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Topical ivermectin improves allergic skin inflammation

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atopic dermatitis; ivermectin; skin inflammatory diseases; T-cell immunity; topical treatment.

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Abstract

Background: Ivermectin (IVM) is widely used in both human and veterinary medicine to treat parasitic infections. Recent reports have suggested that IVM could also have anti-inflammatory properties.
Methods: Here, we investigated the activity of IVM in a murine model of atopic dermatitis (AD) induced by repeated exposure to the allergen *Dermatophagoides farinae*, and in standard cellular immunological assays.
Results: Our results show that topical IVM improved allergic skin inflammation by reducing the priming and activation of allergen-specific T cells, as well as the production of inflammatory cytokines. While IVM had no major impact on the functions of dendritic cells *in vivo* and *in vitro*, IVM impaired T-cell activation, proliferation, and cytokine production following polyclonal and antigen-specific stimulation.
Conclusion: Altogether, our results show that IVM is endowed with topical anti-inflammatory properties that could have important applications for the treatment of T-cell-mediated skin inflammatory diseases.

Ivermectin (IVM) has been used as a broad-spectrum antiparasitic agent for over 20 years in both veterinary and human medicine (1). IVM derives from a group of natural macrocyclic lactones, named avermectins, which were isolated from the soil actinomycete, *Streptomyces avermectinius*. The antiparasitic effects of IVM are mediated through inhibition of the neuronal and muscular activity of parasites, which leads to paralysis and death. The drug binds selectively and with high affinity to glutamate-gated chloride ion channels, which are expressed on the nerves and muscles of invertebrates. Of note, IVM also binds to numerous ligand-gated ion channels expressed by vertebrates, as well as to diverse ion transporters or the nuclear receptor farnesoid X receptor (FXR) (2–4). However, with the exception of the FXR (3), the affinity of IVM for these receptors/transporters is low (2), which supports the good tolerability of IVM in mammals, with minimal side-effects (5).

In addition to its antiparasitic activity, IVM is also endowed with immunomodulatory properties. Hence, when administered to healthy animals, IVM has been shown to stimulate immune responses triggered by exogenous antigens, such as xenoantigens and haptens (6–8). However, IVM is also endowed with anti-inflammatory effects that have been observed in a murine model of allergic asthma and on the cellular and humoral responses of lambs injected with human erythrocytes or ovalbumin (9, 10). Similar pro- or anti-inflammatory effects were obtained *in vitro* with IVM, which was shown to inhibit the production of nitric oxide and prostaglandin E2 (PGE2) in LPS-stimulated RAW 264.7 macrophages (11) and, conversely, to enhance the secretion of IL-1β or PGE2 triggered by ATP in mouse peritoneal macrophages (12, 13). These seemingly contradictory findings thus indicate that the downstream effects of IVM treatment can differ depending on the initial trigger, the host, or the drug regimen.

Abbreviations
AD, atopic dermatitis; BMDC, bone marrow dendritic cell; BMV, betamethasone valerate; Derf, *Dermatophagoides farinae*; dLN, draining lymph node; FXR, farnesoid X receptor; IFN-γ, interferon gamma; IVM, ivermectin; PBLs, peripheral blood lymphocytes.

Interestingly, in recent Phase III clinical trials, IVM 1% cream demonstrated a robust efficacy to reduce the number of skin inflammatory lesions seen in patients with moderate to severe papulopustular rosacea (14), illustrating the potential of the drug to be a new topical treatment for skin inflammatory diseases. Nevertheless, so far, it is not known whether the therapeutic effects of IVM in patients with rosacea rely on an anti-inflammatory effect or on an antiparasitic effect leading to the elimination of the mite *Demodex folliculorum,* a major trigger of rosacea, or both (15).

In this study, we tested the effect of topical IVM in a mouse model of AD-like skin inflammation, induced by repeated exposure to the allergen *Dermatophagoides farinae* (Derf) (16). Our results show that IVM improves the symptoms of allergic skin inflammation, via inhibition of T-cell activation, proliferation, and cytokine production.

Methods

A brief description of the Methods is provided below. See additional details in the supplemental Methods, in the article's Supporting information.

Drug compounds

IVM and betamethasone valerate (BMV) were provided by Galderma (Galderma R&D, Sophia Antipolis, France). IVM was reconstituted, respectively, in DMSO for *in vitro* experiments and in acetone for mice ear painting.

For information about *animals, human cells, mAbs,* or *reagents*, see the supplemental Materials.

Mouse model of AD

Eczema was induced by topical application of Derf house dust mite extract (250 μg of protein allergen solution per ear) provided by P. Moingeon (Stallergenes, Antony, France) to the left ears of mice once a week for six consecutive weeks, as previously described (16). Vehicle (70% DMSO in water) was applied to the left ears of control mice.

In separate experiments, mouse eczema was induced by a single application (250 μg of protein extract) of Derf extract to the left ears of animals immunized 5 days before by subcutaneous (s.c.) injection containing 1.5×10^6 bone marrow-derived dendritic cells (BMDCs) pulsed with 1 mg/ml Derf extract (for 90 min at 37°C, 5% CO_2) (17).

Drug treatment

Drug treatment was performed by applying 10 μl of IVM (0.1%) or BMV (0.01%) to the site of allergen exposure, on both sides of the left ears of the mice. Vehicle (acetone) was applied on the contralateral ear. Drug treatment was started 1 day after the third application of Derf (i.e., day 16) and repeated on alternate days.

In some experiments, BMDCs were co-incubated with 3 μM IVM and 1 mg/ml Derf extract (for 90 min at 37°C, 5% CO_2), before their use for inducing mouse eczema.

For information about *cell culture, T-cell purification, BMDC generation, and flow cytometry analysis*, see the supplemental Methods.

In vivo DC migration

Derf extract was labeled with a PE-CF594 labeling kit (Life Technologies, Carlsbad, California, USA) and used to track *in vivo* skin dendritic cell migration.

In vitro BMDC stimulation assays

BMDCs were stimulated for 6 hr (37°C, 5% CO_2) in 24-well plates (Costar, Sigma, Saint-Quentin-Fallavier, France) at a concentration of 2×10^6/ml with the indicated concentrations of Derf protein extract, in the presence or absence of the indicated concentrations of IVM.

