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CD1d deficiency limits tolerogenic properties of peritoneal macrophages

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Running title: Immunosuppressive effect of peritoneal macrophages

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ABSTRACT

Invariant natural killer T (iNKT) cells are involved in various autoimmune diseases. Although iNKT cells are arthritogenic, transforming growth factor beta (TGFβ)-treated tolerogenic peritoneal macrophages (Tol-pMφ) from wild-type (WT) mice are more tolerogenic than those from CD1d knock-out iNKT cell-deficient mice in a collagen-induced arthritis (CIA) model. The underlying mechanism by which pMφ can act as tolerogenic antigen presenting cells (APCs) is currently unclear. To determine cellular mechanisms underlying CD1d-dependent tolerogenicity of pMφ, *in vitro* and *in vivo* characteristics of pMφ were investigated. Unlike dendritic cells or splenic Mφ, pMφ from CD1d$^{+/-}$ mice showed lower expression levels of costimulatory molecule CD86 and produced lower amounts of inflammatory cytokines upon lipopolysaccharide (LPS) stimulation compared to pMφ from CD1d-deficient mice. In a CIA model of CD1d-deficient mice, adoptively transferred pMφ from WT mice reduced the severity of arthritis. However, pMφ from CD1d-deficient mice were unable to reduce the severity of arthritis. Hence, the tolerogenicity of pMφ is a cell-intrinsic property that is probably conferred by iNKT cells during pMφ development rather than by interactions of pMφ with iNKT cells during antigen presentation to cognate T cells.

Keywords: CD1d, NKT cells, Rheumatoid Arthritis, CIA, peritoneal macrophage

INTRODUCTION

T cell receptor (TCR) complexes of conventional $CD4^+$ and $CD8^+$ T cells are known to generate essential biochemical signals to initiate T cell immunity upon recognizing antigenic peptides derived from lysosomal or proteosomal proteolytic processing. Antigenic peptides are presented by major histocompatibility complex (MHC) molecules widely expressed on professional antigen presenting cells (APCs) including dendritic cell (DCs), macrophages, and B cells (1,2). In contrast to conventional T cell activation, invariant natural killer T (iNKT) cells can recognize lipid or glycolipid antigens presented on CD1d, an MHC class I (MHC-I)-like molecule, and show prolonged cytokine production upon activation (3). CD1d is a cell surface glycoprotein comprising a heavy chain in non-covalent association with a β2-microglobulin light chain. It is broadly expressed in lymphoid and myeloid cells (4). iNKT cells can be distinguished based on their TCR expression. They share some markers such as CD161 (NK1.1 in mice) and NKR-P1 that are characteristics of natural killer cells (3). iNKT cells can produce a wide variety of cytokines including proinflammatory and anti-inflammatory cytokines with multiple effects on the outcome of immune reactions (5). iNKT cells can also be activated in the absence of foreign microbial challenges, suggesting that they might occupy some immunological niches under immunologically quiet time and inflammatory condition (6).

During the past several years, regulatory and autoimmune roles of iNKT cells have been characterized. However, contrasting results have been observed using various approaches pertaining to iNKT cell-targeted treatments. It is currently unclear whether their effects are beneficial or detrimental to the host (5,7,8). Differing effects of iNKT cells in various systems reflect their ability to inform or influence functions of APCs (6). It has been recently shown that

iNKT cells can reverse suppressive types of regulatory APCs known as myeloid-derived suppressor cells (MDSCs) into DCs to stimulate Th1 T cell responses (9). However, repeated administration of α-Galcer can result in an exhausted phenotype of iNKT cells that provides altered signals to DC and induces regulatory DC phenotypes that can prevent the onset of autoimmunity and silence autopathogenic T cells (10). Other studies have shown that CD1d-dependent iNKT cells play crucial roles in reducing joint inflammation (11). These effects were correlated with other autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) (12).

Macrophages play pivotal roles in rheumatoid arthritis (RA). They are prevalent in inflamed synovial membranes and at the cartilage–pannus junction. These cells possess broad proinflammatory, destructive, and remodeling capacities that contribute to acute and chronic phases of RA. Previously, it has been demonstrated that augmented recruitment and enhanced function of APCs are key steps associated with innate and adaptive immunity (13).

We have previously reported that TGFβ-treated tolerogenic pMφ from CD1d$^{+/-}$ mice, but not from CD1d KO (iNKT cell-deficient) mice, can facilitate APC-mediated suppression of CIA (14). In this study, we expanded these initial findings and investigated capabilities of pMφ. We found that their characteristics as tolerogenic APCs to suppress CIA were mostly cell-intrinsic rather than caused by TGFβ treatment.

RESULTS

Involvement of iNKT cells in anti-inflammatory phenotypes of peritoneal macrophages

