cells in draining LNs after DNTB treatment by FACS, we found unaltered frequencies and activation of CD4⁺ and CD8⁺ T cells in WT and CD73^{-/-}

DNBS-pulsed BMDCs (at day 3). Proliferation and surface marker expression were analyzed in CD4⁺ or CD8⁺ cells. A typical experiment is shown; numbers indicate percentage of cells in quadrant. (e) As in d, except CD73^{-/-} animals were used. (f) Mice were treated as in d and e and re-stimulated at day 1 or day 3 after DNTB application. Expression of CD69 by CD4⁺ or CD8⁺ cells is shown. Means ± standard error of the mean are shown. **P* < 0.05, *t* test; *n* = 3. BMDC, bone marrow-derived dendritic cell; CFSE, carboxyfluorescein succinimidyl ester; ctrl, control; d, day; DNFB, 2,4-dinitrofluorobenzene; DNTB, 2,4-dinitrothiocyanobenzene; pos., positive; solv, solvent; WT, wild type.

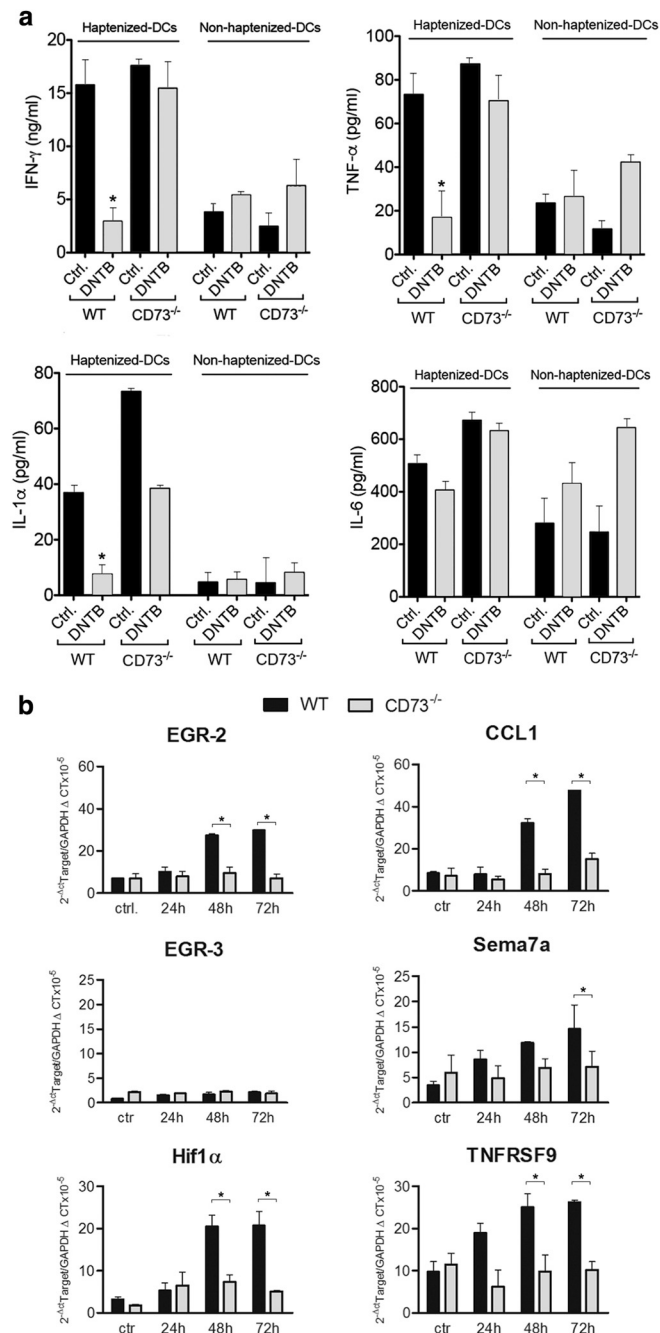


Figure 6. DNTB suppresses cytokine release in re-stimulated T cells and up-regulates anergic markers. (a) WT or CD73^{-/-} animals were treated with DNTB or solvent (ctrl); 6 days later, draining LNs were removed, and isolated T cells were re-stimulated with DNBS-pulsed or nonpulsed DCs. Cytokine production was analyzed 96 hours later. Means $\pm$ standard deviation are shown. * $P < 0.05$ indicates a significant difference between DNTB and control, t test; $n = 3$. A typical experiment is shown. (b) WT or CD73^{-/-} animals were treated with DNTB. Draining LNs were harvested at indicated time points, and mRNA from isolated T cells was prepared and subjected to real-time quantitative reverse transcriptase-PCR. Means $\pm$ standard deviation are shown. * $P < 0.05$, t test; $n = 3$. ctrl, control; DC, dendritic cell; DNFB, 2,4-dinitrofluorobenzene; DNTB, 2,4-dinitrothiocyanobenzene; h, hour; LN, lymph node; WT, wild type.

mice, regardless of DNTB application (see [Supplementary Figure S6](#) online). However, CD4⁺ and CD8⁺ T cells isolated from the skin of DNTB-treated WT mice displayed

substantial up-regulation of LAG3 ([Figure 5a–c](#)). LAG3⁺ T cells were present in WT mice over a prolonged period of time (6 days), whereas in CD73^{-/-} mice, the number of LAG3⁺ skin T cells had already dropped to control levels already on day 4 ([Figure 5d](#)).

To assess the function of the T cells after DNTB painting, LN T cells were isolated, stained with carboxyfluorescein succinimidyl ester and co-cultured for 3 days with DNBS-pulsed BMDCs. Thereafter, T-cell proliferation and expression of activation markers were analyzed. [Figure 5e](#) shows that CD4⁺ and CD8⁺ T cells from DNTB-treated WT mice proliferated significantly less and expressed lower levels of the activation marker CD25 compared with T cells from the solvent control group. Moreover, diminished production of granzyme B and perforin by CD8⁺ T cells suggested impaired effector functions. In contrast, T cells from CD73^{-/-} mice displayed similar activation levels, effector functions, and proliferative capacities after DNTB application compared with solvent controls ([Figure 5e](#)). The development of hyporeactive T cells in WT mice required at least 3 days ([Figure 5f](#)), because T cells isolated from WT mice 1 day after DNTB treatment showed activation comparable to controls, indicating that anergic instruction has not yet been fully accomplished. The appearance of T-cell hyporeactivity after 2–3 days coincided with the expression of CD73 by CD207⁺ smDCs in draining LNs that peaked also around 2–3 days after application of DNTB ([Figure 4d](#)).

We further analyzed the production of immune stimulatory cytokines such as IFN- γ , tumor necrosis factor- α , IL-1 α , and IL-6 in DC/T-cell cultures ([Figure 6a](#)). All of these cytokines, except for IL-6, were reduced in cultures of T cells derived from DNTB-treated WT animals. In contrast, T cells derived from CD73^{-/-} mice showed a robust secretion of cytokines, irrespective of treatment with DNTB or solvent.

Finally, T cells isolated from DNTB-treated animals were tested for markers indicative for anergy. Similar to the results obtained with OTII cells in vitro, elevated levels of *ERG2*, *CCL1*, *Hif1 α* , *Sema7a*, and *TNFRSF9* were measured 48–72 hours after DNTB application in WT animals ([Figure 6b](#)). In contrast, CD73^{-/-} animals did not up-regulate these genes upon DNTB treatment. In summary, we conclude that tolerization by DNTB up-regulates expression of CD73 on DCs, which leads to conversion of ATP to Ado, rendering T cells refractory to subsequent activation by DNFB.

DISCUSSION

At least five different subsets of skin DCs are involved in shaping contact hypersensitivity reactions ([Henri et al., 2010](#)). Among them are immunizing and tolerizing subsets ([Idoyaga et al., 2013](#); [Igyarto et al., 2009](#); [Kaplan et al., 2012](#)). In a DNTB-driven tolerance model, we detected up-regulation of the ectoenzyme CD73 in CD207⁺ smDCs that was accompanied by elevated levels of extracellular Ado in the draining LNs. When smDCs were unable to express CD73, the tolerogenic effects of DNTB ceased. Therefore, we concluded that the production of Ado by CD73 provides a crucial mechanism for the induction of tolerance.

In T cells, lack of co-stimulation during TCR engagement leads to anergy, rendering them unresponsive to recurring TCR stimuli ([Appleman and Boussiotis, 2003](#); [Schietinger and](#)

Greenberg, 2014). Immature DCs inherently express T-cell co-stimulatory molecules only weakly; thus, they are prone to inducing anergic T cells (Pletinckx et al., 2015; Vendelova et al., 2018). However, our data indicate that weak co-stimulation of T cells by iDCs alone is not sufficient to mediate T-cell hyporeactivity, because production of extracellular Ado by CD73 was mandatory.

