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Cutting Edge: PD-1 Regulates Imiquimod-Induced Psoriasiform Dermatitis through Inhibition of IL-17A Expression by Innate γδ-Low T Cells

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Programmed cell death 1 (PD-1) is a key regulatory molecule that has been targeted in human cancers, including melanoma. In clinical testing, Abs against PD-1 have resulted in psoriasiform dermatitis (PsD). To determine whether PD-1 regulates PsD, we compared skin responses of PD-1–deficient (PD-1KO) mice and wild-type (WT) controls in an imiquimod (IMQ)-induced murine model of psoriasis. PD-1KO mice showed severe epidermal hyperplasia, greater neutrophil infiltration, and higher expression of Th17 cytokines (versus WT mice). IMQ exposure increased PD-1 expression by skin γδ-low (GDL) T cells and enhanced expression of PD-L1 by keratinocytes. Three-fold increases in the percentage of IL-17A+ GDL T cells were observed in skin cell suspensions derived from IMQ-treated PD-1KO mice (versus WT controls), suggesting that the lack of PD-1 has a functional effect not only on αβ T cells, but also on GDL T cells, and that PD-1 may play a regulatory role in PsD. The Journal of Immunology, 2015, 195: 000–000.

Programmed cell death 1 (PD-1) is a membrane receptor that delivers inhibitory signals to T cells and other immune cells through interactions with two major ligands, programmed death ligand 1 and 2 (PD-L1 and PD-L2) (1). Treatment with nivolumab, an anti–PD-1 mAb, in combination with ipilimumab (anti–CTLA-4) for patients with melanoma has been reported to lead to impressive improvements in clinical responses, including overall survival (2). When combined with ipilimumab, the use of nivolumab results in up to 65% of patients developing an uncharacterized skin rash, depending on the dosing of the two agents. When used alone, nivolumab resulted in a 15% incidence of skin eruption in patient cohorts from Europe, North and South America, and the Middle East (3). Interestingly, ~3% of melanoma patients treated in Japan with nivolumab developed a psoriasiform dermatitis (PsD) (4). Given that the estimated prevalence of psoriasis in general Japanese populations is only 0.3% (5), treatment with a PD-1 antagonist resulted in a dramatic increase of a psoriasis-like skin eruption in Japanese patients.

PD-1 genetic deficiency in mice leads to the development of autoimmune dilated cardiomyopathy or lupus-like autoimmune phenotypes, depending on the genetic background (1, 6). Mutations in PD-1 in humans have been associated with autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and type 1 diabetes among others (7). PD-1 and its ligand, PD-L1, are involved in controlling contact dermatitis (8) and graft-versus-host disease (9), but the role of PD-1 axis in PsD has not been established. We and others have shown that unconventional γδ T cells migrate into skin and express cytokines such as IL-22 and IL-17A, which play critical roles in development of PsD induced by IL-23 and imiquimod (IMQ), a TLR7 agonist (10–14). In contrast to resident Vγ5+ γδ T cells in mouse epidermis that do not express significant levels of IL-17A and IL-22, dermal and epidermal γδ T cells in IMQ- and IL-23–treated mouse skin express Vγ4 (15) and low to intermediate levels of the γδ receptor and thus have been termed γδ-low (GDL) T cells (13). GDL T cells are the major producers of IL-17A and IL-22 in the psoriatic epidermis (10–14). Mice that are defective in the transcription factor Sox13 were shown to selectively lack Vγ4+ γδ T cells and were partially protected from IMQ-induced PsD (15).

In this study, we evaluated the role of PD-1 in the mouse model of psoriasis. Our data show that the genetic deficiency of PD-1 enhanced the phenotype of psoriasis-like inflammatory skin disease. Moreover, we show that GDL T cells in the skin constitutively express PD-1, and that PD-1 level is further upregulated upon IMQ treatment. PD-1 genetic deficiency

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Abbreviations used in this article: GDL, γδ-low; IMQ, imiquimod; PD-1, programmed cell death 1; PD-1KO, PD-1−/−; PD-L1, programmed death ligand 1; PD-L1−/−, programmed death ligand 1−/− fusion protein; PD-L2, programmed death ligand 2; PsD, psoriasiform dermatitis; RT-PCR, quantitative real-time PCR; WT, wild-type.
promoted psoriatic inflammation by enhancing the production of IL-17A and IL-22 by γδ T cells and by greatly increasing neutrophil infiltration into the epidermis.