iNKT cells not only can regulate local immune effector functions, but also can promote or inhibit priming of adaptive immune responses by releasing cytokines to induce APCs toward immunogenic or tolerogenic phenotypes (10). Tolerogenic effects of TGF-β_2-treated APCs (Tol-APCs) require iNKT cells to mediate the suppression of CIA (14). Based on these findings, we initially determined whether pMφ from CD1d$^{+/-}$ mice and CD1d$^{-/-}$ (iNKT cell-deficient) mice possessed intrinsic variances that might contribute to differential responses of CIA. Results validated previous findings, demonstrating that transfer of pMφ from CD1d$^{+/-}$ mice lowered incidences and clinical CIA scores compared to transfer of pMφ from CD1d$^{-/-}$ mice (Figure 1). These results suggest that iNKT cells might be involved in the tolerogenicity of CD1d$^{+/-}$ pMφ. Therefore, we further assessed characteristics of these cells from CD1d$^{+/-}$ and CD1d$^{-/-}$ mice by observing changes in costimulatory molecule expression with or without LPS stimulation. Results showed that CD86 levels were significantly lower in pMφ from CD1d$^{+/-}$ mice. However, there was no significant difference in MHC II level (Figure 2A). Other markers such as CD11c, CD80, CD206, F4/80, PD-L1, and PD-L2 showed no significant difference in their expression between pMφ from CD1d$^{+/-}$ mice and pMφ from CD1d$^{-/-}$ mice (Supplemental Figure 1). There were no significant differences in the expression of CD1d, MHC II, CD11b, CD80, or CD86 either between pMφ and Tol-pMφ (data not shown). Secretion of inflammatory cytokines such as TNF-α and IL-6 was markedly reduced in pMφ from CD1d$^{+/-}$ mice compared to that in pMφ from CD1d$^{-/-}$ mice after LPS stimulation (Figures 2B). In contrast, secretion of anti-inflammatory IL-10 in pMφ from CD1d$^{+/-}$ mice was significantly higher than that in pMφ from CD1d$^{-/-}$ mice

(Figure 2B), further suggesting that differences in pMφ between CD1d$^{+/-}$ and CD1d$^{-/-}$ mice were probably associated with the presence or absence of iNKT cells during pMφ maturation.

CD1d$^{+/-}$ peritoneal macrophages attenuate *in vitro* CD4^{+} T cell activation

To confirm tolerogenic phenotypes of pMφ from CD1d$^{+/-}$ mice, *in vitro* CD4 T cell-stimulating capacities of pMφ as APCs were measured. To this end, we isolated and cocultured OVA-specific TCR transgenic OT-II CD4 T cells with OVA-loaded pMφ from CD1d$^{-/-}$ and CD1d$^{+/-}$ littermate mice for 72 h *in vitro*. TGFβ$_2$-treated pMφ from CD1d$^{+/-}$ mice exhibited lower percentage of OT-II cell proliferation (30.02 ± 2.73%). However, stronger proliferation of OT-II cells was observed in TGF-β$_2$-treated pMφ from CD1d$^{-/-}$ mice (48.13 ± 2.86%). Similarly, OVA-loaded pMφ from CD1d$^{+/-}$ mice showed decreased proliferation of OT-II cells (33.5 ± 3.44%) in comparison with pMφ from CD1d$^{-/-}$ mice (50.13 ± 1.47%) in CFSE-dilution assay (Figures 3A and 3B). IFN-γ and IL-4 are widely used markers of CD4^{+} T cell effector function in immune regulation (15). ELISA measurements of IFN-γ in culture supernatants revealed that both CD1d$^{+/-}$ pMφ and Tol-pMφ-stimulated OT-II cells, showing significantly lower IFN-γ production than CD1d$^{-/-}$ pMφ and Tol-pMφ-stimulated OT-II cells, respectively (Figure 3C). Neither pMφ nor Tol-pMφ-stimulated OT-II produced measurable levels of IL-4 (data not shown). These results suggest that the presence of CD1d-restricted NKT cells might be a factor conferring the ability of pMφ to attenuate CD4^{+} T cell activation.

Differential CD4 T cell responses were observed upon stimulation with antigen-loaded pMφ matured in environments with differential CD1d expression. Therefore, we determined whether

DCs from peritoneum/macrophages and DCs from other tissues had similar differential characteristics depending on CD1d expression. Splenic DCs and splenic Mφ showed no significant differences in CD1d-dependent CD80 or CD86 expression (data not shown). $CD1d^{-/-}$ peritoneal dendritic cells (pDCs) showed no increase in CD4 T cell stimulation compared to $CD1d^{+/-}$ pDCs either in CFSE dilution assays. These results suggest that iNKT cell-mediated tolerogenicity of APCs is CD1d-dependent and uniquely evident in pMφ.

$CD1d^{+}$ peritoneal macrophages exhibit tolerogenic effects in a CIA model of CD1d KO host

We have previously shown that iNKT cells are critical for the induction of Tol-APC-mediated suppression of CIA (14). Thus, we further investigated whether the tolerizing potential was an intrinsic character of pMφ rather than an acquired phenotype following interactions with iNKT cells during immune responses. To exclude iNKT cells' involvement, we adoptively transferred $CD1d^{+/-}$ Tol-pMφ or $CD1d^{-/-}$ Tol-pMφ cells into CIA-induced CD1d KO DBA/1 mice that lacked iNKT cells. When disease progression in mice was compared, treatments by $CD1d^{+/-}$ Tol-pMφ showed significantly reduced percentages of incidence (Figure 4A, n = 4-5 mice) and clinical scores (4.8 ± 1.8 vs. 7.4 ± 2.0 at day 48) compared to treatments by $CD1d^{-/-}$ Tol-pMφ (Figure 4A). These data validated the ability of $CD1d^{+/-}$ Tol-pMφ to ameliorate CIA by attenuating $CD4^{+}$ T cell activation. In parallel, we compared effects of pMφ in $CD1d^{+/-}$ and $CD1d^{-/-}$ mice with CIA. $CD1d^{+/-}$ pMφ showed significantly lower incidence and severity of arthritis than those of $CD1d^{-/-}$ pMφ (6.0 ± 1.1 vs. 9.7 ± 0.7 at day 50; Figure 4B). Taken together with data from experiments using Tol-pMφ, these results clearly demonstrate that CD1d-expressing pMφ have intrinsic

138 immunosuppressive functions, suggesting that iNKT cells possibly can confer these tolerogenic
139 activities during the development of pMφ.

