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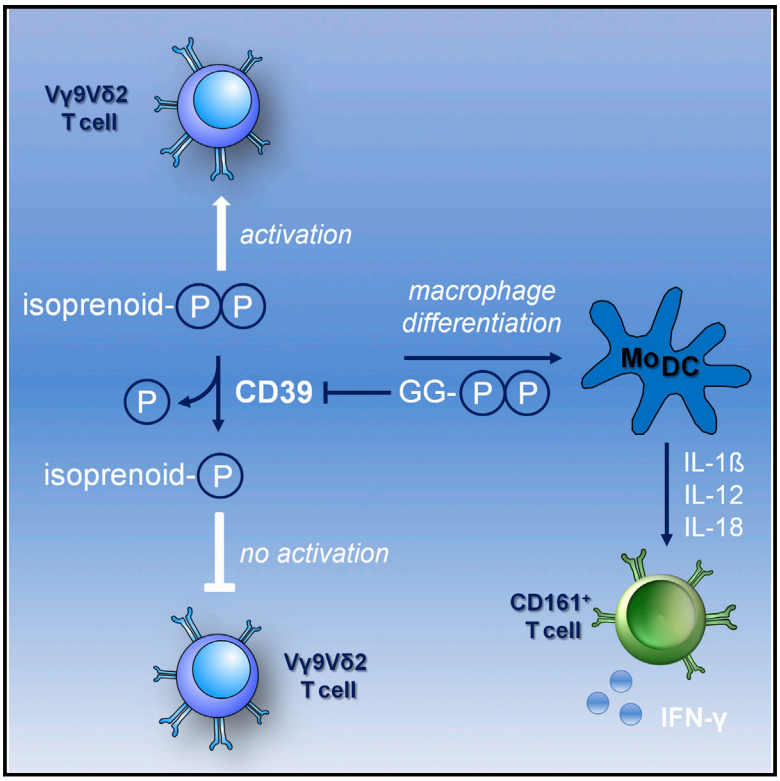
Ecto-ATPase CD39 Inactivates Isoprenoid-Derived Vγ9Vδ2 T Cell Phosphoantigens

Graphical Abstract



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In Brief

CD39 has so far only been known as an ecto-ATPase. Gruenbacher et al. demonstrate that CD39 also dephosphorylates and thus inactivates self- and pathogen-associated phosphoantigens (pAgs) of Vγ9Vδ2 T cells. Only geranylgeranyl diphosphate (GGPP) acts as a CD39 regulator and facilitates macrophage-dependent, innate-like effector T cell responses.

Highlights

- The ecto-ATPase CD39 inactivates isoprenoid-derived Vγ9Vδ2 T cell phosphoantigens
- CD39-resistant GGPP regulates CD39 expression and activity
- GGPP upregulates CCL2 and IL-1β in IL-15 differentiated macrophage-like cells
- GGPP-imprinted macrophage-like cells promote innate-like effector T cell responses

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Ecto-ATPase CD39 Inactivates Isoprenoid-Derived Vγ9Vδ2 T Cell Phosphoantigens

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SUMMARY

In humans, Vγ9Vδ2 T cells respond to self and pathogen-associated, diphosphate-containing isoprenoids, also known as phosphoantigens (pAgs). However, activation and homeostasis of Vγ9Vδ2 T cells remain incompletely understood. Here, we show that pAgs induced expression of the ecto-ATPase CD39, which, however, not only hydrolyzed ATP but also abrogated the γδ T cell receptor (TCR) agonistic activity of self and microbial pAgs (C_5 to C_{15}). Only mevalonate-derived geranylgeranyl diphosphate (GGPP, C_{20}) resisted CD39-mediated hydrolysis and acted as a regulator of CD39 expression and activity. GGPP enhanced macrophage differentiation in response to the tissue stress cytokine interleukin-15. In addition, GGPP-imprinted macrophage-like cells displayed increased capacity to produce IL-1β as well as the chemokine CCL2 and preferentially activated CD161-expressing $CD4^+$ T cells in an innate-like manner. Our studies reveal a previously unrecognized immunoregulatory function of CD39 and highlight a particular role of GGPP among pAgs.