Materials and Methods

Mice

C57BL/6j mice (8–12 wk of age) were purchased from The Jackson Laboratory or Charles River Laboratories and used with approval by the Animal Care and Use Committees at the Medical College of Wisconsin. PD-1–deficient (PD-1KO) mice were provided by T. Honjo (6).

IMQ-induced psoriasis model

Mice were treated daily for 5 d on each ear with 5 mg 3.5% IMQ cream, which was diluted from 5% IMQ cream (Taro Pharmaceuticals, New York, NY) with control vehicle cream (Vanicream; Pharmaceutical Specialties, Cleveland, GA) (15).

Ab treatment

Mice received i.p. injections with 200 μg/mouse of either anti–PD-1 (clone J43) or control hamster IgG (Bio X Cell, West Lebanon, NH) in a total volume of 0.2 ml 2 h before application of IMQ at days 0, 2, and 4 (8).

In vitro plate-bound T cell activation assay

Skin cells (200,000 cells/well) were cultured in 96-well flat-bottom plates in the presence of either PD-L1 lg fusion protein (PD-L1-lg; BPS Bioscience, San Diego, CA) (16) or IgGl isotype control (ALX-804-133, Alexi Biochemicals, San Diego, CA). Replicate cultures were in complete RPMI 1640 medium supplemented with 10% FBS. Cultures were analyzed after overnight incubation at 37°C.

Flow cytometry

Anti-mouse γδ-TCR (clone GL3), CD45 (30-F11), Sca-1 (D7), Vy6 (UC3-10A6), PD-1 (29F.1A12), PD-L1 (10F.9G2), and PD-L2 (TY25) Abs were purchased from BioLegend (San Diego, CA). Anti–IL-17A (eBio17B7) mAb was purchased from eBioscience (San Diego, CA). Ear skin was digested to obtain skin cell suspensions (15). Intracellular staining was done after incubating cells for 4 h with brefeldin A and PMA/ionomycin as described (13, 14). Flow cytometry was performed using an Acuri C6 or LSR II (BD Biosciences, San Jose, CA) in conjunction with FlowJo version 10.0.7 analysis software (Tree Star, San Carlos, CA).

Quantitative real-time PCR

Total RNA of mouse skin was prepared using an RNeasy Fibrous Tissue Kit (Qiagen, Hilden, Germany), and quantitative real-time PCR (RT-PCR) was performed via a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA) (13).

Histopathological analysis

Routine H&E staining was performed on formaldehyde-fixed, paraffin-embedded skin samples. For immunohistochemistry, we stained paraffin-embedded skin specimens with Abs specific for anti-phospho-STAT3 (9131, Cell Signaling Technology, Beverly, MA) (17). Images were acquired using an Infinity3-1C digital camera (Lumenera, Ottawa, Canada) attached to a Carl Zeiss microscope. Epidermal thickness was measured at four different points on the image using Infinity Analyze version 5.0.3 software (Lumenera). Abscess area was measured by ImageJ version 1.48 software.

Statistical analysis

All data are expressed as mean ± SEM. Data were analyzed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA). Simple comparisons of means and SEM of data were made by using a two-sided Student’s t test. A p value < 0.05 was considered statistically significant.

Results and Discussion

PD-1 genetic deficiency results in enhanced dermal inflammation, epidermal acanthosis, and neutrophilic abscess formation following application of IMQ

To determine whether PD-1 regulates the development of PsD, we treated PD-1KO mice and wild-type (WT) control littermates with topical IMQ in a well-known model of PsD. Untreated (Supplemental Fig. 1A) or treated with vehicle alone (data not shown), PD-1KO mice under 8 wk of age had no obvious inflammatory or epidermal thickness changes in skin compared with WT mice. Whereas application of a 5% IMQ resulted in no significant difference between PD-1KO mice and WT mice (Supplemental Fig. 1B), application of 3.5% IMQ to mouse ears resulted in clearly enhanced ear swelling in PD-1KO mice when compared with the WT controls (Fig. 1A). Next, we blocked PD-1 in WT mice with PD-1–specific mAb to exclude the possibility that the result in PD1-KO mice was due to broader immunologic abnormalities in PD-1KO animals. Mice treated with anti–PD-1 mAb developed enhanced ear swelling compared with mice treated with isotype Abs (Fig. 1B). Histological examination of WT mice showed that 3.5% IMQ treatment resulted in mild epidermal acanthosis and relatively little neutrophil infiltration into the epidermis and cornified layer. In contrast, PD-1KO mice developed marked acanthosis of the epidermis as well as dense epidermal neutrophilic abscesses (Fig. 1C). In human psoriatic skin, such neutrophilic abscesses are called Munro’s abscesses and are a histologic hallmark of human psoriasis. Quantitative histological analysis revealed an ∼2-fold increase in epidermal thickness (epidermal acanthosis) in the PD-1KO mice compared with WT mice (Fig. 1D). Additionally, the epidermis of PD-1KO mice accumulated ∼20-fold more surface area composed of neutrophilic abscesses (Fig. 1E). Taken together, these data suggest that PD-1 blockade either by genetic knockout or mAb treatment pro-
motes psoriasiform skin inflammation induced by topical IMQ treatment.