140

DISCUSSION

Selective moderation of macrophage activation remains an attractive therapeutic approach to diminish local and systemic inflammation for preventing irreversible joint damage because the activation of monocytic lineage is not locally restricted, but extended to systemic parts of the mononuclear phagocyte system (16). Activation of APCs following interactions with iNKT cells may occur during immune activation and quiescence. Thus, interactions of iNKT cells with APCs might have proinflammatory or tolerizing outcomes, suggesting that effector and regulatory iNKT cells can coexist (6).

Induction of antigen-specific tolerance is critical for preventing autoimmunity and maintenance of immune tolerance. TGF-β_2-treated Tol-APCs are known to induce anterior chamber-associated immune deviation (ACAID)-like tolerance (17). ACAID is a peripheral tolerance that protects eye tissues from destructive inflammation. It is mainly mediated by eye-derived APCs and B cells (18), αβ T cells (19), and NKT cells (20). Earlier data have shown that ACAID tolerance can be induced by Tol-APCs by inhibiting CIA and its related systemic immune responses in murine arthritis models following a single injection of Tol-APCs where iNKT cells are associated with a shift from Th1 to Th2 responses of CII-specific T and B cells (14).

CD1d antigen presentation is defective in some patients with RA, showing reduced iNKT cell numbers and altered functions (21). APCs express functional CD1d molecules on their surface to retain suppressive capacities following burn injury-induced immune suppression (22). B cells expressing CD1d are also required for iNKT cells to facilitate enhanced antibody production (23). The ensuing mechanism is probably restricted to T-independent antigens (24). Sonoda et al. (20) have also demonstrated that CD1d on APCs is required to generate immunologic tolerance and

shown that CD1d-positive APCs could induce iNKT cell-dependent efferent T cell tolerance of antigens inoculated via eyes. Although the requirement of CD1d expression on APCs in previous studies has highlighted the direct role of iNKT cells, results of the present study using iNKT cell-deficient mice suggest that in addition to direct interactions of APCs with iNKT, maturation of APCs in the presence of iNKT cell also contributes to the suppressive phenotype of pMφ, thereby suppressing CIA.

Our *in vivo* studies showed that both $CD1d^{+/-}$ pMφ and Tol-pMφ contributed to lower clinical scores and incidences in iNKT cell-deficient CIA model, thus ruling out the direct action of iNKT cells on these APCs. LPS-induced expression of costimulatory molecules and cytokine secretions corroborated suppressive or tolerogenic capacities of $CD1d^{+/-}$ pMφ. Because we ruled out the direct contribution of iNKT cells at the time of antigen recognition on pMφ using $CD1d^{-/-}$ hosts where iNKT cells were deficient, long-term education by iNKT cells during development of donor $CD1d^{+/-}$ pMφ might have led to intrinsic immunosuppressive properties of pMφ. Because of this cell intrinsic immunosuppressive property of pMφ, we could not exclude the possibility that CD1d expression itself on pMφ may exert an immunosuppressive effect by some unknown mechanisms.

In summary, our results revealed that CD1d-expressing pMφ suppressed $CD4^+$ T cell proliferation after coculture, leading to down-regulation of Ag-specific IFN-γ production by CD4 T cells. We also observed lower CIA severity both in $CD1d^{+/-}$ and $CD1d^{-/-}$ recipient mice after adoptive transfer of $CD1d^{+/-}$ pMφ, but not by such transfer of $CD1d^{-/-}$ pMφ. These observations suggest that CIA suppression is mediated by CD1d-expressing pMφ and that tolerogenicity of pMφ is a cell-intrinsic property probably conferred by iNKT cells during pMφ development. Emerging

evidence indicates that environmental factors can shape the identity of tissue resident macrophages. Therefore, earlier education by NKT cells potentially contributes to tolerogenic programs of pMφ, thus influencing the course of the CIA.

MATERIALS AND METHODS

WT C57BL/6 mice were purchased from Orient Bio (Seongnam-si, Gyeonggi-do, Korea). C57BL/6 CD1d$^{-/-}$ mice used in this study were provided by Albert Bendelac's lab (3). All animal experiment protocols adapted in this study were approved by the Institutional Animal Care and Use Committee of Korea University (KUIACUC-2018-25). DBA/1 mice were purchased from Charles River Laboratories (Japan) and backcrossed more than eight times with C57BL/6 CD1d$^{-/-}$ mice to generate DBA/1CD1d$^{-/-}$ mice. Mice with DBA background were used for *in vivo* arthritis induction. All other experiments were performed using mice with C57BL/6 background. OT-II TCR transgenic (Tg) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Complete Freund's adjuvant (CFA), *Mycobacterium tuberculosis*, ovalbumin (OVA), and carboxyfluorescein succinimidyl ester (CFSE), a fluorescent cell staining dye, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chicken OVA$_{323–339}$ peptide (H-2^{d} restricted; amino acid sequence ISQAVHAAHAEINEAGR) was purchased from GenScript (Piscataway, NJ, USA). Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies, Rockville, MD, USA) supplemented with 2-mM L-glutamine, 50-U/ml penicillin, 50-μg/ml streptomycin, 10-μg/ml gentamicin sulfate, 50-μM β-mercaptoethanol (Life Technologies), and 10% heat-inactivated FBS (Hyclone, Logan, UT, USA). IL-4 and IFN-γ concentrations were measured using OPTEIA Mouse IL-4 and IFN-γ enzyme-linked immunoassay kits (BD Pharmingen, San Diego, CA, USA), respectively.