INTRODUCTION

γδ T cells are unconventional T cells (Godfrey et al., 2015) that constitute a first line of defense against pathogens and tumors (Chien et al., 2014; Kabelitz et al., 2013; Vantourout and Hayday, 2013). γδ T cells can be classified according to their expression of T cell receptor (TCR) variable (V) region segments (Kabelitz and He, 2012). Whereas Vδ1 T cells reside in epithelial tissues and join the battle against viruses, fungi, and tumor cells, Vδ2 T cells co-expressing Vγ9 are the major γδ T cell population in adult human blood. Although Vγ9Vδ2 T cells represent a minority among all T cells (<10%), their frequency in the blood of patients may rise significantly during infections (up to 60% of total T cells) (Chien et al., 2014). The Vγ9Vδ2 TCR functions as a pattern recognition receptor to detect self and pathogen-associated phosphoantigens (pAgs), which are organic molecules often consisting of one or more C_5 isoprene units and a diphosphate group (also known as the pyrophosphate group) (Bonneville et al., 2010; Thurnher and Gruenbacher, 2015). Butyrophilin 3A1 (BTN3A1) has recently been reported to bind pAgs and mediate activation of Vγ9Vδ2 T cells (Harly et al., 2012; Sandstrom et al., 2014; Vavassori et al., 2013; Wang et al., 2011). Vγ9Vδ2 T cells have long been considered to be primate specific as they do not exist in mice. However, the genes encoding Vγ9, Vδ2, and BTN3A1 have been found to be co-conserved across a variety of placental mammals (Karunakaran et al., 2014).

In tumor cells, pAgs frequently accumulate due to a dysregulated mevalonate pathway (Thurnher and Gruenbacher, 2015; Thurnher et al., 2012). Nitrogen-containing bisphosphonates (N-BPs) such as zoledronate inhibit a key enzyme of the mevalonate pathway and thus induce accumulation of isopentenyl diphosphate (IPP), a prototypic Vγ9Vδ2 T cell pAg, which is also produced by microbial pathogens including mycobacteria via the non-mevalonate pathway of isoprenoid biosynthesis. Consistent with their ability to perform innate-like responses, Vγ9Vδ2 T cells express CD161 (*KLRB1*) and the transcription factors T-bet and eomesodermin (Dimova et al., 2015; Fergusson et al., 2014; Gruenbacher et al., 2014; Ribot et al., 2014).

γδ T cells have promising anti-tumor potential (Silva-Santos et al., 2015). In a recent study, which integrated tumor gene expression profiles and overall survival data from nearly 18,000 patients, intra-tumoral γδ T cell signatures emerged as the most significant favorable cancer-wide prognostic population, and at the molecular level, CD161 has been identified as the top favorable pan-cancer prognostic gene (Gentles et al., 2015). Both indirect and direct evidence for the anti-leukemic potential of γδ T cells have also been obtained (Airoldi et al., 2015; Godder et al., 2007; Wilhelm et al., 2014).

Inflammatory and immune responses are associated with the controlled or lytic release of nucleotides, particularly ATP (Idzko et al., 2014). Extracellular ATP can function as a potent signaling molecule through triggering of purinergic P2 receptors (P2Rs) (Burnstock and Boeynaems, 2014; Eltzschig et al., 2012). Given the biological potency of ATP, the control of the duration and magnitude of the cellular responses to ATP is crucial (Antonioli et al., 2013; Dwyer et al., 2007). Therefore, immune cells either constitutively express or upregulate CD39, an ecto-ATPase, which converts ATP and ADP into AMP. The ecto-5′-nucleotidase CD73 can complete the dephosphorylation process and convert the monophosphate into adenosine. Whereas ATP