Despite the fact that many cell types express CD73 and may therefore contribute to the anergic instruction of T cells, our data indicate that Ado derived from smDCs is crucial. For instance, the half-life of Ado is short (5–10 seconds) (Moser et al., 1989), and long-distance diffusion is unlikely. Therefore, Ado produced in skin will not reach the draining LNs. Moreover, Ado has to be present during the cognate DC/T-cell interaction (Zarek et al., 2008). This is achieved best by local production of Ado by CD73⁺ DCs during antigen presentation to T cells. Finally, antigen-loaded CD73^{-/-} DCs failed to render OTII hyporeactive even in WT animals, indicating that bystander CD73⁺ cells are not sufficient to overcome the CD73 deficiency of DCs.

The suppressive effects of Ado on T cells are well documented (Kepp et al., 2017), because its binding to adenosine receptor prevents functional maturation of CD8⁺ T cells and blocks secretion of type 1 cytokines by CD4⁺ T cells (Linnemann et al., 2009; Romio et al., 2011; Zarek et al., 2008). In line with these results, our data indicate that CD73^{-/-} iDCs, which are defective in producing extracellular Ado, stimulate T cells better than Ado-producing WT iDCs. Thus, signaling by Ado, locally generated by CD73-expressing iDCs during T-cell priming, may act as brake for T-cell activation and is mandatory for the induction of anergic T cells.

During DNTB-induced tolerization, induction of Tregs may be one mechanism of contact hypersensitivity suppression (Riemann et al., 2005). Our data support this concept, because T-cell anergy precedes T-cell conversion into bona fide Tregs (Pletinckx et al., 2015). Moreover, the transcription factor EGR2 that we found up-regulated in anergic T cells induces expression of LAG3, which is a characteristic molecule for Tregs (Nakachi et al., 2017; Safford et al., 2005). Thus, anergic T cells induced as early as 6 days after DNTB treatment may serve as precursors for Tregs later on.

Ado prevents the phosphorylation of Akt in T cells (Linnemann et al., 2009). In support of this, we recorded that Akt phosphorylation was augmented in CD73^{-/-} iDC/T-cell cocultures. Here, CD73^{-/-} iDCs stimulated Akt- and S6 kinase phosphorylation as well as WT mDCs. Therefore, the lower production of suppressive Ado by CD73^{-/-} iDCs may compensate for the weak expression of costimulatory molecules by iDCs. Moreover, iDCs that express only low amounts of co-stimulating molecules anyway can further dampen activating signals in T cells by locally producing Ado. As a consequence, activation of T cells is avoided, and anergic T cells are induced. mDCs, on the other hand, are unable to induce anergic T cells, because strong T-cell coactivating signals provided by CD80, CD86, and cytokines may overrule suppressive Ado signaling.

Additionally, Ado-producing iDCs triggered the expression of EGR2 and NDRG1, which are prototypic markers for anergic T cells (Nakachi et al., 2017; Oh et al., 2015; Safford et al., 2005). The transcription factor EGR2 up-regulates suppressive molecules such as LAG3 and Tim3 during

tumor development in T cells (Okamura et al., 2009; Williams et al., 2017). Among these candidate molecules affected by EGR2, we detected only up-regulation of LAG3 in WT iDC/T-cell cocultures. This may indicate a differential activation of defined EGR2-dependent pathways or a distinct programming of T cells during the induction of anergy compared with T cells in a tumor environment.

Although there is no evidence of direct regulation of EGR2 by Ado in leukocytes so far, in neurons and endothelial cells cyclic AMP (cAMP) can activate EGR1, a sister molecule of EGR2 (James et al., 2004; Simo-Cheyrou et al., 2017). Because the adenosine receptors A_{2a} and A_{2b} induce cAMP in leukocytes, activation of EGR2 via engagement of adenosine receptors is feasible. A second pathway stimulated by Ado involves the guanosine triphosphate-binding protein Rap1. Rap1 can be activated by binding of Ado to A_{2a} adenosine receptor (Almahariq et al., 2015; Ring et al., 2015) and has been shown to induce an anergic state in Jurkat T cells (Boussiotis et al., 1997). Therefore, Rap1 and cAMP are both possible mediators of Ado-induced induction of T-cell anergy.

In summary, our data indicate that skin tolerogens such as DNTB mediate the up-regulation of CD73 on CD207⁺ smDC subsets. This, in turn, leads to rising levels of Ado in skin draining LNs, which renders T cells anergic. As a consequence, tolerance against the allergen DNFB ensues. Thus, the regulated surface expression of CD73 by defined subsets of DCs and the ensuing production of Ado provide a thus far unknown mechanism by which DCs can promote tolerance and may prevent autoimmunity.

MATERIALS AND METHODS

For a comprehensive description, see the [Supplementary Materials and Methods](#) online.

Preparation of T cells

Ear halves were incubated in phosphate buffered saline, 0.5% Trypsin, 5 mmol/L EDTA at 37 °C for 30 minutes and then filtered. A total of 2×10^6 cells/500 μ l were stained with antibodies as indicated. Samples were analyzed by FACS. For LN T cells, LNs were ground through a cell strainer, resuspended in MACS buffer (phosphate buffered saline, 1% fetal calf serum, 1 mmol/L EDTA) and isolated by magnetic beads.

BMDCs and co-cultures with T cells

BMDCs were prepared using granulocyte-macrophage colony-stimulating factor and IL-4 (Mahnke et al., 2000) in a 5-day culture protocol. Developing DCs were then recultured for 24 hours in a new culture dish without lipopolysaccharide, yielding iDCs, whereas mDCs were obtained by maturation with lipopolysaccharide (1 ng/ml).

In cocultures, 5×10^4 CD4⁺CD25⁻OTII cells and 1.5×10^4 iDCs or mDCs in the presence of 100 ng/ml Ova peptide, were cultured for 7 days in 96-well plates (Pletinckx et al., 2015). T cells were separated from DCs by depletion of CD11c⁺ cells or by FACS sorting. Next, 1×10^5 OTII cells/200 μ l/well were cultured in 96-well plates and stimulated with 1 μ g/ml anti-CD3 and 1 μ g/ml anti-CD28. Proliferation was determined by pulsing with ³H-thymidine, and IL-2 content was determined by ELISA.

Real-time PCR

RNA was extracted from LNs and OTII cells by RNeasy (Qiagen, Hilden, Germany) columns. cDNA synthesis was performed with

cDNA synthesis kits. Quantitative real-time reverse transcriptase–PCR analysis was performed with a LightCycler (Roche, Mannheim, Germany), using SYBR green master mix, and quantification was made in comparison to GAPDH (Livak and Schmittgen, 2001). Primers were from Eurofins (Ebersberg, Germany) according to sequences from Williams et al. (2017).

Contact hypersensitivity and tolerance induction

We used 25 μ l 0.5% DNFB sensitization. Challenge was with 5 μ l of 0.20% DNFB/side to one ear, and swelling was measured after 24 hours. Tolerance was induced by application of 100 μ l 1% DNTB to shaved abdomen. At 7 days thereafter, sensitization and challenge were performed with DNFB (Riemann et al., 2005). For transfer experiments, LNs were taken from DNTB-treated mice. CD11c⁺ cells were isolated by MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) beads or sorting. We subcutaneously injected 1×10^6 cells/mouse. Animal protocols were approved by the state of Baden Württemberg (#G73/15) and experiments were carried out according to the guidelines for animal welfare.

Quantifying Ado, Akt, and S6 phosphorylation

Ado in tissues and cell culture supernatants was determined by a commercially available kit. Phosphorylation of Akt and S6 kinases was determined using ELISA kits.

Flow cytometry of smDCs

smDC were isolated and stained according to established protocols (Neuberger et al., 2017). Briefly, LNs were enzymatically digested, and cells were strained (70- μ m sieve). For staining, cells were fixed and permeabilized using commercially available kits. For granzyme B detection, the cells were fixed and permeabilized with the anti-mouse Foxp3 staining kit. Samples were analyzed by FACS.

Restimulation of LN T cells by DNBS-pulsed BMDCs

CD3⁺ cells were purified by magnet beads. A total of 1×10^5 CD3⁺ cells were co-cultured in 96-well plates with 1×10^4 BMDCs and pulsed with DNBS. Analysis of CD4⁺ and CD8⁺ T-cell activation, proliferation, Treg frequencies, granzyme B production, and cytokine secretion were performed at day 3 of co-culture. For assessment of proliferation, CD3⁺ cells were labeled with carboxyfluorescein succinimidyl ester before the co-culture. Cytokine secretion was measured using the LEGENDplex kit (BioLegend, San Diego, CA).

Statistical analysis

The data were analyzed using GraphPad Prism software (GraphPad Software, La Jolla, CA).

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CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) grant CRC156 to CSV, HCP, FM, HJS, AE, and KM.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org and at <https://doi.org/10.1016/j.jid.2018.10.016>.