In vitro T-cell culture

CD8 + and CD4 + T cells, co-purified from the splenocytes of naïve mice by magnetic separation (Miltenyi Biotech, Bergisch Gladbach, Germany), and human peripheral blood lymphocytes (PBLs) isolated from blood samples by standard density centrifugation, were stained with 1 μM CFSE before culture; 2×10^5 isolated mouse T cells or human PBLs were then seeded in flat-bottomed 96-well plates (Costar, Sigma, Saint-Quentin-Fallavier, France). Polyclonal stimulations were performed with indicated concentrations of coated anti-CD3ε mAbs (eBioscience, San Diego, California, USA) in the presence of soluble anti-CD28 mAbs (2 μg/ml, eBioscience). T cells were stimulated *in vitro* in the presence or absence of IVM at the indicated concentrations and cultured at 37°C, 5% CO_2.

For information about the methods we used to assess (i) production of the inflammatory mediator by skin cells at the peak of Derf response, (ii) Derf-specific T-cell responses in the dLNs of sensitized animals, (iii) *in vivo* skin dendritic cell migration, (iv) *in vitro* BMDC maturation and cytokine production, and (v) *in vitro* T-cell proliferation and differentiation, see the supplemental Methods.

Results

Topical ivermectin inhibits skin inflammation in a mouse model of AD

To test for the effect of IVM on allergic skin inflammation, we used a mouse model of AD in which C57BL/6 animals were repeatedly exposed to a Derf protein extract [onto the ear, once a week for six consecutive weeks, (Fig. 1A)], as previously described (16). This experimental model led to the progressive development of a robust skin inflammatory reaction (Fig. 1B, closed circles), which displayed the major characteristics of AD inflammation, that is, thickening of the epidermis, blood vessel enlargement, major infiltration of mononuclear cells in the dermis, and more importantly prominent recruitment of eosinophils (Fig. 1C1). To examine the therapeutic effects of IVM in this model, the drug