In vitro generation of tolerogenic pMφ

Peritoneal exudate cells (PECs) were prepared after collecting peritoneal washes of C57BL/6 or DBA/1 mice at three days after intraperitoneal (i.p.) injections of 3 ml of 3% thioglycolate solution (Sigma-Aldrich). Isolated PECs (pMφ) were then cultured overnight in a serum-free medium. For the generation of Tol-pMφ, 5 ng/ml TGF-β_2 (R&D systems, Minneapolis, MN, USA) was added into the culture medium. After culture, pMφ and Tol-pMφ were washed three times with phosphate buffer saline (PBS). Remaining adherent cells were subjected to cold stress at 4°C in PBS for 2 h and then collected by vigorous pipetting. Cells were then washed three times with PBS and resuspended in PBS to cell density of 1×10^6 cells/ml (17). Subsequently, cells were stained with anti-CD11b and CD11c antibodies. Typical pMφ phenotypes ($CD11b^+CD11c^+$) were confirmed in more than 90% of cells. To transfer pMφ into CIA-induced mice, 100 µl of cell suspensions containing 1×10^6 cells CII in incomplete Freund's adjuvant (IFA; Sigma-Aldrich) was injected into tail veins at 7 days after the second immunization.

CIA induction and assessments of clinical scores

DBA/1 mice were immunized intradermally (i.d.) at the base of the tail with 100 µg of chicken type CII (Sigma-Aldrich) emulsified with an equal volume (50 µl) of CFA (Sigma-Aldrich) according to a standard method (25). Mice were boosted by i.d. injections with 100 µg of CII emulsified in IFA on day 21. Seven days later, mice received intravenous (i.v.) injections of either pMφ or Tol-pMφ at 1×10^6 cells/mouse. Mice were then monitored on alternate days for the development of arthritis until the end of the experiment. Arthritis severity was graded as follows: 0 = normal paws; 1 = edema and erythema in only one digit; 2 = slight edema or erythema in multiple digits; 3 = slight edema involving the entire paw; 4 = moderate edema and erythema

231 involving the entire paw; and 5 = severe edema and erythema involving the entire paw and
232 subsequent ankylosis. Cumulative values were determined for all paws, with a maximum score
233 of 20. Average macroscopic scores were then calculated.
234
235 ***In vitro* OVA-specific CD4$^+$ T cell responses**
236 Effector CD4$^+$ T cells were obtained from OT-II transgenic mice immunized subcutaneously (s.c.)
237 using 100 μg of OVA protein in CFA. After two weeks, primed CD4$^+$ T cells were sorted using
238 antibody-coated magnetic beads and labeled with 5 μM CFSE. Purified CD4$^+$ T cells (5×10^5
239 cells/well) were then added into 24-well plates containing OVA-loaded pMφ or Tol-pMφ ($5 \times$
240 10^4 cells/well). After three days, culture supernatants were collected and analyzed for cytokines
241 using enzyme-linked immunosorbent assay (ELISA) kits. Cultured cells were then harvested for
242 proliferation assays using CFSE dilution.
243
244 **Measurements of costimulatory molecules and cytokine secretions**
245 In addition to pMφ, splenic macrophages and dendritic cells were isolated using anti CD11b- and
246 CD11c-magnetic beads through magnetic-activated cell sorting (MACS). Expression levels of
247 CD11c, CD11b, F4/80, CD80 (B7-1), CD86 (B7-2), CD1d (1B1), CD206, and MHC II (IA^b)
248 were analyzed using a FACSVerse flow cytometer (BD). Culture supernatants were then assayed
249 for IL-6, IL-10, and TNF-α levels using relevant enzyme-linked immunoassay kits (BD
250 Pharmingen, San Diego, CA, USA) after stimulation with LPS from *Escherichia coli* 055:B5 at
251 various concentrations.
252

Flow cytometric analysis

Cells were stained with anti-FcR-γ mAb (2.4G2) at 4°C for 20 min in FACS staining buffer (PBS containing 0.1% BSA and 0.01% sodium azide). Cells were then stained with the following mAbs (BD Biosciences) for an additional 30 min: TCRβ (H57), CD4 (RM4-5), CD8α (53-6.7), IFN-γ (XMG1.2), and IL-4 (11B11). Stained cells were then analyzed using a FACS Calibur or FACS Verse and analyzed with FlowJo program.

Statistical analysis

Differences in clinical data between groups were assessed by Kruskal-Wallis test followed by Dunn's Multiple comparison post-test (clinical score) or Student's t-test using Prism 7 software (GraphPad Software, La Jolla, CA, USA). Statistical significance was considered at $p < 0.05$.

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CONFLICTS OF INTEREST

The authors have no potential conflict of interest relevant to this article to disclose.

Figure legends

Figure 1. CD1d$^{+/-}$ pMφ suppresses collagen-induced arthritis.

(A) Experimental schedules. To induce CIA, CD1d$^{+/-}$ mice were immunized using intradermal tail base injections of 100 μg of chicken CII emulsified with an equal volume of CFA. Three weeks later, mice were i.d. boosted with 100 μg of CII in IFA. Seven days later, mice received intravenous injections of 1×10^6 CD1d$^{-/-}$ pMφ (○), CD1d$^{+/-}$ pMφ (□), or PBS as CIA control (◆). (B) Incidence of arthritic mice; (C) Clinical scores of arthritis in each group. Paws were scored from 0 to 5 according to the severity of arthritis, with a maximum cumulative score of 20. Results are presented as means ± SEM from three independent assessments (5 mice per group) of inflammation scores (0–5).