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Supplemental Methods

Mice.

C57BL/6 and OTII mice were purchased from Charles River (Sulzfeld, Germany). CD73 deficient (CD73^{-/-}) mice were a gift from J. Schrader, Düsseldorf. All mice were housed at the central animal facility of the University of Heidelberg cared for in accordance with the institutional guidelines for animal welfare.

Reagents.

Cell culture medium RPMI 1640 (with glutamine) and MEM and supplements (Pen/Strep, HEPES, glutamine) were purchased from Lonza (Biozym, Hessisch Oldendorf, Germany). FCS was from Hyclone (VWR, Bruchsal, Germany). Complete medium (CM) was RPMI supplemented with 10% FCS (v/v), 1% Pen/Strep, PBS and HBSS were purchased from Biochrom (Berlin, Germany). Standard chemicals were purchased from Carl Roth (Karlsruhe, Germany). Adenosine (Ado; 2-Chloroadenosine) and PSB12379 were from Tocris (Biotechnie, Wiesbaden, Germany). EDTA, Bovine Serumalbumin (BSA), 1-Fluoro-2,4-dinitrobenzene (DNFB) and 2,4-Dinitrobenzene sulfonic acid (DNBS) were from Sigma-Aldrich (Schnelldorf, Germany). 2,4-Dinitrothiocyanobenzene (DNTB) was from Alfar Aesar (formerly Lancaster Synthesis; ThermoFisher, Karlsruhe, Germany). The Ova peptide (Aminoacid 323-339) was from Genaxxon, Ulm, Germany).

Preparation of T cells.

For FACS analysis of skin T cells, ears of respective mice were removed, washed in ethanol and split into two halves with forceps. Ear halves were then incubated in PBS, 0.5% Trypsin (ThermoFisher), 5 mM EDTA (Applichem, Darmstadt, Germany) at 37°C for 30 min. Thereafter an equal volume of PBS, 0.1% DNase I (wt/vol)(Sigma), 20% FCS (vol/vol) was added for 10 min. Remaining tissue is then cut by scissors, put through a strainer (Corning, Wiesbaden, Germany) and washed 3 times with FACS buffer. Aliquots of 2x10⁶ cells/500µL were then stained with the following antibodies: anti-CD45 FITC, 1:200; anti-CD4 BV500, 1:500; anti-CD8 AF700 1:200; anti-CD69 PE-Dazzle594, 1:150; anti-CD272 APC, 1:200; anti-LAG3 BV450 1:200; anti-CD137 PE, 1:100; anti-PD-1 PE-Cy7, 1:200 (all from Biolegend), 20 min, 4°C in presence of TruStain fcX™. After 2 washes samples were analyzed by a Gallios flow cytometer

(Beckman Coulter, Krefeld, Germany) and data were analyzed with FlowJo software v10.2 (TreeStar).

For LN T cells LN of mice were removed and grinded through a 40 µm Falcon cell strainer (Corning). After 2 washes with MEM cells were resuspended in MACS buffer (PBS, 1%FCS (vol/vol), 1 mM EDTA) to a density of 1×10^8 /ml and CD4⁺ T cells were isolated by magnetic bead sorting (Mojo, Biolegend, Fell, Germany) according to manufacturer's instructions. Briefly, per 10^7 cells 10 µl of Biotin-Antibody cocktail: Biotin anti- CD8α, CD11b, CD11c, CD19, CD24, CD25, CD44, CD45R/B220, CD49b, CD105, TER-119/Erythroid, TCR-γ/δ was added, mixed and incubated for 15 min on ice. Thereafter 10 µl per 10^7 cells Streptavidin Nanobeads were added. After 15 min incubation 2.5 ml Buffer was added and tubes were placed into an appropriate magnetic field. After 5 min isolated cells were poured of the tube and collected. After 2 washings with MACS buffer cells were counted and aliquots were stained for CD4 and analyzed for purity by flow cytometry. In average 90% to 95% of all cells were CD4⁺.

BMDC and cocultures with T cells.

For preparation of BMDC, bone marrow cells were flushed out of the tibias of mice with a syringe and MEM. Bone marrow cells were put through a sieve (70 µm; Corning), centrifuged (360g, 4°C, 7 min) and red blood cell were lysed by 3 min incubation with ACK buffer (ThermoFisher). After 3 washes with MEM cells were resuspended (1.2×10^6 cells/ml) in CM supplemented with 10 µg/ml GM-CSF and 10 µg/ml IL-4 (Miltenyi, Bergisch Gladbach, Germany). Cells were cultivated (37°C, 5% CO₂) in 24 well dishes (Falcon) at 1 ml/well for 5 d. Intermitted feeding was at d 2 and d 4. At d 5 iDC were collected. For maturation iDC were recultivated for 24 h in CM medium supplemented with 1 ng/ml LPS (Sigma, Schnelldorf, Germany). For FACS analysis aliquots of 1×10^5 BMDC were stained with a cocktail of anti-MHCII FITC, 1:200; anti-CD11c APC, 1:100; anti-CD86 PECy5, 1:200; anti-CD73 BV421, 1:200; anti-CD39 PE, 1:100; together with TruStain fcX™ (all from Biolegend) and analyzed by a Gallios flow cytometer (Beckman Coulter).

Cocultures were prepared according to standard protocols. Briefly, 5×10^4 CD4⁺CD25⁻OTII cells were cultivated (37°C, 5% CO₂) together with 1.5×10^4 iDC or mDC in presence of 100 ng/ml Ova peptide (Genaxxon, Ulm, Germany) for 7 d in 96 round bottom well plates (Corning). To some cultures Ado in doses as indicated was added. Cells were harvested and purified from DC by magneto-depletion of CD11c⁺ cells (Biolegend) or by FACS sorting (FACS-Melody; BD,

Heidelberg, Germany) using CD11c-PE as marker for depletion. Duplicates of 1×10^5 OTII cells/200 μ l/well were cultured in 96 well plates (Corning) and stimulated with 0.5 μ g/ml anti-CD3 (clone 145-2C11) and 0.5 μ g/ml anti-CD28 (clone 37.51) (Biolegend). After 3 d wells were pulsed with ^{3} H-Thymidine (Hartmann Analytics, Braunschweig, Germany) over night and harvested thereafter. From some wells tissue culture supernatant was removed before pulsing with ^{3} H-Thymidine to analyze IL-2 content by ELISA (Ebioscience; Frankfurt, Germany).

Injection of OTII cells and footpad swelling

CD4⁺ OTII T cells were prepared by MACS beads (Miltenyi, Germany) from cells suspensions of LN and spleens of OTII mice. 5×10^6 T cells in 200 μ l PBS/mouse were injected into the tail veins of recipient mice. After 24h 1×10^6 iDC or mDC per mouse as indicated (preparation see above) were injected intraperitoneally. After 7 d footpads were measured using a caliper (Kroeplin, Schlüchtern, Germany) and 100ng Ova (peptide synthesis; Genaxxon, Germany) in 50 μ l PBS were injected into the left hind footpad. PBS injection served as control. 24h later, footpad thickness was measured with a caliper and lymph node cells were analyzed in FACS for donor OTII T cells (TCR V α 2, clone B20.1; V β 5, clone MR9.4) and for Ki67 (clone SolA15) and CD69 (clone H1.2F3) expression (all from Biolegend) as indicated.

Real time PCR.

OTII cells were isolated after coculture with DC as described. LN T cells were isolated from skin draining LN 24 h, 48 h or 72 h after DNTB painting. Briefly, single cell suspensions of LN cells were prepared by grinding LN through a sieve (corning) and CD4⁺ T cells were prepared by Mojosort magneto beads (Biolegend) as described for OTII cells. Aliquots of 1×10^6 cells were subjected to RNA extraction using RNeasy columns (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Quality and quantity of RNA were determined by measuring the absorbance at 260 nm and 280 nm using a Denovix Micro-Spectrophotometer (Biozyme). cDNA synthesis was performed with first strand cDNA synthesis kit from ThermoFisher (Karlsruhe, Germany) in a standard PCR cycler (^{3} Prime, Techne, Germany) according to the manufacturer's instructions. Samples were tested for integrity with standard GAPDH primers and stored at -20 °C. RT-qPCR analysis was performed with a Roche LightCycler, using SYBR green master mix (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. To verify the specificity of each primer, a melting-curve analysis was performed. Further controls

were firstly without reverse transcriptase (one for each RNA) and secondly no DNA template for each primer pair. All RT-qPCRs were run in duplicate and the RNA was quantified by $2^{-\Delta\Delta CT}$ method in comparison to GPDH. All primers were purchased from Eurofins (Ebersberg, Germany) and sequences were as follows: **Lag3**: 1. tgctttgggaagctccagt, 2. gctgcaggaagatggac; **Tnfrsf9**: 1. ccggtcttaagcacagacct, 2. gaacgggtactggcgtctgtc; **Egr2**: 1. ctacccgggtggaagacctc 2. aatgttgatcatgccatctcc; **Sema7a**: 1. tcaatcggctgcaagatgt, 2. cgcagacagctgagtagttcc; **Hif1a**: 1. gctgctcactgtgaaggaagt, 2. tggggaatgcattttaccat; **Egr3**: 1. caatctgtaccccgaggaga, 2. ccgatgtccatcacattctct; **Ccl1**: 1. tcaccatgaaacccactgc, 2. agcagcagctattggagacc. **Gapdh**: 1. tgcaccaccaactgcttagc 2. ggcatggactgtggcatgag.