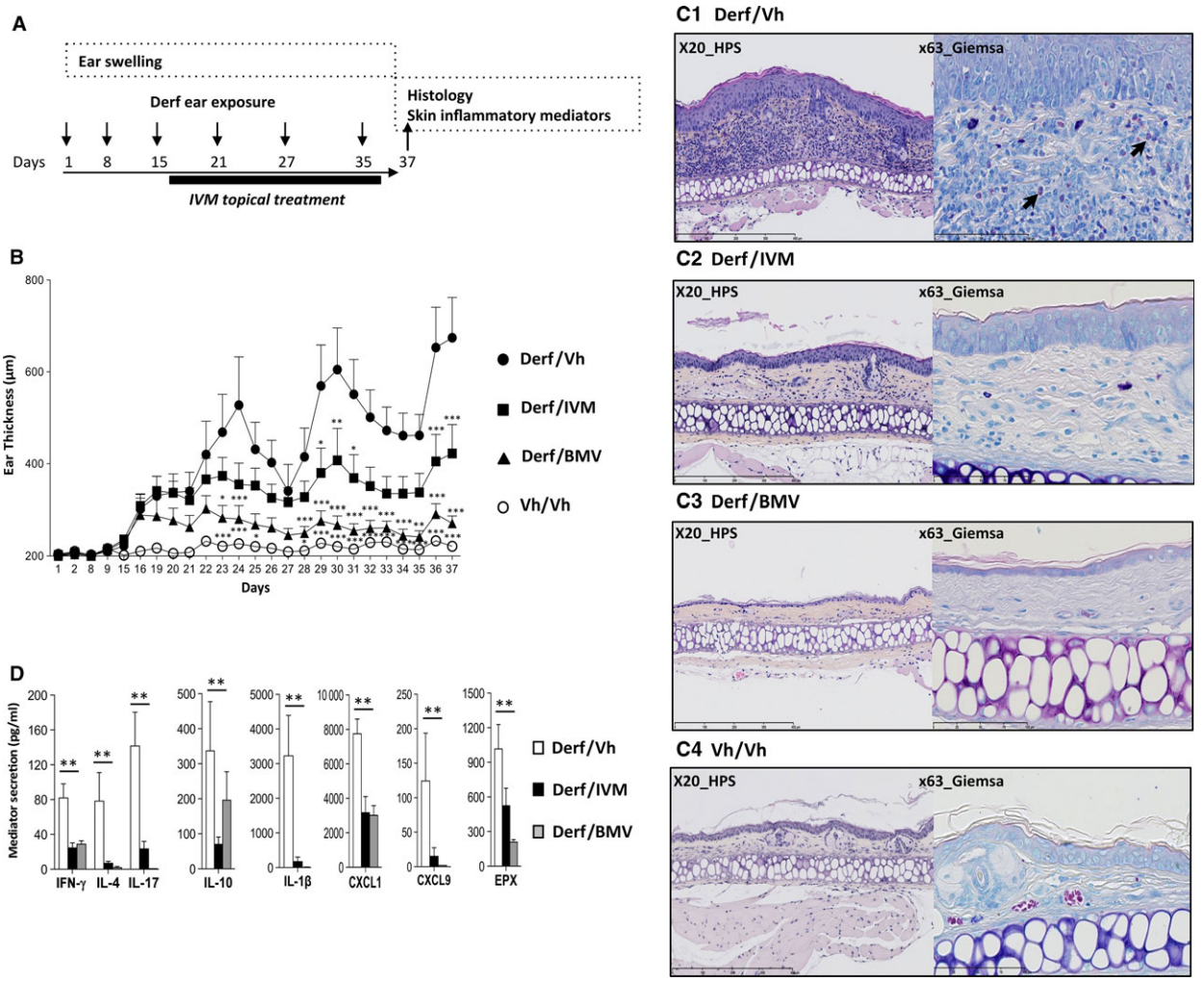

Figure 1 IVM inhibits skin inflammation in a mouse model of AD. (A) Protocol 1 – Epicutaneous Derf immunization. The left ears of C57BL/6 mice were painted with a solution of Derf weekly for five consecutive weeks (day 1, D8, D15, D21, D27, and D35). In parallel, animals were treated on the same ear with either IVM (0.1%), BMV (0.01%), or their respective vehicle from days 16 to 37. (B) Skin inflammation. Derf-induced skin inflammation was measured throughout the protocol and was expressed as the mean ear thickness (µm) ± SEM in either vehicle (closed circles, Derf/Vh)-, IVM (closed squares, Derf/IVM)-, or BMV (closed triangles, Derf/BMV)-treated animals. Data from control (unsensitized and vehicle-treated) animals are also shown (open circle, Vh/Vh). (C) Histological analysis of inflamed skin. Illustrations of representative ear (collected 2 days after the sixth round of Derf exposure = day 37) sections after HPS (hematoxylin–phloxin–safran) and Giemsa colorations in vehicle (C1)-, IVM (C2)-, or BMV (C3)-treated animals, or unsensitized and vehicle-treated controls (C4). Arrows in C1 depict eosinophil skin infiltration. (D) Production of inflammatory mediators by skin cells. IFN-γ, IL-4, IL-17, IL-10, IL-1β, CXCL1, CXCL9, and eosinophil peroxidase (EPX) were titrated into the supernatants of skin cells, collected at day 37, and cultured for 24 h at 37°C. Results are expressed as mean values (pg/ml) ± SD in vehicle (white bars)-, IVM (black bars)-, or BMV (gray bars)-treated animals. All results are representative of three independent experiments ($n = 5$–8 per group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus Derf-immunized and vehicle-treated group; two-way ANOVA test (A) or Mann–Whitney *U*-test (B–D).

was applied every day onto the site of allergen exposure, by starting the treatment at D16 (i.e., 1 day after the third application of Derf and the onset of skin inflammation). Control animals received either the IVM vehicle (acetone) or a reference topical corticosteroid (betamethasone valerate, BMV 0.01%). Figure 1B shows that IVM (0.1%) treatment (closed squares) markedly improved the skin inflammation with a 50% reduction in ear thickness at day 37. Histological analysis of IVM-treated mice at day 37 (Fig. 1C2) showed a dramatic improvement of all the Derf-induced skin changes: normalization of the epidermal thickness, major reduction in the cellular inflammatory infiltrate, and decreased eosinophil infiltration [confirmed by the significant diminution of eosinophil peroxidase (EPX) in the skin of IVM-treated animals (Fig. 1D)]. Similar results were observed when the dose of IVM was significantly decreased by 10-fold (0.01%; data not

shown), suggesting that IVM has strong anti-inflammatory properties in this parasite-independent inflammatory setting. As expected, the topical corticosteroid (Fig. 1B, closed triangles; and Fig. 1C3) was very efficient, with an almost complete clearing of the skin inflammation at day 37, but with noticeable adverse effects including thinning of the epidermis and dermis, when compared with unsensitized and vehicle-treated animals (Fig. 1C4).

AD-like inflammation in mice is mediated by allergen-specific T cells, which are primed in lymph nodes draining the sensitization site and which migrate to the skin. Upon challenge, skin-infiltrating T cells release IFN-γ, IL-4, and IL-17, and cytotoxic mediators (granzyme B, Fas-L, Trail...) (16–21). We then tested for the presence of type 1, type 2, or type 17 responses in the skin of Derf-induced allergic inflammation and analyzed the impact of IVM treatment. The production of IFN-γ, IL-4, IL-10, and IL-17 from skin cells collected at day 37 was significantly reduced in IVM-treated mice compared with vehicle-treated mice, with a level of reduction close to that seen with BMV (Fig. 1D). We also investigated the production of several inflammatory mediators known to be present in AD lesions [IL-1β (22), chemokine motif ligand 1 (CXCL1) (22), CXCL9 (23), and EPX (24)] and observed that they were all significantly reduced following IVM or BMV treatment (Fig. 1D).

Collectively, these results indicate that IVM restrains the Derf-induced allergic skin inflammation and T-cell responses in the skin.

IVM inhibits the priming and activation of Derf-specific T cells *in vivo*

We next evaluated the number of allergen (Derf)-specific T cells in the dLNs of sensitized mice at day 37 by ELISPOT assay (Fig. 2A1). A significant decrease in the number of IFN-γ- and IL-4-producing T cells was seen in IVM-treated compared to vehicle-treated mice (Fig. 2A2), supporting the hypothesis that the suppression of skin allergic responses is secondary to the inhibition of allergen-specific T-cell activation in dLNs.

To confirm this hypothesis, we analyzed the impact of IVM treatment in another model in which Derf sensitization is induced by the subcutaneous transfer of Derf-loaded BMDCs in naïve animals (Fig. 2B1). As previously shown, mice immunized with Derf-loaded BMDCs develop acute AD-like inflammation following a single epicutaneous Derf challenge at day 5, which is mediated by allergen-specific cytotoxic type 1 CD8 + T cells that are primed in the dLNs 3–5 days postinjection (17). We then examined the impact of IVM on T-cell priming by treating mice topically for 6 days (from day −1 to day 4) at the site of the BMDC injection. Figures 2B2 and S1 show a significant decrease in the percentage and the numbers of proliferating T cells in dLNs (as revealed by FACS analysis, using the cell cycle protein Ki67 and the differentiation marker CD44) in immunized mice treated with IVM versus vehicle-treated animals. An important reduction was also observed in the number of Derf-specific CD8 + T cells at day 5 postimmunization (Fig. 2B3), as revealed by ELISPOT assay following *in vitro* Derf re-activation of dLN CD8 + T cells. Interestingly, in parallel experiments, the addition of IVM directly into ELISPOT cultures of dLN CD8 + T cells recovered from vehicle-treated mice immunized by Derf-pulsed DC also led to a significant reduction in the frequency of IFN-γ-producing cells after Derf reactivation (Fig. 2B4), suggesting that IVM treatment not only prevents the priming of allergen-specific effector T cells, as shown in Fig. 2B3, but also inhibits their effector functions.