Figure 2. Comparison of responses of CD1d$^{-/-}$ and CD1d$^{+/-}$ pMφ to LPS stimulation.

CD11b- and CD11c-positive pMφ were isolated after magnetic-activated cell sorting. (A) CD1d, MHC II, and CD86 expression levels were measured using flow cytometry gated on CD11b$^+$ I-A^{b+} cells after treatments with various concentrations of LPS for 24 h. (B) Supernatants from cultured pMφ were collected after 24 h of LPS stimulation. TNF-α, IL-6, and IL-10 levels were measured using ELISA. CD1d$^{-/-}$ (□) vs. CD1d$^{+/-}$ (■) macrophages; NA, no antigen stimulation. Data are representatives of four independent experiments. Differences were considered significant at ***, $P < 0.001$; **, $P < 0.01$; and *, $P < 0.05$ (Student's t-test).

Figure 3. Inhibitory effects of CD1d$^+$ pMφ on OVA-specific CD4$^+$ T cell activation.

pMφ from CD1d$^{+/-}$ and CD1d$^{-/-}$ mice were loaded with or without OVA protein, treated with TGF-β_2 if necessary, and co-cultured for 72 h with CFSE-labeled CD4$^+$ T cells from OVA-primed OT-II mice. (A) CFSE profiles of OT-II cell. Histogram shows CFSE profile of TCRVα2$^+$ -gated cells; (B) Percentages of proliferating CD4$^+$ T cells gated on TCRVα2$^+$ cells; (C) Supernatants from cocultures were collected and levels of IFN- γ were analyzed using ELISA. Data are representatives of four independent experiments. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ (Student's t-test).

Figure 4. CD1d$^+$ pMφ display tolerogenic phenotypes in CIA-induced CD1d KO hosts.

CD1d KO mice were immunized by i.d. injections of 100 μg of chicken CII emulsified with an equal volume of CFA at the base of the tail to develop CIA. On day 21, mice were i.d. boosted with 100 μg of IFA. Seven days later, mice received i.v. injections of 1×10^6 TGF-β_2-treated pMφ (Tol pMφ; ○), TGF-β_2-untreated (pMφ; □), or PBS transfer as CIA control (◆). (A) Incidence and clinical scores of arthritis in groups received Tol pMφ; (B) Incidence and Clinical scores of arthritis in groups received pMφ. Each paw was scored from 0 to 5 according to the severity of arthritis, with a maximal cumulative score of 20. Results are representatives of three independent experiments. Data are presented as mean inflammatory scores (0–5) ± standard errors of the mean (SEM; 4–5 mice per group); *, $P < 0.05$ (Student's t-test).

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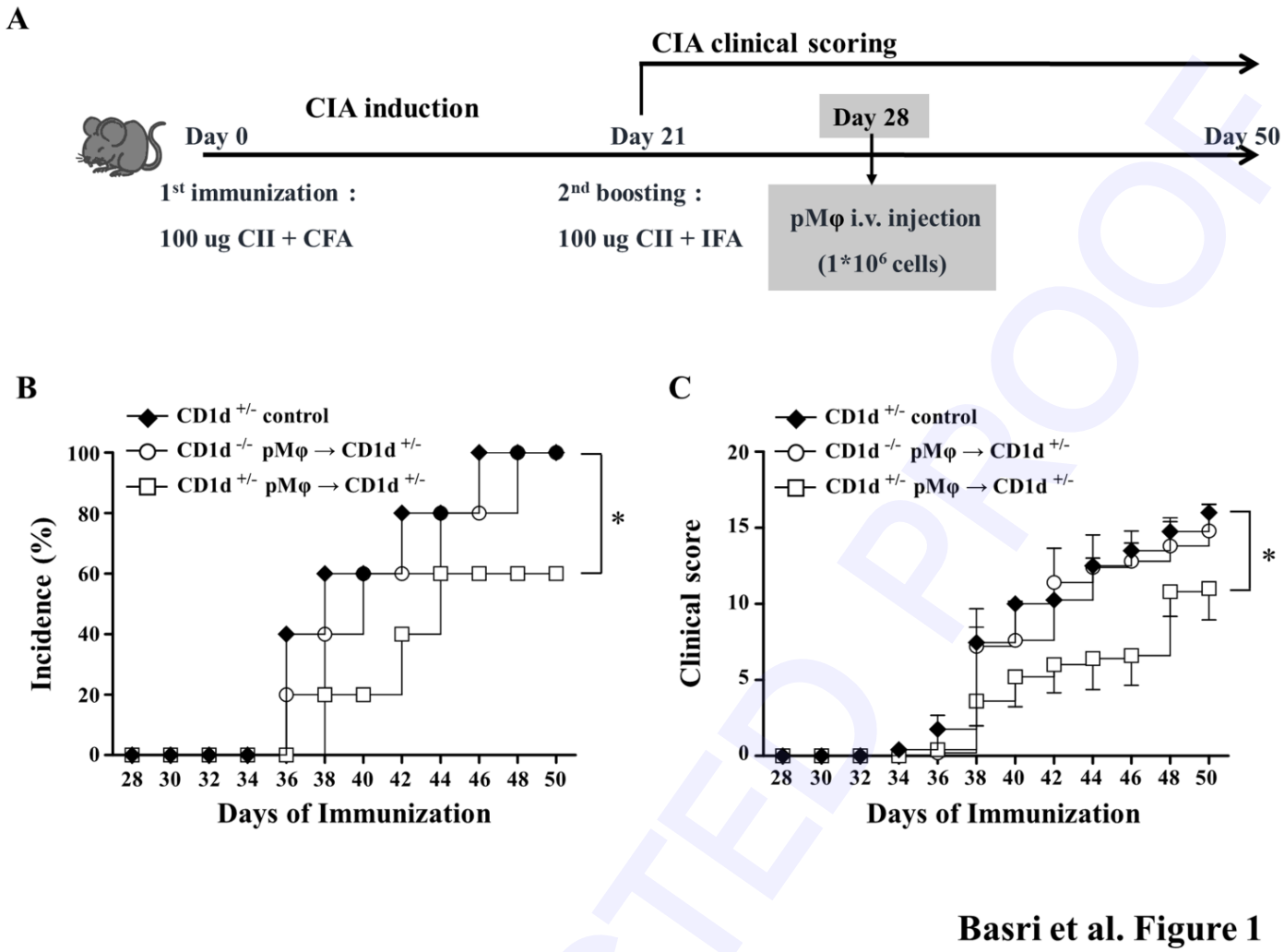