Contact hypersensitivity and tolerance induction.

Mice were epicutaneously sensitized by applying 25 μ l of 0.5% DNFB to the shaved abdomen. The vehicle consisted of acetone/olive oil (Aoo; 4:1, v/v). Challenge was carried out after 5 d by topical application of 5 μ l of 0.10 % DNFB to one ear. Control ears were treated with solvent. Ear swelling was measured by a caliper (Oditest, Kroeplin, Germany) after 24 h and is displayed as mean difference of thickness of challenged – thickness solvent treated ears. For induction or tolerance 100 μ l 1% DNTB in Aoo was applied to shaved abdomen. After 7 d sensitization and challenge was performed with DNFB.

For transfer experiments mice were treated with DNTB and 48 h later draining LN were taken and cut into small pieces followed by a digestion for 1 h at 37°C in DMEM medium supplemented with 10% of FCS in the presence of 400 U/ml of Collagenase IV (Cell systems, Troisdorf, Germany) and 50 U/ml of DNase I (Sigma). The digestion was stopped with 10 μ M of EDTA (AppliChem) for 10 min at 37°C. Cells were washed 3 times with MACS buffer (PBS, 1% FCS (vol/vol), 1 mM EDTA) and adjusted to a density of 1×10^8 cell/400 μ l. 100 μ l anti-CD11c magneto beads (Miltenyi) and TruStain fcX™ (anti-mouse CD16/32) antibodies (Biolegend) diluted 1:100 were added, incubated at 4°C and cells were washed thereafter. For positive selection LS columns (Miltenyi) were used according to the manufacturer's instructions. After 3 washings cells were resuspended in PBS and 1×10^6 cells/mouse were s.c. injected.

Western blotting.

Cells were isolated as indicated and washed thoroughly with PBS. Aliquots of 5×10^6 cells were lysed in PBS containing 1% Triton X-100 (Sigma) and protease inhibitor cocktail (Roche,

Mannheim, Germany) on ice for 30 min with intermittent shaking. Samples were spun down (13.000 rpm, 4°C, 10 min) and supernatants were stored at -20°C. Protein content was determined by BCA-test (Pierce). 1 µg - 10 µg total protein (depending on the total amount of protein) was loaded on 7.5% standard Polyacrylamide gels (Roth) and transferred onto PVDF membranes (Merk-Millipore, Darmstadt, Germany) by semi-dry blotting (VWR, Darmstadt, Germany). Membranes were blocked by either 5% non-fat dry milk powder in TBS or by 5% BSA/TBS, according to the antibodies later used for staining. Anti-NDGR1 antibody (Cell signaling, Frankfurt, Germany) was used at 1:500, anti-EGR2 antibody (Biolegend) was used at 1:800 and anti-β-Actin (Santa Cruz Biotech, Heidelberg, Germany) was used at 1:200 for overnight incubation at 4°C. After 3 washes with TBS, blots were incubated with mouse- or rabbit-specific HRP-labeled secondary reagents (Dianova, Hamburg, Germany) for 4 h at a 1:3000 dilution. Thereafter blots were washed 3 times, overlayed with ECL reagent (GE-Healthcare, Freiburg, Germany) and analyzed (Chemidoc, Biorad, München, Germany).

Quantifying Ado, Akt and S6 phosphorylation.

For quantification of Ado, cells or whole LN as indicated were cultivated in DMEM without phenol red (ThermoFisher, Darmstadt, Germany) supplemented with 50 µM ATP (Tocris) for 3 h to 4 h at 37°C, 5% CO₂. Tissue culture supernatants were harvested and Ado content was determined by a commercially available kit (Biocat, Heidelberg, Germany) according to the manufacturers protocol. Briefly, tissue culture supernatants were subjected to adenosine deaminase degradation followed by a multistep enzymatic reaction, leading to formation of a fluorescent product, measured at Ex535/Em587 nm in a fluorometer (Biozyme). Phosphorylation of Akt and S6 kinases was determined using instantOne ELISA (Ebioscience) kits according to the manufacturer's manual. Triplicates of samples were prepared and analyzed by an ELISA reader (ThermoFisher). Positive controls and negative samples were provided by the kit.

Flow cytometry of smDC.

For isolation of smDC, the draining LN (dLN) were taken and cut into small pieces followed by a digestion for 80 min at 37°C and 900 rpm in DMEM medium (BioWhittaker™ Lonza) supplemented with 10% of FCS in the presence of 800 U/ml of Collagenase IV (Worthington) and 50 U/ml of DNase I (Sigma-Aldrich). The digestion was stopped by incubating with 10 µM

of EDTA (AppliChem) for 10 min at 37°C and 900 rpm. The cells were passed through a 70 µm cell strainer, washed and stained as described before.

Surface staining: performed for 25 min at 4°C using Hanks' Salt Solution (Biochrom) supplemented with 0.5% BSA and 2 mM EDTA (AppliChem). Fc receptors were blocked by pre-incubating the cells with TruStain fcX™ (anti-mouse CD16/32) antibody (Biolegend) diluted 1/100 prior to staining. mAbs were purchased from BioLegend, unless otherwise indicated. Alexa Fluor 488-conjugated mAbs: anti-CD86 (GL-1). PE-conjugated mAbs: anti-CD73 (TY/11.8), anti-CD80 (Michel-19). PE/Dazzle 594-conjugated mAbs: anti-CD3 (17A2), anti-CD19 (6D5), anti-CD69 (H1.2F3). PE/Cy7 conjugated mAbs: anti-CD39 (24DMS1, Thermo Fisher Scientific). APC/R700 conjugated mAbs: anti-CD11b (M1/70, BD Biosciences). APC/eFluor 780-conjugated mAb: anti-CD11c (N418, ThermoFisher). APC/Fire 750-conjugated mAb: anti-CD25 (PC61). Brilliant Violet 421- conjugated mAb: anti-CD103 (2E7). eFluor 450- conjugated mAb: anti-CD8 (53-6.7, ThermoFisher). Brilliant Violet 510- conjugated mAb: anti-I-A/I-E (M5/114.15.2), anti-CD4 (RM4-5).

Intracellular staining: the cells were fixed and permeabilized using IC Fixation buffer and Permeabilization buffer (ThermoFisher). CD207 staining was performed for 35 min at RT using anti-CD207-Alexa Fluor 647-conjugated mAb (929F3.01, dendritics, Lyon, France) in the presence of TruStain fcX™ (anti-mouse CD16/32) antibody. For Granzyme B detection, the cells were fixed and permeabilized using the ThermoFisher anti-mouse Foxp3 staining set according to the manufacturer's instructions. Anti-granzyme B-Alexa Fluor 647-conjugated mAb (GB11, Biolegend) was used.

Nuclear staining of Foxp3 and Ki67 was performed using the Thermo Fisher Scientific anti-mouse Foxp3 staining set according to the manufacturer's instructions. Anti-Foxp3-APC (FJK-16s, ThermoFisher) and anti-Ki67-PerCP-eFluor 710 (SolA15, ThermoFisher) antibodies were used. Samples were measured in Gallios flow cytometer (Beckman Coulter) and data were analyzed with FlowJo software v10.3 (TreeStar).

Restimulation of LN T cells by DNBS-pulsed BMDC.

Single cell suspensions from dLNs of solvent control or treated-mice were obtained by gently mashing and passing through a 40 µm cell strainer. For co-culture of CD3⁺ T cells with DNBS-pulsed BMDC, total CD3⁺ cells were negatively selected using the EasySep™ Mouse T cell Isolation kit (StemCell Technologies) according to the manufacturer's instructions. 1×10^5 CD3⁺

cells were co-cultured in round bottom 96-well plates together with 1×10^4 day 8 BMDC pulsed with DNBS (1 mM, 30 min, 37°C). Analysis of CD4⁺ and CD8⁺ T cells activation, proliferation, Treg frequencies, granzyme B production and cytokines secretion were performed at d3 of co-culture. For assessment of proliferation, CD3⁺ cells were CFSE-labeled prior to the co-culture using 2 μ M CFSE (Thermo Fisher Scientific) diluted in PBS.