Ivermectin does not inhibit DC migration and functions

To gain insight into the mechanisms of the impaired T-cell activation, we next explored the impact of IVM on DC phenotype and functions. First, we tested the effect of IVM on the migration of skin DCs toward the dLNs. To this aim, animals were painted with a Derf protein extract that was fluorescently labeled with PE-CF594 (Fig. 3A1), and skin migrating DCs were analyzed in dLNs 2 days later by FACS after gating on PE-CF594 + CD11c^{int} MHC-II^{hi} cells, as previously reported (skin migrating DCs were determined by high expression of MHC class II markers and intermediate expression of CD11c [data not shown]) (25, 26). Figure 3A2 shows that IVM did not hamper skin DC migration to LNs. Indeed, 48 hrs after Derf painting, skin migrating PE-CF594 + DCs represented 0.28% and 0.38% of live LN cells in vehicle- and IVM-treated mice, respectively. Moreover, similar levels of the maturation markers CD40 and CD86 were recorded on skin migrating PE-CF594 + DCs in the different settings (data not shown).

We next examined the effect of IVM on DC maturation and the production of cytokines *in vitro*. Immature BMDCs were incubated with Derf protein extract for 6 h in the presence or in the absence of the drug. Derf promoted a robust maturation of BMDCs, as revealed by the increased expression of CD40, CD86, PD-L1, and MHC class II co-stimulation markers (Fig. 3B1). The addition of graded doses of IVM (0.3 and 3 μM; of note, IVM was toxic at 10 μM) did not impact Derf-induced maturation (Fig. 3B1). In addition, IVM did not alter the amounts of cytokines (IL-12p40, IL-23, IL-6, and IL-10) secreted by BMDCs upon Derf stimulation (Fig. 3B2).

Finally, we immunized C57BL/6 animals with Derf-pulsed BMDCs treated *in vitro* with IVM (3 μM) or with vehicle, and Derf-specific T-cell responses in dLNs were analyzed 5 days later (Fig. 3C1). IVM did not alter the capacity of BMDC to prime CD8 + T cells and even induced a slight increase in their numbers compared to the control untreated group (Fig. 3C2).

Collectively, our *in vivo* and *in vitro* results show that the epicutaneous application of IVM has no major effect on DC migration, phenotype, and maturation that could explain the impaired T-cell priming and activation.