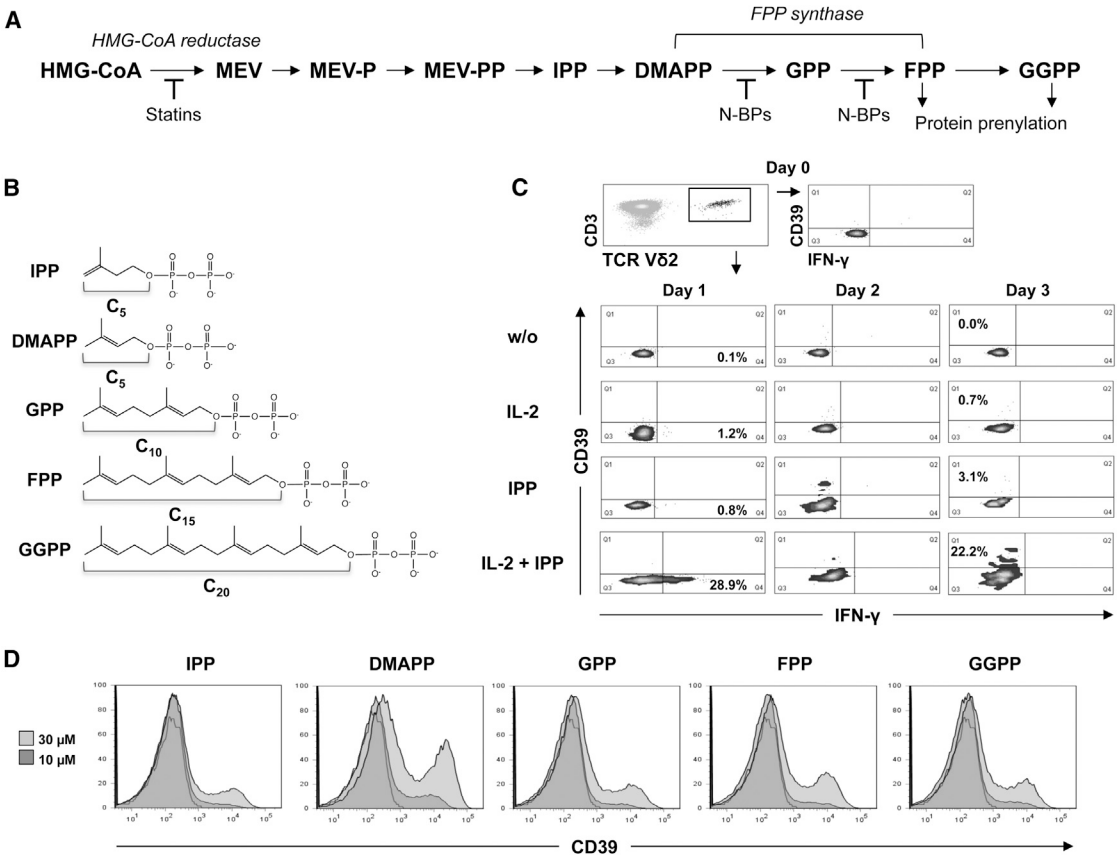

Figure 1. Mevalonate-Derived pAgs Induce Early IFN-γ and Delayed CD39 Expression in Vγ9Vδ2 T Cells

(A) pAg generation in the mevalonate pathway: HMG-CoA reductase, the target of statins, generates mevalonate (MEV). Two kinases and a decarboxylase convert mevalonate into IPP. An isomerase catalyzes the interconversion of IPP and its isomer DMAPP. FPP synthase catalyzes sequential condensation reactions of DMAPP with two units of IPP to form FPP. GGPP synthase catalyzes yet another condensation reaction with IPP to form GGPP. FPP and GGPP serve as activated isoprenoid donor substrates in posttranslational protein prenylation. By inhibiting FPP synthase, nitrogen-containing bisphosphonates (N-BPs) induce depletion of downstream FPP and GGPP and thus inhibition of prenylation as well as the accumulation of upstream IPP.

(B) Structures of mevalonate-derived pAgs with varying isoprenoid chain lengths (C_5 to C_{20}).

(C) IL-2 primed T cell cultures ($CD3^+$ PBMCs) were stimulated with isopentenyl diphosphate (IPP, 30 μM) and then stained for surface CD3/Vδ2/CD39 as well as for intracellular IFN-γ at the times indicated. $CD3^+V\delta2^+$ cells were gated and selectively analyzed for CD39 and IFN-γ expression. Gates were set according to isotype controls. A flow cytometry plot representative of two independent experiments with two different donors is shown.

(D) IL-2 primed T cell cultures ($CD3^+$ PBMCs) were stimulated for 3 days with pAgs at either 10 μM (dark gray) or 30 μM (light gray) and then stained for surface CD3/Vδ2/CD39. $CD3^+V\delta2^+$ cells were gated and selectively analyzed for CD39 expression. Isotype controls are indicated by open histograms; an experiment representative of three experiments with three different donors is shown.