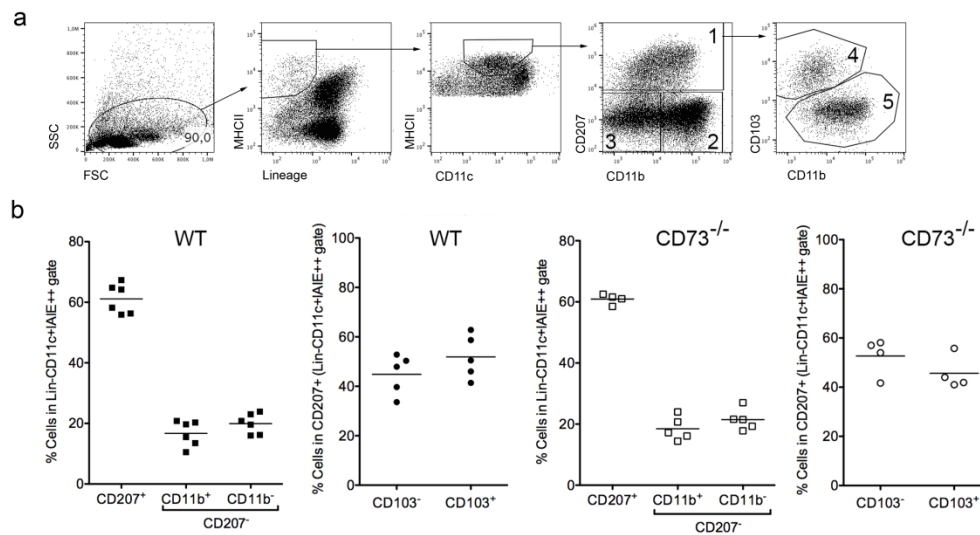
For measurement of granzyme B production after the 3 days of co-culture, the cells were re-stimulated for 5 hours with 0.5 μ g/ml anti-CD3 (145-2C11, Biolegend) plus 0.5 μ g/ml anti-CD28 antibodies (37.51, Biolegend) in the presence of Brefeldin A (ThermoFisher). The staining for FACS analysis was performed as previously described.

Cytokine secretion was measured after 3d of co-culture. Supernatants were collected, centrifuged at 10,000 $\times$ g for 10 min and transferred to new tubes. Samples were stored at -20°C until measurement using the LEGENDplex™ Mouse Inflammation Panel (13-plex, Biolegend).

Statistical analysis

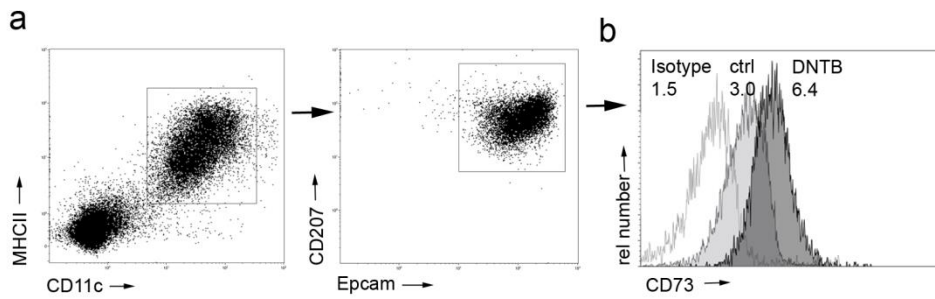
The data were analyzed utilizing Student t-test or One-way Anova using Graph Pad Prism (Witzenhausen, Germany) software.

Figure S1



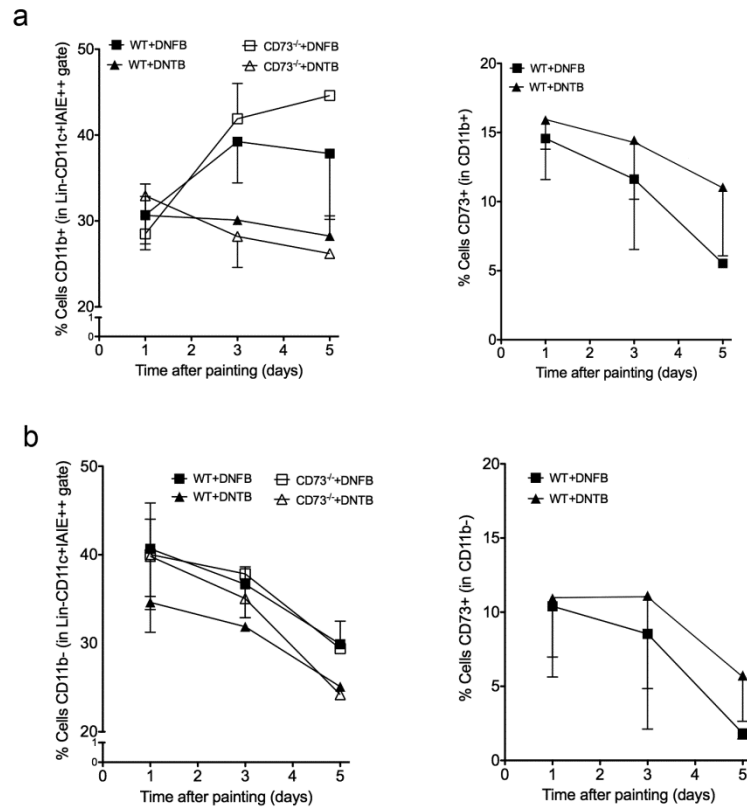
Comparable frequencies of DC subsets in WT and CD73^{-/-} mice. **a**, gating strategy to distinguish skin migrating (sm) DC subsets. Numbers in quadrants indicate DC subsets: 1=CD207⁺; 2=CD207⁻CD11b⁺, referred to as CD11b⁺; 3=CD207⁻CD11b⁻, referred to as CD11b⁻; 4=CD207⁻CD103⁺; 5=CD207⁺CD103⁺. **b**, the relative distribution of smDC subsets in inguinal LN in WT and CD73^{-/-} mice is shown. Dots indicate individual experiments, bars represent means.

Figure S2



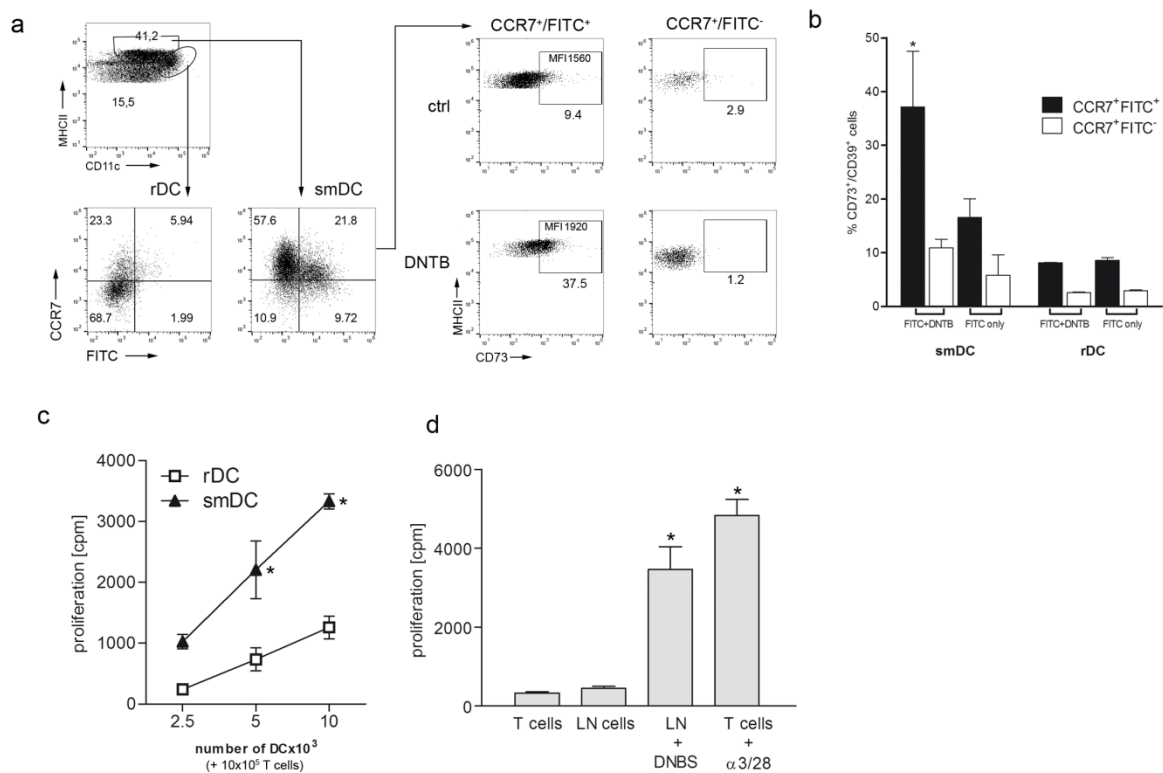
LC upregulate CD73 in skin 6h after DNTB. **a**, Epidermal cells were prepared using trypsin. Cells were stained for viability, CD45 and for markers shown. For analysis in FACS, cells were pre-gated for viable, CD45⁺ cells (not shown) and further analyzed as indicated. **b**, shows the expression of CD73 6h after solvent treatment (light grey histogram) and after DNTB treatment (dark grey histogram). Isotype of DNTB treatment is shown in white. Numbers indicate mean fluorescence intensities ($\times 10^3$). Shown is a typical experiment out of 4.