Fig. 1. CD1d+/- pM

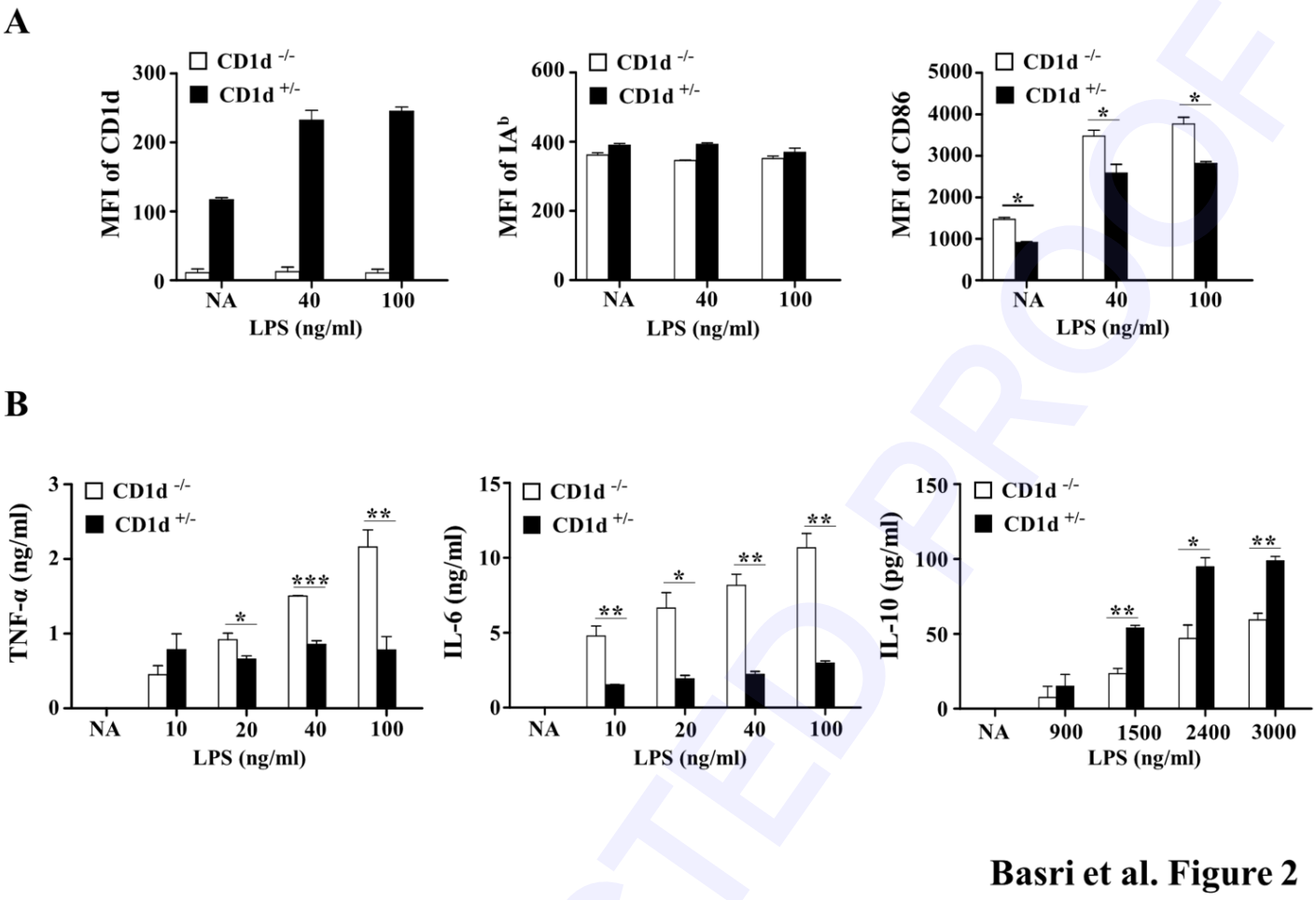


Fig. 2. Comparison of responses of CD1d-/- and CD1d+/- pM

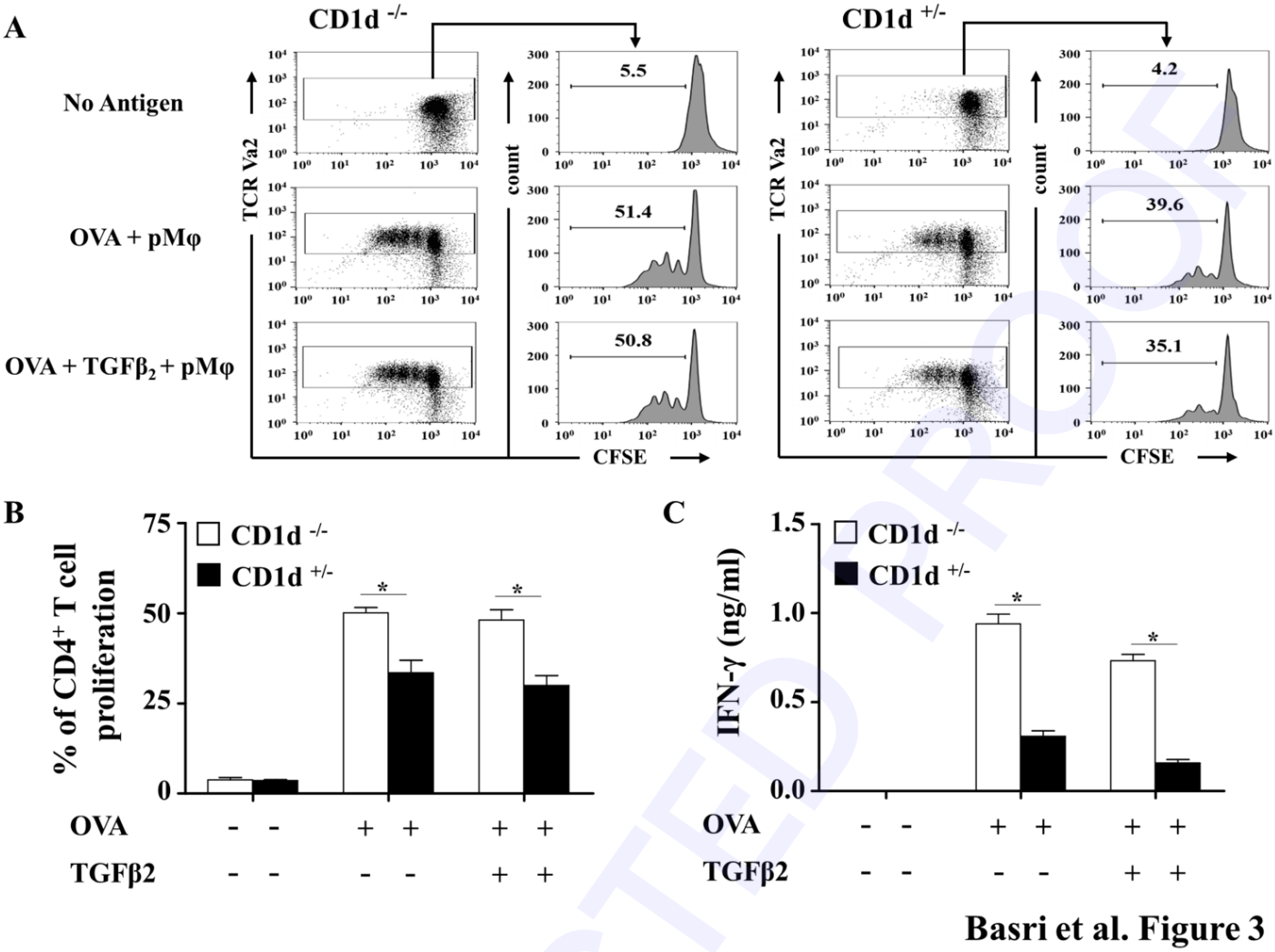


Fig. 3. Inhibitory effects of CD1d+ pM

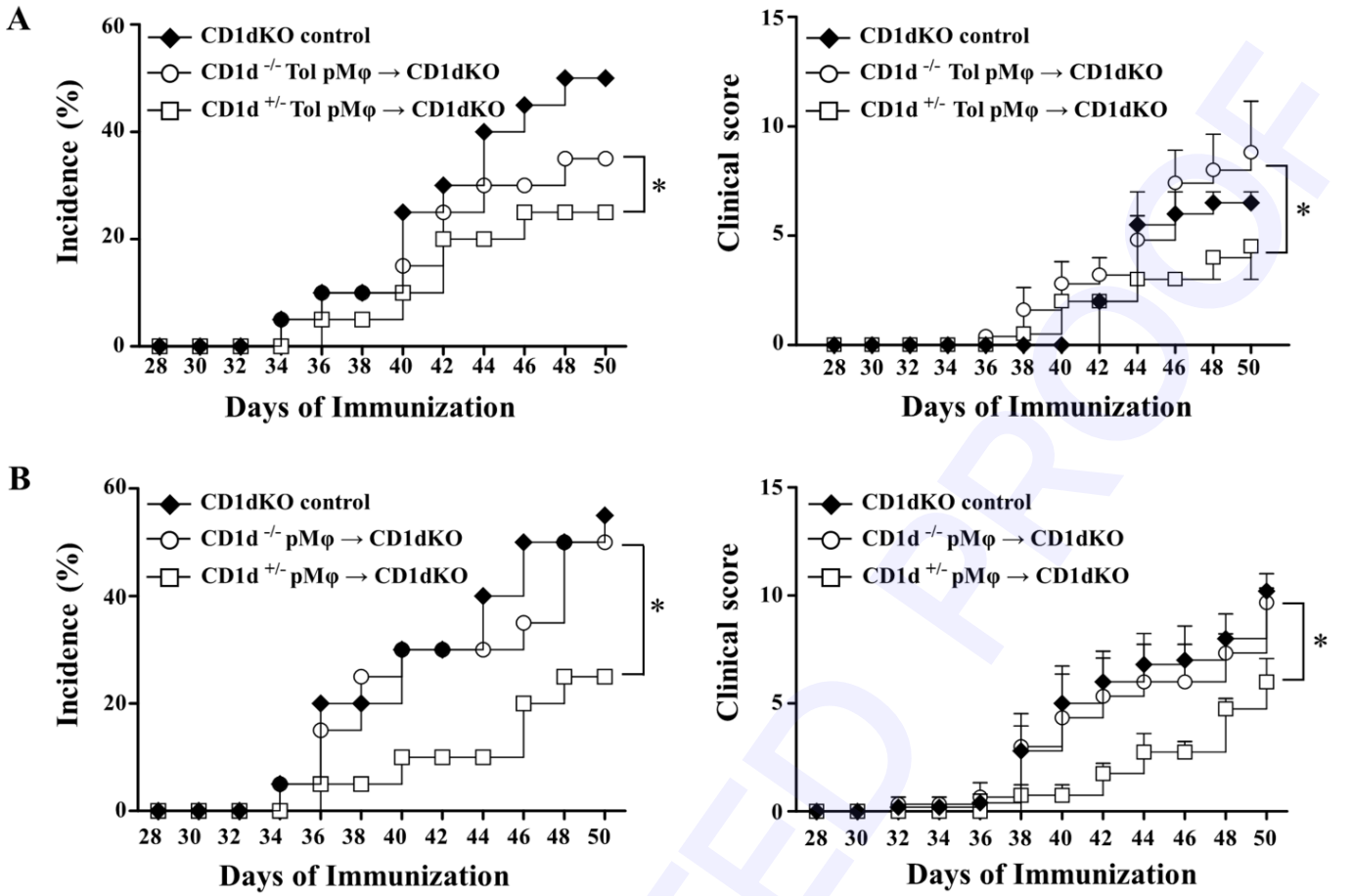
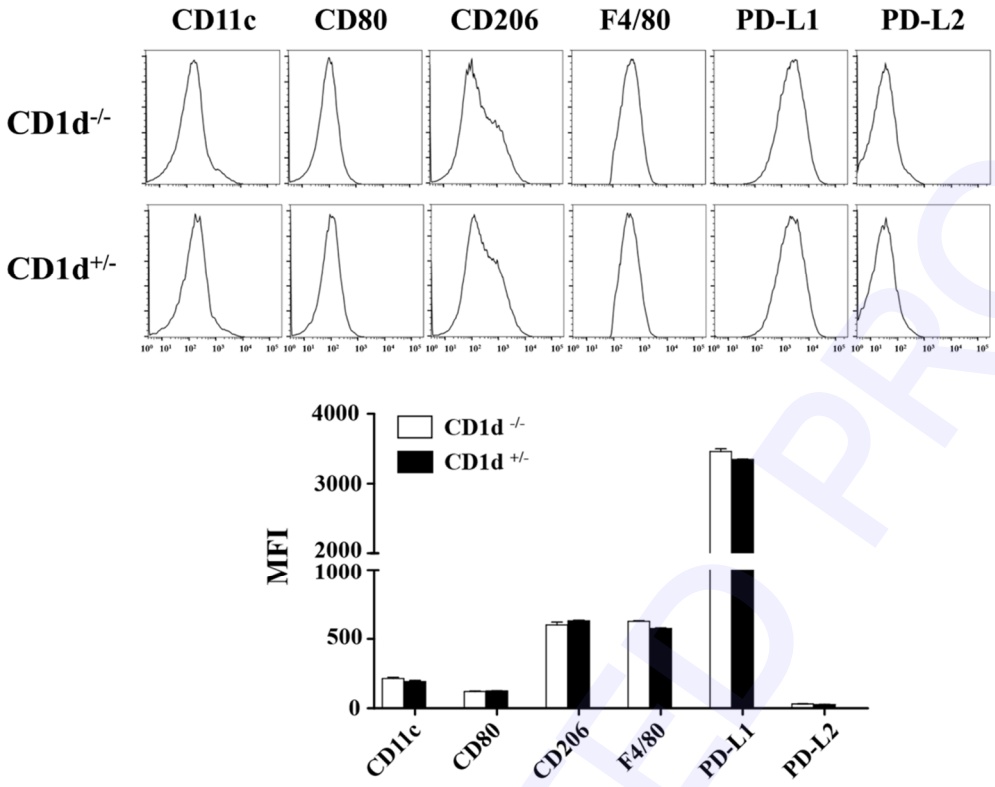


Fig. 4. CD1d+ pM



Supplemental Figure 1. Surface expression patterns of pMφ from CD1d$^{-/-}$ and CD1d$^{+/-}$ mice.

Peritoneal exudate cells (PECs) from CD1d$^{-/-}$ (□) and CD1d$^{+/-}$ (■) mice were harvested at 3 days after induction with 3% thioglycollate. Cells were cultured overnight and subjected to cold stress for 2 h before being resuspended by vigorous pipetting. CD11c, CD80, CD206, F4/80, PD-L1, and PD-L2 expression levels were assessed using flow cytometry gated on CD11b^{+} CD11c^{+} cells. Three independent experiments produced similar results.