See also Figure S1.

exerts predominantly proinflammatory effects, adenosine has potent immunosuppressive effects (Eltzschig et al., 2012). Genetic deletion of *CD39* is associated with impaired ATP phosphohydrolysis (Enjyoji et al., 1999). Increased CD39 expression in T cells with age has been reported to promote T cell apoptosis and to contribute to impaired vaccine responses in older individuals (Fang et al., 2016). Although CD39 is considered to be an activation marker of B and T cells (Dombrowski et al., 1998; Dwyer et al., 2007), Vγ9Vδ2 T-cell-associated CD39 has not been reported so far. In the present work, we therefore sought to examine expression and function of CD39 in Vγ9Vδ2 T cell activation and homeostasis and provide evidence that CD39 serves a previously unrecognized purpose.

RESULTS

pAgs Induce Expression of the Ecto-ATPase CD39 on Vγ9Vδ2 T Cells

To examine whether CD39 is upregulated during Vγ9Vδ2 T cell activation by mevalonate-derived pAgs (Figures 1A and 1B), we combined CD39 cell surface staining with intracellular IFN-γ detection. Data shown in Figure 1C demonstrate that freshly isolated Vγ9Vδ2 T cells (day 0) lacked CD39 expression. Vγ9Vδ2 T cells produced IFN-γ early (day 1) in response to pAg plus interleukin-2 (IL-2), whereas CD39 upregulation was clearly delayed and started only on day 3. Treatment with various mevalonate-derived pAgs with isoprenoid chain lengths ranging