Figure S3



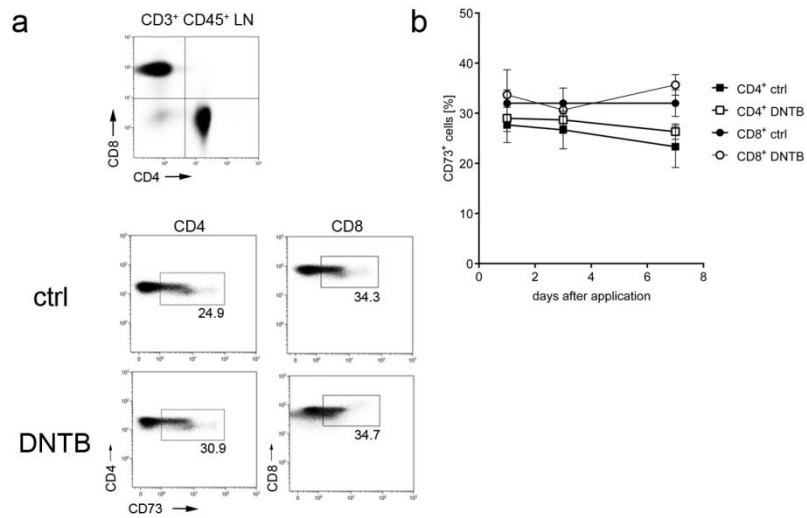
No upregulation of CD73 expression after DNTB treatment by CD11b⁺ and CD11b⁻ smDC. **a**, CD11b⁺ smDC were analyzed for migration and for expression of CD73 1d - 5d after DNTB or DNFB treatment. Shown are means $\pm$ SEM, n=4. **b**, as in a, except CD11b⁻ smDC were analyzed. Shown are means $\pm$ SEM, n=4.

Figure S4



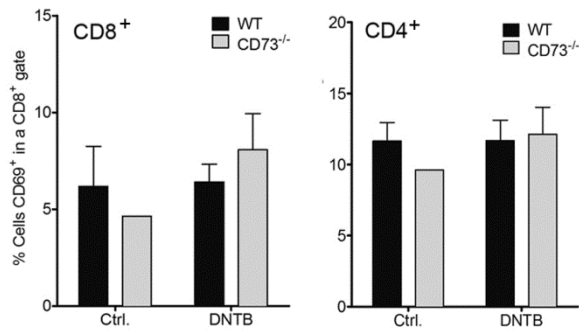
DNTB induces expression of CD73 by smDC. **a**, shows a representative gating strategy. A typical experiment out of 4 is shown. Mice were painted with DNFB and FITC or FITC alone. 48h later draining LN were removed and single cell suspensions were analyzed by FACS as indicated. Skin migrating DC (smDC) and resident DC (rDC) were analyzed for CCR7 and FITC expression. From the smDC fraction dot plots of CD73 expression vs MHC class II are shown (positive cells were gated according to CD73^{-/-} controls (not shown). The numbers indicate the percentage of cells in the depicted gates. Mean fluorescence (MFI) is given for CD73 expression. **b**, as in **a**, percentage of CD73⁺ cells from FACS gates as indicated are shown. Mean percentage $\pm$ SD, n=2; *indicates a significant difference (t-test p<0.05) to other groups. **c**, smDC and rDC as indicated were sorted from WT mice **d** after DNTB painting and cultivated together with 1x10⁵ purified CD4⁺ T cells. T cells were from DNFB primed animals, harvested 7d after DNFB application. T cell proliferation was determined by ³H-Thymidine pulse for 12 h at d3 of culture. *indicates a significant difference (t-test p<0.05; n=3) between rDC and smDC. **d**, controls for **e**. Briefly, 1x10⁵ purified cells as indicated were either left untreated or were incubated with anti-CD3/CD28 antibodies (1ug/ml) or DNBS (water soluble form of DNFB), respectively. Proliferation was determined as in **d**. *indicates significant differences (t-test p<0.05; n=3) between control and stimulated.

Figure S5



Expression of CD73 by T cells is not affected by DNTB treatment. **a**, WT mice were treated with solvent (ctrl) or DNTB and LN were isolated 24h later (a) or in a time course study at timepoints as indicated (b). Expression of CD73 by CD4⁺ and CD8⁺ cells was analyzed by FACS. **b**, shows mean percentages $\pm$ SEM of CD73⁺ cells (n=3).

Figure S6



No differences in activation of LN T cells after DNTB treatment. LN T cells were analyzed 3d after solvent (ctrl) or DNTB treatment by FACS for CD69 expression. Shown are means $\pm$ SEM, n=3.

Supplemental Table S1. Alleles, genotypes and inferred phenotypes observed in patients with NECD and control individuals.

Alleles	NECD (No)	NECD (%)	Controls (No)	Controls (%)	OR (adjusted): Wald	Intergroup comparison values. p-value (adjusted): LRT global
CYP2C8*3 C/C	242	72.89%	434	71.15%	–	0.029
CYP2C8*3 C/T	87	26.20%	155	25.41%	1.01 (0.74-1.37)	
CYP2C8*3 T/T	3	0.90%	21	3.44%	0.24 (0.07-0.83)	
Total	332		610			
CYP2C8*4 G/G	303	90.72%	520	88.29%	–	0.452
CYP2C8*4 C/G	29	8.68%	66	11.21%	0.75 (0.47-1.18)	
CYP2C8*4 C/C	2	0.60%	3	0.51%	1.07 (0.18-6.47)	
Total	334		589			
CYP2C9*2 C/C	248	73.81%	441	71.94%	–	0.064
CYP2C9*2 C/T	85	25.30%	153	24.96%	0.99 (0.72-1.34)	
CYP2C9*2 T/T	3	0.89%	19	3.10%	0.28 (0.08-0.95)	
Total	336		613			
CYP2C9*3 A/A	280	87.50%	513	86.66%	–	0.954
CYP2C9*3 A/C	38	11.88%	75	12.67%	0.95 (0.62-1.44)	
CYP2C9*3 C/C	2	0.63%	4	0.68%	0.85 (0.15-4.71)	
Total	320		592			
CYP2C19*2 A/A	245	72.49%	449	72.65%	–	0.673
CYP2C19*2 A/G	87	25.74%	162	26.21%	0.98 (0.73-1.33)	
CYP2C19*2 G/G	6	1.78%	7	1.13%	1.65 (0.55-4.97)	
Total	338		618			
CYP2C19*17 C/C	217	65.76%	376	62.77%	–	0.648
CYP2C19*17 C/T	99	30.00%	194	32.39%	0.89 (0.66-1.19)	
CYP2C19*17 T/T	14	4.24%	29	4.84%	0.82 (0.42-1.59)	
Total	330		599			

Genotypes	NECD (No)	NECD (%)	Controls (No)	Controls (%)	OR (adjusted): Wald	Intergroup comparison values. p-value (adjusted): LRT global
CYP2C8*1/*1	215	65.55%	351	60.52%	—	0.065
CYP2C8*1/*3	80	24.39%	140	24.14%	0.94 (0.68-1.3)	
CYP2C8*1/*4	22	6.71%	55	9.48%	0.65 (0.38-1.1)	
CYP2C8*3/*3	3	0.91%	21	3.62%	0.22 (0.06-0.75)	
CYP2C8*3/*4	6	1.83%	10	1.72%	0.98 (0.35-2.75)	
CYP2C8*4/*4	2	0.61%	3	0.52%	1.01 (0.17-6.14)	
Total	328		580			
CYP2C9*1/*1	202	63.72%	354	60.10%	—	0.247
CYP2C9*1/*2	73	23.03%	137	23.26%	0.93 (0.67-1.3)	
CYP2C9*1/*3	33	10.41%	62	10.53%	0.95 (0.6-1.5)	
CYP2C9*2/*2	3	0.95%	19	3.23%	0.27 (0.08-0.94)	
CYP2C9*2/*3	4	1.26%	13	2.21%	0.55 (0.18-1.71)	
CYP2C9*3/*3	2	0.63%	4	0.68%	0.82 (0.15-4.53)	
Total	317		589			
CYP2C19*1/*1	146	44.51%	248	41.61%	—	0.759
CYP2C19*1/*2	66	20.12%	120	20.13%	0.93 (0.64-1.33)	
CYP2C19*1/*17	77	23.48%	155	26.01%	0.84 (0.6-1.19)	
CYP2C19*2/*2	6	1.83%	6	1.01%	1.82 (0.57-5.77)	
CYP2C19*2/*17	20	6.10%	40	6.71%	0.85 (0.48-1.51)	
CYP2C19*17/*17	13	3.96%	27	4.53%	0.79 (0.39-1.59)	
Total	328		596			
Inferred Phenotypes	NECD (No)	NECD (%)	Controls (No)	Controls (%)	OR (adjusted): Wald	Intergroup comparison values. p-value (adjusted): LRT global
CYP2C8 RM	215	65.55%	351	60.52%	—	0.107
CYP2C8 IM	102	31.10%	195	33.62%	0.86 (0.64-1.15)	