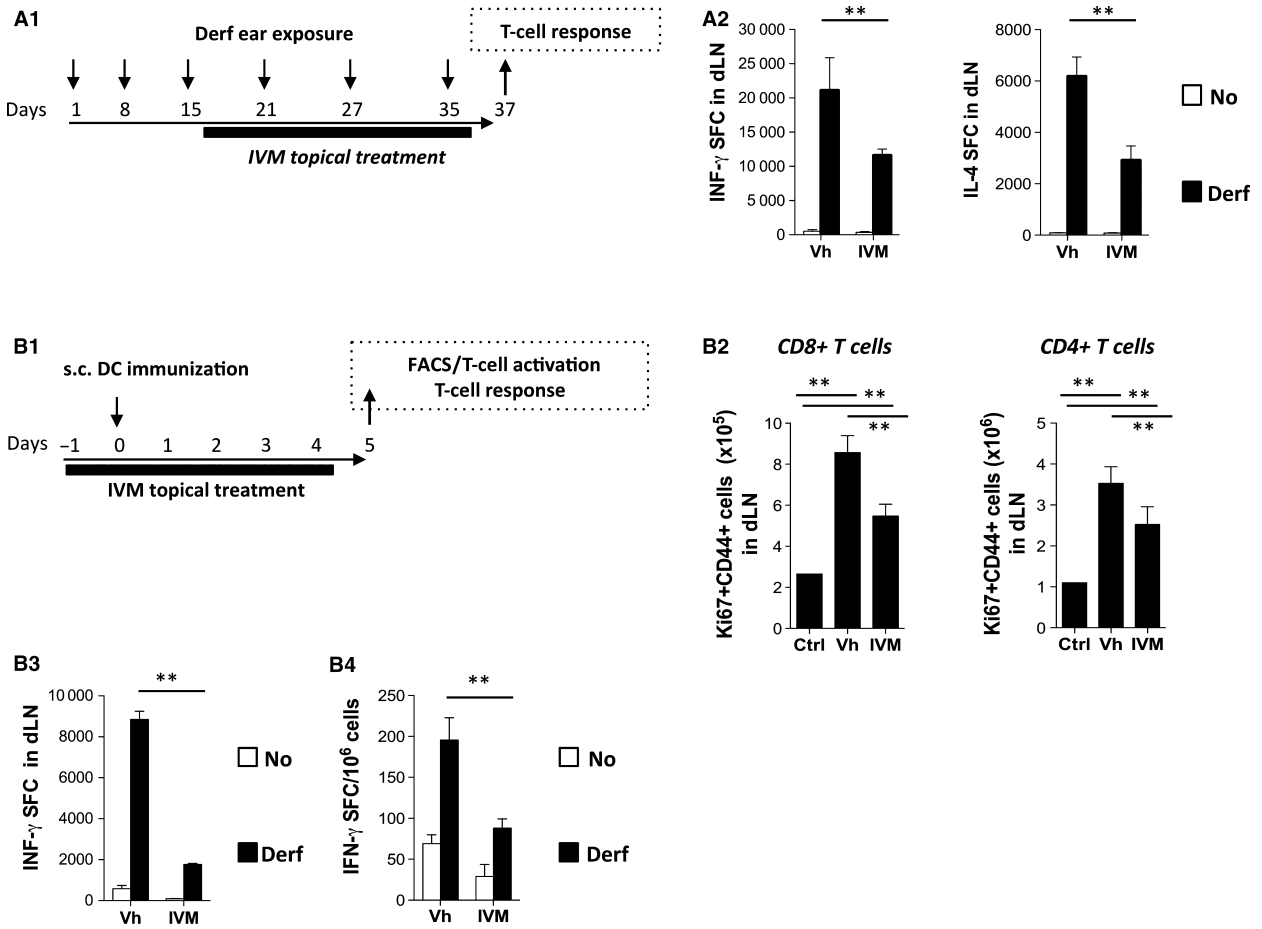

Figure 2 IVM prevents the priming and effector functions of Derf-specific T cells *in vivo*. (A1) Protocol 2. C57BL/6 mice were sensitized and treated with IVM or vehicle alone as in Fig. 1. (A2) Derf-specific T cells in dLNs. dLN cells were collected at day 37 and restimulated overnight with Derf (0.5 mg/ml; black bars) or left untreated (white bars). The following day, the number of IFN-γ- and IL-4-producing T cells was enumerated by ELISPOT assay. Results are expressed as the mean number of spot-forming cells (SFC)/dLN ± SD in vehicle- and IVM-treated mice. (B1) Protocol 3 – s.c. DC immunization. C57BL/6 mice immunized via s.c. injection of 1.5×10^6 Derf-pulsed BMDCs were treated topically with IVM (0.1%) or vehicle for 6 days (from day −1 to day 4) at the site of BMDC injection. (B2) Proliferating cells in dLNs. At day 5, dLNs from Derf-immunized mice, treated with either IVM or vehicle, were analyzed for the number of CD8 + Ki67 + CD44 + and CD4 + Ki67 + CD44 + T cells. Histograms depict mean cell numbers/dLN ± SD. Data from nonimmunized and untreated controls (Ctrl) are also shown. (B3) Derf-specific CD8 + T cells in dLNs. CD8 + T cells, purified from the dLNs of day 5 Derf-immunized animals, were restimulated overnight with Derf (0.5 mg/ml; black bars) or left untreated (white bars). The following day, the number of Derf-specific CD8 + T cells producing IFN-γ was determined by ELISPOT assay. Results are expressed as the mean number of SFC/dLN ± SD in either vehicle- or IVM-treated mice. (B4) Derf-specific CD8 + T-cell response following the addition of IVM into ELISPOT cultures. CD8 + T cells, purified from the dLNs of day 5 vehicle-treated and Derf-immunized animals, were restimulated overnight with Derf (0.5 mg/ml; black bars) or left untreated (white bars). The frequency of Derf-specific, IFN-γ-producing CD8 + T cells (mean SFC/10^6 dLN cells ± SD) was determined after the addition of IVM (3 μM) into overnight restimulated cultures. All results are representative of three independent experiments, (n = 3–4 mice per group). *$P < 0.05$, **$P < 0.01$, Mann–Whitney *U*-test.

IVM inhibits T-cell activation *in vitro*

We next investigated the direct effect of IVM (1 or 3 μg/ml) on the proliferation and functional properties of mouse and human T cells.

In a first set of experiments, purified mouse spleen T cells were labeled with CFSE and co-cultured with graded doses of anti-CD3 (0.3 or 1 μg/ml) + anti-CD28 (2 μg/ml) mAbs (Fig. 4A). Robust T-cell proliferation was observed after 72 hrs of culture, with, respectively, 76% and 98% of T cells having diluted CFSE when stimulated with 0.3 and 1 μg/ml anti-CD3 mAb. Interestingly, IVM strongly decreased T-cell proliferation in a dose-dependent manner (Fig. 4A1). However, T-cell proliferation was not fully inhibited, with only the last rounds of cell division being reduced in the presence of IVM. Importantly, this effect was observed for both CD4 +