**Enhanced cutaneous expression of inflammatory cytokines in PD-1KO mice**

Multiple inflammatory cytokines, such as IL-17A and IL-22, which are produced by Th17 CD4⁺ helper cells and γδ⁺ T cells, as well as neutrophil-attracting chemokines, are critical for the pathogenesis of psoriasis (18). To determine whether the IMQ-induced cytokine milieu in the skin was altered with PD-1 deficiency, we examined the mRNA level of inflammatory cytokines in the ear skin of PD-1KO and WT mice using RT-PCR. Unstimulated PD-1KO skin showed similar (low) cytokine/chemokine levels as WT mice (data not shown). Both IL-17A and IL-22 were enhanced in the PD-1KO mice after application of IMQ (Fig. 2). IL-22 has been linked to epidermal acanthosis (11), possibly explaining the increased epidermal thickening in PD-1KO mice following IMQ treatment. Next, to gain mechanistic insight regarding the elevated neutrophil influx in the epidermis of PD-1KO mice, we quantified the levels of neutrophil-attracting chemokines and chemokine receptors via RT-PCR analysis. Marked increases of neutrophilic chemokines CXCL1, CXCL2, CXCL5, CXCR2, a chemokine receptor expressed on neutrophils, as well as the neutrophil surface marker Ly6g were observed in the PD-1KO mice (Fig. 2). We also found similar trends in anti–PD-1 mAb-treated mice (Supplemental Fig. 1C). Taken together, these data suggest that IMQ treatment in PD-1KO mice results in the production of enhanced levels of inflammatory cytokines and chemokines, which in turn recruit more neutrophils to the inflamed skin lesion to amplify psoriatic inflammation.

**Expression of PD-1 is upregulated in the skin, but not lymph node, after treatment with topical IMQ**

We and others have previously reported that Vγ4⁺ GDL T cells migrate into skin in response to IL-23– or IMQ-induced inflammation and become the predominant IL-17/IL-22–producing cells that drive PsD in murine models (10–14). PD-1 expressed on GDL T cells might directly control their activation. To determine whether GDL T cells express PD-1, ear skin cells were harvested from untreated mice as well as mice following application of IMQ and examined by flow cytometry. PD-1 expression was detected on GDL T cells from untreated mice but not resident epidermal γδ-high T cells (Fig. 3A). Expression level of PD-1 was higher on GDL T cells when compared with CD45⁺, γδ⁻ cell types (Fig. 3A). Furthermore, compared with vehicle-treated mice, GDL T cells from skin, but not lymph node, of IMQ-treated mice showed enhanced expression of PD-1 (Fig. 3B). Thus, PD-1 shows steady-state expression in GDL T cells in the skin, which is elevated on these cells following treatment with IMQ.

**Increased expression of inflammatory IL-17A in GDL T cells of PD-1KO mice following IMQ treatment**

PD-L1, the ligand for PD-1, has been shown to be expressed by keratinocytes in models of inflammatory skin disease, including graft-versus-host disease (9) and contact dermatitis (19), but not in PsD. Expression of activated phospho-STAT3, a key mediator of IL-22–induced epidermal proliferation, was confirmed by immunohistochemistry in IMQ-treated skin (Fig. 3C). Because phospho-STAT3 has been shown to induce expression of PD-L1 (20), further flow cytometry analysis was carried out to examine the level of PD-L1. Of note, PD-L1, but not PD-L2, was upregulated on IMQ-treated keratinocytes (Fig. 3D). These data suggest that during IMQ treatment, PD-L1 is upregulated on keratinocytes, potentially suppressing activation of GDL T cells via its interaction with PD-1. Consistent with this hypothesis, PD-1KO GDL T cells isolated from the cervical lymph node (Fig. 3E, 3F) and ear skin (Fig. 3G, 3H) expressed ~2- to 3-fold more IL-17A when compared with WT GDL T cells.