CYP2C8 PM	11	3.35%	34	5.86%	0.51 (0.25-1.03)	
Total	328		580			
CYP2C9 RM	202	63.72%	354	60.10%	–	0.436
CYP2C9 IM	109	34.38%	218	37.01%	0.88 (0.66-1.17)	
CYP2C9 PM	6	1.89%	17	2.89%	0.62 (0.24-1.59)	
Total	317		589			
CYP2C19 UR	13	3.96%	27	4.53%	0.79 (0.39-1.59)	0.638
CYP2C19 RM	77	23.48%	155	26.01%	0.84 (0.6-1.19)	
CYP2C19 NM	146	44.51%	248	41.61%	–	
CYP2C19 IM	86	26.22%	160	26.85%	0.91 (0.65-1.27)	
CYP2C19 PM	6	1.83%	6	1.01%	1.82 (0.57-5.77)	
Total	328		596			

Supplemental Table S2. Alleles, genotypes and inferred phenotypes observed in patients with NERD and control individuals.

Alleles	NERD (No)	NERD (%)	Controls (No)	Controls (%)	OR (adjusted): Wald	Intergroup comparison values. p-value (adjusted): LRT global
CYP2C8*3 C/C	39	68.42%	434	71.15%	–	0.601
CYP2C8*3 C/T	17	29.82%	155	25.41%	1.23 (0.67-2.23)	
CYP2C8*3 T/T	1	1.75%	21	3.44%	0.52 (0.07-3.99)	
Total	57		610			
CYP2C8*4 G/G	48	87.27%	520	88.29%	–	0.732
CYP2C8*4 C/G	7	12.73%	66	11.21%	1.14 (0.49-2.62)	
CYP2C8*4 C/C	0	0.00%	3	0.51%	0 (0-Inf)	
Total	55		589			
CYP2C9*2 C/C	41	70.69%	441	71.94%	–	0.975
CYP2C9*2 C/T	15	25.86%	153	24.96%	1.06 (0.57-1.96)	
CYP2C9*2 T/T	2	3.45%	19	3.10%	1.14 (0.26-5.05)	
Total	58		613			
CYP2C9*3 A/A	44	80.00%	513	86.66%	–	0.365
CYP2C9*3 A/C	10	18.18%	75	12.67%	1.59 (0.76-3.3)	
CYP2C9*3 C/C	1	1.82%	4	0.68%	2.79 (0.3-25.61)	
Total	55		592			
CYP2C19*2 A/A	49	84.48%	449	72.65%	–	0.084
CYP2C19*2 A/G	8	13.79%	162	26.21%	0.45 (0.21-0.98)	
CYP2C19*2 G/G	1	1.72%	7	1.13%	1.31 (0.16-10.85)	
Total	58		618			
CYP2C19*17 C/C	31	55.36%	376	62.77%	–	0.550
CYP2C19*17 C/T	22	39.29%	194	32.39%	1.38 (0.78-2.44)	
CYP2C19*17 T/T	3	5.36%	29	4.84%	1.24 (0.36-4.32)	

Total	56		599			
Genotypes	NERD (No)	NERD (%)	Controls (No)	Controls (%)	OR (adjusted): Wald	Intergroup comparison values. p-value (adjusted): LRT global
CYP2C8*1/*1	33	61.11%	351	60.52%	–	0.463
CYP2C8*1/*3	14	25.93%	140	24.14%	1.07 (0.56-2.06)	
CYP2C8*1/*4	3	5.56%	55	9.48%	0.58 (0.17-1.94)	
CYP2C8*3/*3	1	1.85%	21	3.62%	0.5 (0.06-3.81)	
CYP2C8*3/*4	3	5.56%	10	1.72%	3.15 (0.82-12.02)	
CYP2C8*4/*4	0	0.00%	3	0.52%	0 (0-Inf)	
Total	54		580			
CYP2C9*1/*1	32	58.18%	354	60.10%	–	0.737
CYP2C9*1/*2	12	21.82%	137	23.26%	0.97 (0.49-1.94)	
CYP2C9*1/*3	6	10.91%	62	10.53%	1.09 (0.44-2.72)	
CYP2C9*2/*2	1	1.82%	19	3.23%	0.59 (0.08-4.52)	
CYP2C9*2/*3	3	5.45%	13	2.21%	2.62 (0.71-9.7)	
CYP2C9*3/*3	1	1.82%	4	0.68%	2.66 (0.29-24.59)	
Total	55		589			
CYP2C19*1/*1	23	41.07%	248	41.61%	–	0.219
CYP2C19*1/*2	7	12.50%	120	20.13%	0.63 (0.26-1.5)	
CYP2C19*1/*17	21	37.50%	155	26.01%	1.46 (0.78-2.73)	
CYP2C19*2/*2	1	1.79%	6	1.01%	1.81 (0.21-15.67)	
CYP2C19*2/*17	1	1.79%	40	6.71%	0.27 (0.04-2.05)	
CYP2C19*17/*17	3	5.36%	27	4.53%	1.18 (0.33-4.2)	
Total	56		596			
Inferred Phenotypes	NERD (No)	NERD (%)	Controls (No)	Controls (%)	OR (adjusted): Wald	Intergroup comparison values. p-value (adjusted): LRT global

CYP2C8 RM	33	61.11%	351	60.52%	–	0.892
CYP2C8 IM	17	31.48%	195	33.62%	0.93 (0.5-1.71)	
CYP2C8 PM	4	7.41%	34	5.86%	1.23 (0.41-3.7)	
Total	54		580			
CYP2C9 RM	32	58.18%	354	60.10%	–	0.301
CYP2C9 IM	19	34.55%	218	37.01%	0.97 (0.54-1.76)	
CYP2C9 PM	4	7.27%	17	2.89%	2.63 (0.83-8.29)	
Total	55		589			
CYP2C19 UR	3	5.36%	27	4.53%	1.18 (0.33-4.2)	0.179
CYP2C19 RM	21	37.50%	155	26.01%	1.46 (0.78-2.73)	
CYP2C19 NM	23	41.07%	248	41.61%	–	
CYP2C19 IM	8	14.29%	160	26.85%	0.54 (0.23-1.23)	
CYP2C19 PM	1	1.79%	6	1.01%	1.81 (0.21-15.67)	
Total	56		596			

Supplemental Table S3. Alleles, genotypes and inferred phenotypes observed in patients with mixed pattern and control individuals.

Alleles	Mixed pattern (No)	Mixed pattern (%)	Controls (No)	Controls (%)	OR (adjusted): Wald	Intergroup comparison values. p-value (adjusted): LRT global
CYP2C8*3 C/C	43	74.14%	434	71.15%	–	0.879
CYP2C8*3 C/T	13	22.41%	155	25.41%	0.85 (0.44-1.62)	
CYP2C8*3 T/T	2	3.45%	21	3.44%	0.95 (0.21-4.2)	
Total	58		610			
CYP2C8*4 G/G	50	89.29%	520	88.29%	–	0.754
CYP2C8*4 C/G	6	10.71%	66	11.21%	0.94 (0.39-2.27)	
CYP2C8*4 C/C	0	0.00%	3	0.51%	0 (0-Inf)	
Total	56		589			
CYP2C9*2 C/C	46	79.31%	441	71.94%	–	0.399
CYP2C9*2 C/T	10	17.24%	153	24.96%	0.63 (0.31-1.27)	
CYP2C9*2 T/T	2	3.45%	19	3.10%	1.01 (0.23-4.47)	
Total	58		613			
CYP2C9*3 A/A	50	89.29%	513	86.66%	–	0.635
CYP2C9*3 A/C	6	10.71%	75	12.67%	0.84 (0.35-2.02)	
CYP2C9*3 C/C	0	0.00%	4	0.68%	0 (0-Inf)	
Total	56		592			
CYP2C19*2 A/A	43	72.88%	449	72.65%	–	0.127
CYP2C19*2 A/G	13	22.03%	162	26.21%	0.84 (0.44-1.6)	
CYP2C19*2 G/G	3	5.08%	7	1.13%	4.54 (1.13-18.27)	
Total	59		618			
CYP2C19*17 C/C	39	67.24%	376	62.77%	–	0.753
CYP2C19*17 C/T	16	27.59%	194	32.39%	0.8 (0.43-1.46)	
CYP2C19*17 T/T	3	5.17%	29	4.84%	0.98 (0.29-3.38)	
Total	58		599			