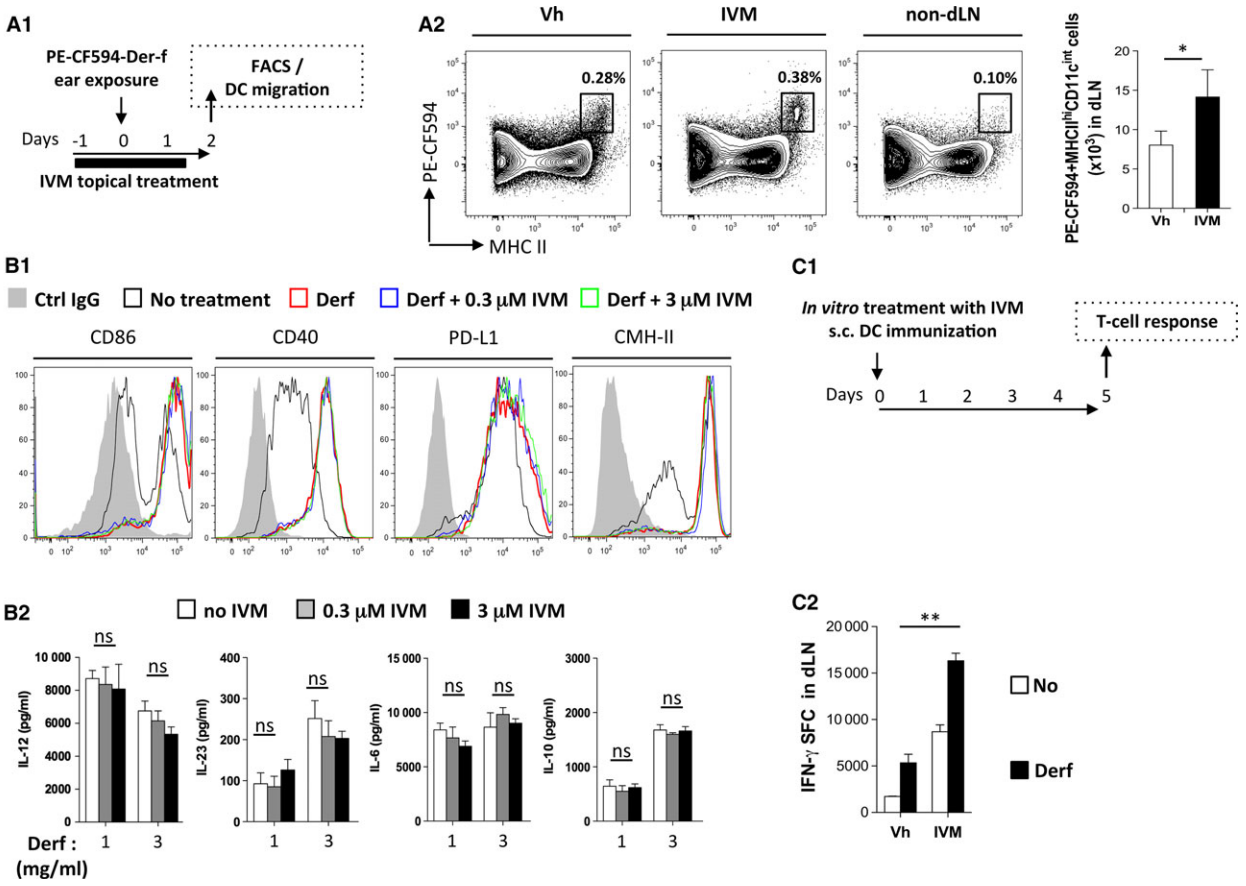

Figure 3 Ivermectin fails to inhibit DC migration and functions. (A1) Protocol 4 – s.c. DC immunization with fluorescent Derf. C57BL/6 mice, epicutaneously sensitized with PE-CF594-labeled Derf (250 µg per ear), were topically treated with IVM (0.1%) or vehicle for 3 days (from day −1 to day 1). (A2) *In vivo* migration of skin DCs. Draining (d)- and non-d-LN cells were collected for analysis of skin migrating DCs, that is, cells co-expressing PE-CF594, MHC-II^{hi} (FACS plots), and CD11c^{int} (data not shown). Histograms depict mean cell numbers/dLN ± SD in either vehicle- or IVM-treated mice. (B1) *In vitro* BMDC maturation. Immature BMDCs were generated with GM-CSF for 6 days. Derf (1 or 3 mg/ml) was added for the last 6 hr of culture in the presence/absence of graded doses of IVM (0.3 or 3 µM). BMDCs were analyzed by flow cytometry, after staining with CD11c, CD86, CD40, PD-L1, and MHC class II or respective isotype mAbs. Histogram overlays represent surface staining gated on CD11c+ cells. (B2) *In vitro* BMDC cytokine secretion. IL-12p40, IL-23, IL-6, and IL-10 were titrated in the supernatant of BMDC cultures. Results are expressed as mean values (pg/ml) SD. (C1) Protocol 5 – s.c. immunization with IVM-treated DC. C57BL/6 mice were immunized with Derf-pulsed BMDCs that have been previously co-incubated with either IVM (3 µM) or vehicle. (C2) Derf-specific CD8 + T cells. CD8 + T cells purified from the dLNs after 5 days were restimulated overnight with Derf (0.5 mg/ml, black bars) or left untreated (white bars). The following day, the number of Derf-specific CD8 + T cells producing IFN-γ was determined by ELISPOT assay. Results are expressed as the mean number of SFC/dLN ± SD. All results are representative of two or three independent experiments. *$P < 0.05$, **$P < 0.01$, Mann–Whitney *U*-test.

and CD8 + T-cell subsets (data not shown), and was not related to IVM toxicity, as no significant difference in the rate of cell death was observed with the concentrations used (0.3 or 3 µM) (data not shown). IVM was found to be toxic for both T cells and BMDCs at a dose of 10 µM. Of note, additional experiments showed that IVM displayed similar inhibitory effects on normal human blood T-cell proliferation induced by anti-CD3 + anti-CD28 mAbs (Fig. 4B).

We next tested for the effect of IVM on effector T-cell functions. IVM significantly reduced the expression of GmzB, a major cytotoxic mediator, by anti-CD3/CD28-activated normal mouse CD8 + T cells. After 5 days of culture, 22% of these cells were GzmB+ in the presence of IVM, compared with 57% in the absence of IVM (Figs 4A2 and S2). Similarly, after 5 days of culture, the ability of activated CD4 + and CD8 + T cells to produce IFN-γ following PMA/ionomycin restimulation was significantly reduced by IVM, with only 50% of CD8 + and 9% CD4 + T cells being IFN-γ+ in the presence of IVM, versus 74% and 32% in the absence of IVM, respectively (Figs 4A2 and S2). In addition to type 1, type 2 responses were also impacted, and a net inhibition of the percentage of IL-4 + CD4 + T cells was recorded following IVM treatment compared to control (2.4% versus 6%, respectively) (Figs 4A2 and S2).