To determine whether PD-1 expressed on GDL T cells plays a suppressive role in the cytokine production of GDL T cells, GDL T cells were isolated from IMQ-treated WT mice after 5 d and subjected to in vitro restimulation in the presence of plate-bound PD-L1–Ig or control Ig. PD-L1–Ig contains the extracellular domain of PD-L1 fused to the human IgG1Fc...
The use of blocking Abs to checkpoint inhibitors such as CTLA-4 and PD-1 has revolutionized the treatment of advanced melanoma and other cancers (2, 3). These agents, however, cause a number of autoimmune-related side effects, including dermatitis (21, 22). Striking increases in the natural prevalence of psoriasis have been reported in Japanese patients who have been treated with PD-1 inhibitors in initial clinical testing (4). Our results show that either PD-1 genetic deficiency or PD-1 blockade by specific mAb markedly exacerbated PsD in mice. Enhancement of PsD was observable at moderate (3.5%), but not high (5%), stimulatory concentrations of IMQ, suggesting that the regulatory roles of PD-1 are most readily detectable when less than maximal levels of IMQ are used. Similarly, enhanced skin graft-versus-host disease was only observed when limiting number of PD-1-deficient OT-1 T cells were adoptively transferred into recipient mice that expressed OVA in the epidermis (9). We hypothesize that subtle mutations of PD-1, PD-L1, or other checkpoint inhibitors may help determine if a given individual develops psoriatic lesions. Although genetic studies in psoriatic patients have revealed multiple genes associated with psoriasis susceptibility, PD-1 has not been implicated to date. One study, however, has identified a psoriasis-associated single nucleotide polymorphism on a RUNX1-binding site located between SLCA93R1 and NAT9 that may interfere with the RUNX1-mediated induction of PD-1 (23).

Although our data suggest that PD-1 on GDL T cells regulates the production of key Th17 cytokines, an adoptive transfer of GDL T cells from PD-1KO mice into WT mice (and vice versa) would be needed to show that GDL T cells are the only cells that are affected by the absence of PD-1. This limitation of the present study will be addressed in future studies, but the increased production of IL-17A in PD-1KO GDL T cells, as well as the suppressive effect of PD-1 engagement via its ligand PD-L1, provides a plausible mechanism for the enhancement of PsD in IMQ-treated PD-1KO mice. Our work also suggests a potential mechanism for the increase of PsD that was observed in patients clinically treated with PD-1 Abs.

Disclosures
The authors have no financial conflicts of interest.

References

FIGURE 3. GDL T cells express PD-1 and keratinocytes express PD-L1. (A) Suspensions of skin cells from untreated WT mice were analyzed by flow cytometry. The cells were gated on CD45+ cells. GDL T cells (circled) express PD-1 at steady-state. (B) Three days after 3.5% IMQ or vehicle cream application, skin suspensions of cells and cervical lymph node (LN) cell were analyzed. Analysis was gated on GDL T cells. Epidermal keratinocytes of 3.5% IMQ-induced psoriasiform lesions show activated STAT3. H&E staining of phospho-STAT3 (Tyr705) in nuclei of psoriatic keratinocytes. 3.5% IMQ-induced psoriasiform lesions show activated STAT3. H&E staining of phospho-STAT3 (Tyr705) in nuclei of psoriatic keratinocytes. Scale bar, 50 μm. (C) Keratinocytes of mice with IMQ-induced PsD express PD-L1 but not express PD-L2. Three days after 3.5% IMQ or vehicle application, keratinocytes (CD45–Sca-1–) were analyzed by flow cytometry. Five days after 5% IMQ cream application, skin suspensions of cells were stimulated with or without 10 μg/ml plate-bound anti-CD3 and anti-CD28 antibodies for 16 h, then analyzed by flow cytometry. Expression of IL-17A was gated on TCR Vγ9+ cells (I). The proportion of IL-17A+ GDL T cells was determined by flow cytometry. The proportion of IL-17A+ GDL T cells was determined by flow cytometry. Figure 3 continued on next page...