Genotypes	Mixed pattern (No)	Mixed pattern (%)	Controls (No)	Controls (%)	OR (adjusted): Wald	Intergroup comparison values. p-value (adjusted): LRT global
CYP2C8*1/*1	36	65.45%	351	60.52%	—	0.952
CYP2C8*1/*3	11	20.00%	140	24.14%	0.77 (0.38-1.55)	
CYP2C8*1/*4	5	9.09%	55	9.48%	0.88 (0.33-2.34)	
CYP2C8*3/*3	2	3.64%	21	3.62%	0.91 (0.2-4.06)	
CYP2C8*3/*4	1	1.82%	10	1.72%	0.96 (0.12-7.76)	
CYP2C8*4/*4	0	0.00%	3	0.52%	0 (0-Inf)	
Total	55		580			
CYP2C9*1/*1	39	70.91%	354	60.10%	—	0.316
CYP2C9*1/*2	8	14.55%	137	23.26%	0.53 (0.24-1.16)	
CYP2C9*1/*3	6	10.91%	62	10.53%	0.88 (0.36-2.17)	
CYP2C9*2/*2	2	3.64%	19	3.23%	0.96 (0.21-4.26)	
CYP2C9*2/*3	0	0.00%	13	2.21%	0 (0-Inf)	
CYP2C9*3/*3	0	0.00%	4	0.68%	0 (0-Inf)	
Total	55		589			
CYP2C19*1/*1	26	44.83%	248	41.61%	—	0.391
CYP2C19*1/*2	10	17.24%	120	20.13%	0.79 (0.37-1.7)	
CYP2C19*1/*17	13	22.41%	155	26.01%	0.8 (0.4-1.6)	
CYP2C19*2/*2	3	5.17%	6	1.01%	4.95 (1.16-21.14)	
CYP2C19*2/*17	3	5.17%	40	6.71%	0.72 (0.21-2.48)	
CYP2C19*17/*17	3	5.17%	27	4.53%	1.03 (0.29-3.65)	
Total	58		596			
Inferred Phenotypes	Mixed pattern (No)	Mixed pattern (%)	Controls (No)	Controls (%)	OR (adjusted): Wald	Intergroup comparison values. p-value (adjusted): LRT global
CYP2C8 RM	36	65.45%	351	60.52%	—	0.762
CYP2C8 IM	16	29.09%	195	33.62%	0.8 (0.43-1.48)	

CYP2C8 PM	3	5.45%	34	5.86%	0.85 (0.25-2.9)	
Total	55		580			
CYP2C9 RM	39	70.91%	354	60.10%	–	0.087
CYP2C9 IM	16	29.09%	218	37.01%	0.67 (0.36-1.22)	
CYP2C9 PM	0	0.00%	17	2.89%	0 (0-Inf)	
Total	55		589			
CYP2C19 UR	3	5.17%	27	4.53%	1.03 (0.29-3.65)	0.268
CYP2C19 RM	13	22.41%	155	26.01%	0.8 (0.4-1.6)	
CYP2C19 NM	26	44.83%	248	41.61%	–	
CYP2C19 IM	13	22.41%	160	26.85%	0.77 (0.39-1.55)	
CYP2C19 PM	3	5.17%	6	1.01%	4.95 (1.16-21.15)	
Total	58		596			

Supplemental Table S4. Alleles, genotypes and inferred phenotypes observed in patients with anaphylaxis and control individuals.

Alleles	Anaphyl axis (No)	Anaphyl axis (%)	Controls (No)	Controls (%)	OR (adjusted): Wald	Intergroup comparison values. p-value (adjusted): LRT global
CYP2C8*3 C/C	21	65.63%	434	71.15%	–	0.187
CYP2C8*3 C/T	11	34.38%	155	25.41%	1.48 (0.7-3.15)	
CYP2C8*3 T/T	0	0.00%	21	3.44%	0 (0-Inf)	
Total	32		610			
CYP2C8*4 G/G	24	82.76%	520	88.29%	–	0.625
CYP2C8*4 C/G	5	17.24%	66	11.21%	1.54 (0.57-4.2)	
CYP2C8*4 C/C	0	0.00%	3	0.51%	0 (0-Inf)	
Total	29		589			
CYP2C9*2 C/C	25	73.53%	441	71.94%	–	0.644
CYP2C9*2 C/T	7	20.59%	153	24.96%	0.81 (0.34-1.91)	
CYP2C9*2 T/T	2	5.88%	19	3.10%	1.85 (0.41-8.44)	
Total	34		613			
CYP2C9*3 A/A	29	90.63%	513	86.66%	–	0.732
CYP2C9*3 A/C	3	9.38%	75	12.67%	0.79 (0.23-2.67)	
CYP2C9*3 C/C	0	0.00%	4	0.68%	0 (0-Inf)	
Total	32		592			
CYP2C19*2 A/A	25	73.53%	449	72.65%	–	0.666
CYP2C19*2 A/G	8	23.53%	162	26.21%	0.89 (0.39-2.01)	
CYP2C19*2 G/G	1	2.94%	7	1.13%	2.79 (0.33-23.81)	
Total	34		618			
CYP2C19*17 C/C	20	71.43%	376	62.77%	–	0.457
CYP2C19*17 C/T	6	21.43%	194	32.39%	0.59 (0.23-1.49)	
CYP2C19*17 T/T	2	7.14%	29	4.84%	1.22 (0.27-5.5)	
Total	28		599			

Genotypes	Anaphyl axis (No)	Anaphyl axis (%)	Controls (No)	Controls (%)	OR (adjusted): Wald	Intergroup comparison values. p-value (adjusted): LRT global
CYP2C8*1/*1	15	53.57%	351	60.52%	—	0.597
CYP2C8*1/*3	8	28.57%	140	24.14%	1.36 (0.56-3.28)	
CYP2C8*1/*4	4	14.29%	55	9.48%	1.63 (0.52-5.12)	
CYP2C8*3/*3	0	0.00%	21	3.62%	0 (0-Inf)	
CYP2C8*3/*4	1	3.57%	10	1.72%	2.09 (0.25-17.62)	
CYP2C8*4/*4	0	0.00%	3	0.52%	0 (0-Inf)	
Total	28		580			
CYP2C9*1/*1	23	69.70%	354	60.10%	—	0.687
CYP2C9*1/*2	5	15.15%	137	23.26%	0.57 (0.21-1.52)	
CYP2C9*1/*3	2	6.06%	62	10.53%	0.55 (0.13-2.41)	
CYP2C9*2/*2	2	6.06%	19	3.23%	1.63 (0.36-7.49)	
CYP2C9*2/*3	1	3.03%	13	2.21%	1.27 (0.16-10.21)	
CYP2C9*3/*3	0	0.00%	4	0.68%	0 (0-Inf)	
Total	33		589			
CYP2C19*1/*1	13	44.83%	248	41.61%	—	0.686
CYP2C19*1/*2	7	24.14%	120	20.13%	1.11 (0.43-2.85)	
CYP2C19*1/*17	5	17.24%	155	26.01%	0.62 (0.22-1.78)	
CYP2C19*2/*2	1	3.45%	6	1.01%	3.8 (0.42-34.69)	
CYP2C19*2/*17	1	3.45%	40	6.71%	0.47 (0.06-3.73)	
CYP2C19*17/*17	2	6.90%	27	4.53%	1.3 (0.28-6.12)	
Total	29		596			
Inferred Phenotypes	Anaphyl axis (No)	Anaphyl axis (%)	Controls (No)	Controls (%)	OR (adjusted): Wald	Intergroup comparison values. p-value (adjusted): LRT global
CYP2C8 RM	15	53.57%	351	60.52%	—	0.537
CYP2C8 IM	12	42.86%	195	33.62%	1.44 (0.66-3.14)	

CYP2C8 PM	1	3.57%	34	5.86%	0.62 (0.08-4.89)	
Total	28		580			
CYP2C9 RM	23	69.70%	354	60.10%	–	0.564
CYP2C9 IM	9	27.27%	218	37.01%	0.66 (0.3-1.45)	
CYP2C9 PM	1	3.03%	17	2.89%	0.94 (0.12-7.4)	
Total	33		589			
CYP2C19 UR	2	6.90%	27	4.53%	1.3 (0.28-6.12)	0.671
CYP2C19 RM	5	17.24%	155	26.01%	0.62 (0.22-1.78)	
CYP2C19 NM	13	44.83%	248	41.61%	–	
CYP2C19 IM	8	27.59%	160	26.85%	0.95 (0.38-2.34)	
CYP2C19 PM	1	3.45%	6	1.01%	3.8 (0.42-34.69)	
Total	29		596			