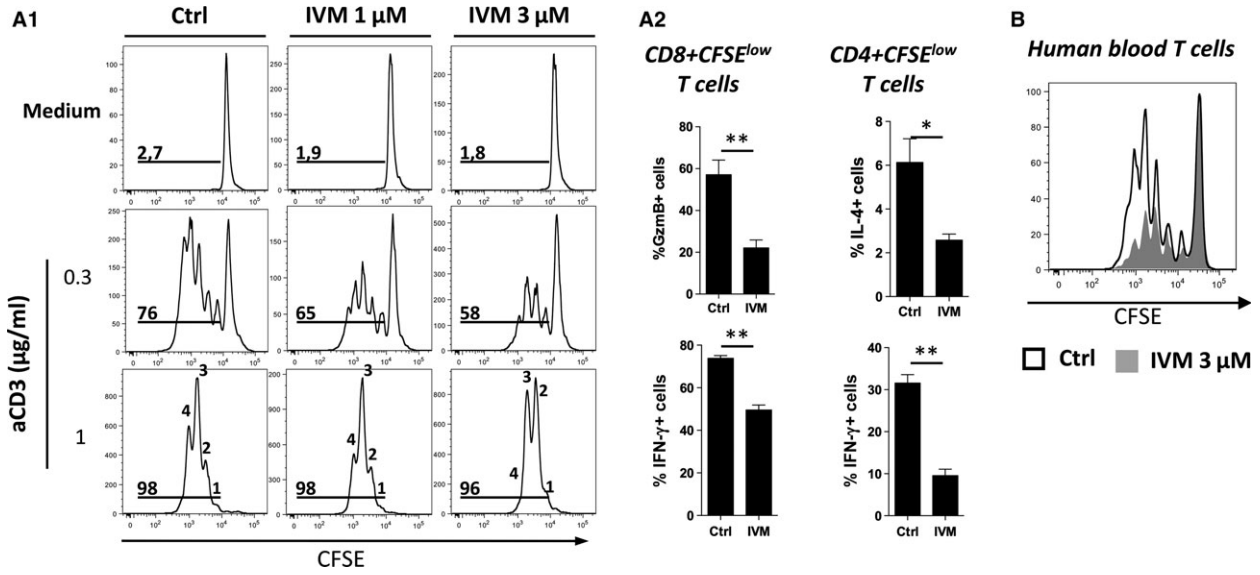

Figure 4 Ivermectin inhibits T-cell proliferation and functions *in vitro*. (A) Polyclonal stimulation/mouse. Both CD4 + and CD8 + T cells were co-purified from the spleen of naïve C57BL/6 mice, stained with CFSE, and stimulated for 3–5 days with graded doses of anti-CD3 (0.3 or 1 µg/ml) and anti-CD28 (2 µg/ml) mAbs, in the presence or not of IVM (1 or 3 µM). *In vitro* cultures were analyzed at day 3 for CFSE dilution in gated live T cells (A1), or at day 5 for GzmB, IFN-γ, and IL-4 expression in gated proliferating CD8 + or CD4 + $CFSE^{low}$ T-cell subsets (A2). Histograms represent the mean values (percentage) ± SD of GzmB+ and cytokine+ cells in either IVM-treated (3 µM) or untreated (control) cultures. (B) Polyclonal stimulation/humans. CFSE-labeled human PBLs were stimulated with anti-CD3 (1 µg/ml) + anti-CD28 (2 µg/ml) mAbs and treated with 3 µM IVM, or untreated. Histograms depict live T cells (gated on both CD4 + and CD8 + T-cell subsets), which have diluted CFSE after 3 days of culture. All the results are representative of three to five independent experiments (with $n = 5$ animals per group for mouse experiments) $^{*}P < 0.05$, $^{**}P < 0.01$, Mann–Whitney *U*-test.

Overall, these results demonstrate that IVM acts directly on T cells, by inhibiting both their proliferation and their activation in type 1, type 2, and cytotoxic effectors.

P2X4R and FXR do not mediate the IVM-induced inhibition of T-cell activation

As mentioned above, IVM is able to bind to several ligand-gated ion channels and ion transporters. To gain insights into the mechanisms by which IVM inhibits T-cell activation, we next investigated its activity on two potential targets expressed by both CD4 + and CD8 + T cells: (i) FXR, because of the relatively high affinity of the drug for this nuclear receptor, and (ii) P2X4R, due to the key role exerted by this receptor in T-cell activation, through its activity on Ca2 + metabolism. Thus, we tested the impact of IVM on the proliferation/differentiation of T cells collected from the spleen of FXR−/− or P2X4R−/− animals. Figure 5 shows that FXR−/− or P2X4R−/− T cells were vigorously activated upon polyclonal stimulation, although proliferation and granzyme B expression were slightly lower for P2X4R−/− cells compared to cells collected in control C57BL/6 animals. IVM hampered T-cell activation to a similar extent, when cells were collected from FXR−/− (Fig. 5A), from P2X4R−/− (Fig. 5B), and from control C57BL/6 animals, demonstrating that these two receptors are not required for the IVM inhibitory activity on T cells.

Discussion

Our current study examined the anti-inflammatory properties of IVM in a relevant mouse model of AD. We report that the epicutaneous application of IVM significantly improved the clinical symptoms and reduced the production of inflammatory cytokines in allergen-exposed skin when applied after the onset of the allergic skin inflammation (therapeutic mode). Besides, IVM prevented the development of skin inflammation and inhibited the priming of allergen-specific effector T cells, induced by immunization with allergen-loaded BMDCs, when applied before the onset of the skin inflammation (preventive mode). Analysis of the mechanisms of the anti-inflammatory properties of IVM showed a direct effect on T cells because *in vitro* IVM treatment of mouse (and human) purified T cells impaired CD4 + and CD8 + T-cell activation and differentiation upon polyclonal and antigen-specific (data not shown) stimulation. Conversely, IVM had no major impact on the activation status of skin DC, both *in vivo* and *in vitro,* and on the migration of skin DC to regional lymph nodes, a phenomenon that is observed with other immunomodulatory drugs used in the treatment for AD, such as tacrolimus, rapamycin, and dexamethasone (27, 28). Our results reveal then an underestimated activity of IVM on T-cell-mediated skin inflammation.

In our study, allergic skin responses were triggered by the repeated administration of a mite protein extract in animals

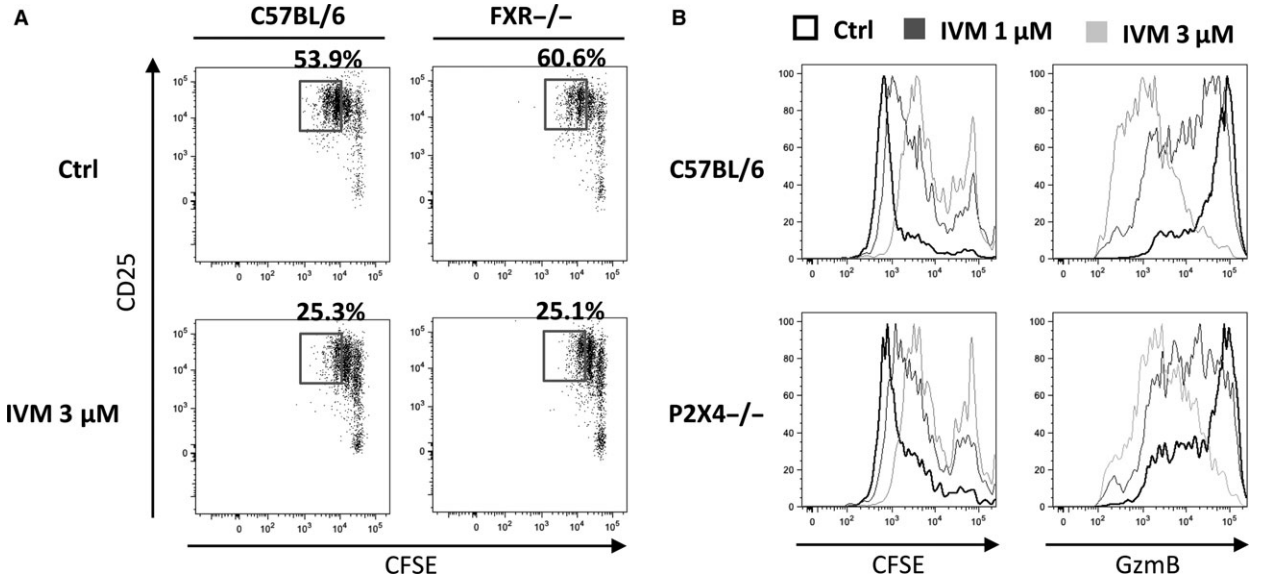

Figure 5 T cells from P2X4R−/− & FXR−/− animals are also sensitive to IVM activity *in vitro*. The anti-inflammatory properties of IVM were assessed using CFSE-labeled mouse T cells from C57BL/6, FXR−/− (A) and P2X4R−/− animals (B). T cells were stimulated, respectively, for 3 (A) or 5 (B) days with a mixture of anti-CD3 (1 µg/ml) + anti-CD28 (2 µg/ml) mAbs, treated with 1 or 3 µM IVM, or untreated. In A, FACS plots represent live T cells that have diluted CFSE and that expressed CD25 (figures represent the percentage of cells that have performed two to three rounds of division after 3 days of culture). In B, histograms depict live T cells that have diluted CFSE or that expressed GzmB markers. Results are representative of two to three independent experiments (n = 3 mice per group).

that were maintained in a specific-pathogen-free (SPF) environment. Hence, compared to a genuine axenic environment, we cannot neglect the fact that repeated epicutaneous applications of IVM could have had an impact on the populations of commensal parasites that colonize murine skin, and indirectly on the activation of skin immunity, as has been suggested for other commensal bacteria (29, 30). Nevertheless, so far, the contribution of saprophyte parasites to the stimulation of skin immunity remains undetermined, and our *in vitro* data clearly show that IVM regulates T-cell activation/reactivation independently of its activity on nematodes or arthropods.

Critical to the effect of a drug are the mode of administration and the respective exposure of target cells. The therapeutic effect of IVM administrated at the onset of the skin inflammation probably relies on inhibition of the reactivation of allergen-specific T cells that have been recruited into the skin to induce the clinical symptoms. Along this line, IVM can block the reactivation of already-primed T cells in ELISPOT cultures (Fig. 2B4). Alternatively, we demonstrated that the administration of the drug in a preventive mode in naïve animals blocks *in vivo* T-cell priming, without inhibition of skin DC migration/functions. This suggests that sufficient amounts of topically applied IVM reached the lymph nodes to hamper T-cell activation.

Our study showing that T cells are targets of IVM does not rule out the participation of other innate cells to the anti-inflammatory effects of IVM. Indeed, as mentioned above, several *in vitro* studies have reported immunomodulatory activities of IVM on mast cells and macrophages (31–33). These two cell types are key for the development of T-cell-mediated skin inflammatory diseases by promoting (i) skin emigration of allergen-loaded DC for T-cell priming (34), (ii) skin recruitment of effector T cells, and (iii) formation of macrophage-induced T-cell/DC clusters essential for the development of dermal skin inflammation (35). Therefore, it is possible that the therapeutic and preventive properties of IVM involve both a direct effect on T-cell stimulation and the ability to regulate the activity of skin mast cells and macrophages. Finally, it will also be interesting to study the effect of IVM on keratinocyte activation, and Derf-induced TSLP production, which initiates the pathology (36).

What are the mechanisms of IVM-induced inhibition of T-cell proliferation and activation? As already mentioned, the antiparasitic effects of IVM are mediated through binding to glutamate-gated chloride ion channels, which are expressed on the nerves and muscles of invertebrates. However, IVM also interacts with numerous ion channels, ion transporters, or FXR expressed by vertebrates (and by T cells) (2–4). Here, we explored the activity of IVM on two potential targets expressed by both CD4 + and CD8 + T cells: (i) FXR, because IVM binds FXR with high affinity and (ii) the purinergic P2X4 receptor (P2X4Rs) that is involved in the regulation of Ca+ metabolism, a critical checkpoint of T-cell activation. Our results showing that T cells from P2XR4−/− and FXR−/− animals are still sensitive to IVM inhibition suggest that the IVM-induced modulation of T-cell activation involves one or several other

potential ligands expressed by T cells. These may include ryanodine receptors (RyRs), critical regulators of Ca2 + influx into human T cells (37), or the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) type A receptors ($GABA_AR$s) and the type 3 5-hydroxytryptamine receptors (5-HT3Rs), as signaling via these receptors modulates cytokine expression and T-cell proliferation (38, 39). However, in large *in vitro* screening studies including binding and enzyme assays (Cerep profiling assays, Cerep, Poitiers, France), IVM (at 10 μM) did not interact with human $GABA_AR$, $5\text{-}HT_3$Rs, or rat RyR3, suggesting that these receptors are unlikely to contribute to IVM-induced T-cell modulation (data not shown). In summary, despite great efforts, we were unsuccessful in characterizing the mechanisms of IVM-induced inhibition of T-cell activation. Therefore, further studies are needed to identify IVM ligands (40, 41).

In conclusion, the present study, showing that topical IVM improves allergic skin inflammation by a mechanism involving T cells, provides important information for considering IVM as a new topical treatment for T-cell-mediated skin inflammatory pathologies.

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Author contributions

Erwan Ventre, Vanina Lenief, Floriane Albert, Léo Laoubi, and Patricia Rossio carried out the experiments and analyzed the data. Aurore Rozières, David Dombrowicz, Bart Staels, Lauriane Ulmann, Valerie Julia, and Emmanuel Vial participated in the interpretation of the data. André Jomard, Fériel Hacini-Rachinel, and Jean-François Nicolas supervised this study. Marc Vocanson and Jean-François Nicolas designed the experiments, analyzed the data, and wrote the manuscript. All authors had final approval of the submitted and published version.

Conflicts of interest

The authors declare no competing financial interests. Patricia Rossio, Valerie Julia, Emmanuel Vial, André Jomard, and Fériel Hacini-Rachinel are employed by Nestlé Skin Health R&D. All other authors are public employees from INSERM, University, and CNRS.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Supplementary Methods.

Figure S1. Proliferating cells in dLNs of C57BL/6 animals immunized with Derf-pulsed BMDCs and treated topically with IVM.

Figure S2. GzmB, IFN-γ and IL-4 expression upon polyclonal stimulation of murine splenocytes.

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