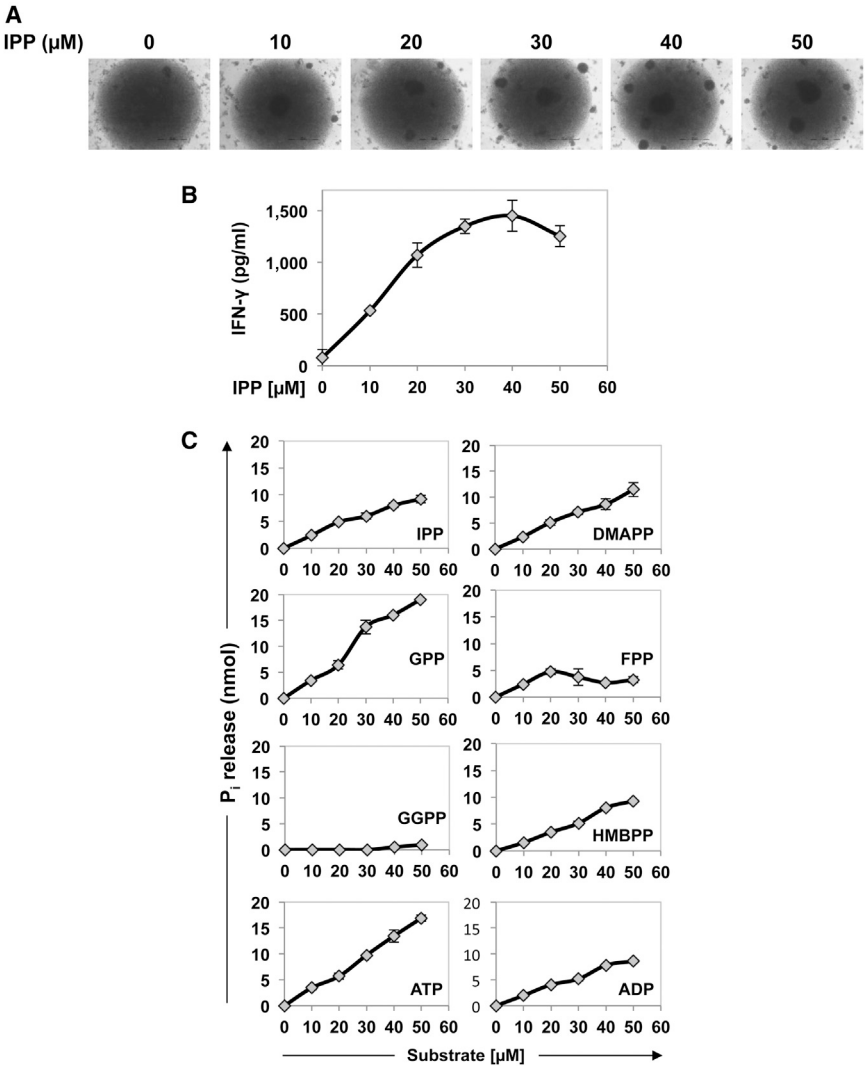

Figure 2. pAgs Are Substrates of CD39

(A) IL-2 primed T cell cultures ($CD3^+$ PBMCs) were stimulated with IPP in round-bottom 96-well plates at the concentrations indicated. Cell aggregation was documented using an Olympus CK2 microscope (magnification, 4 × 10; scale bar, 500 μm) equipped with a ProgRes CT3 digital camera and ProgRes CapturePro 2.5 Software (Jenoptik).

(B) On day 5, IFN-γ was measured in culture supernatants.

(C) Mevalonate-derived self pAgs or pathogen-associated pAg (HMBPP) at 10 to 50 μM (2 to 10 nmol) were incubated for 30 min at 37°C with human rCD39 (0.5 μg/ml) in 0.2 ml of 25 mM Tris, 5 mM $CaCl_2$ (pH 7.5). Inorganic phosphate (P_i) released during CD39-mediated hydrolysis was measured using a colorimetric P_i assay kit. Each pAg was measured at least three times. Controls are ATP and ADP; at 50 μM, maximum P_i release is 20 nmol for ATP and 10 nmol for ADP.

See also Figure S2.

from C_5 to C_{20} partially induced CD39 expression in a dose-dependent manner within 3 days (Figure 1D). In addition, prolonged treatment with other Vγ9Vδ2 T cell agonists that induce intracellular accumulation of pAg or mimic their presence such as the N-BP zoledronate or antibodies against TCR Vγ9 and CD277 (BTN3A1) induced homogenous CD39 expression (Figure S1A).

Consistent with observations in murine Vδ1 T cells (Liang et al., 2014), CD73 expression was usually weak on circulating Vγ9Vδ2 T cells, and if nevertheless present, CD73 expression was silenced upon cell activation (Figure S1B). To exclude the possibility that expanded $CD39^+$ Vγ9Vδ2 T cells are regulatory cells, we compared the phenotypes of expanded Vγ9Vδ2 T cells and regulatory T cells (T_{reg} cells). $CD39^+$ Vγ9Vδ2 T cells expressed markers of effector cells such as CD56 and CD161 (Fergusson et al., 2014; Gentles et al., 2015) but lacked molecules typically associated with T_{reg} cells such as CD73, Foxp3, CD25, and CD152 (Figure S1C).

CD39 Exhibits Isoprenoid Diphosphate Phosphohydrolase Activity

Mevalonate-derived pAgs share a diphosphate (= pyrophosphate) group with ATP (Figure 1B). This raised the question of whether pAgs might be substrates of the ecto-ATPase CD39. To test this intriguing possibility, we first used recombinant CD39 (rCD39) and measured inorganic phosphate (P_i) released during CD39-mediated hydrolysis. pAgs generated in the mevalonate pathway were tested at a range of concentrations (10 to 50 μM) known to induce Vγ9Vδ2 T cell activation (Figures 2A and 2B) (Tanaka et al., 1995). As expected, rCD39 effectively hydrolyzed ATP and ADP (Figure 2C). In addition, rCD39 hydrolyzed IPP, DMAPP, and GPP. In contrast, the protein prenylation donor substrates FPP and geranylgeranyl diphosphate (GGPP), which can also activate Vγ9Vδ2 T cells (Gruenbacher et al., 2014; Thurnher and Gruenbacher, 2015), were either poor substrates (FPP) or not hydrolyzed at all (GGPP). Importantly, HMBPP (also known as HDMAPP), a metabolite of the bacterial non-mevalonate pathway (Chien et al., 2014), was also a substrate of rCD39 (Figure 2C). Whereas the artificial pAg IPPP could also be hydrolyzed, isopentenyl monophosphate (IP) could not be dephosphorylated by rCD39 (Figure S2A). We also performed a side-by-side comparison of human and murine rCD39, respectively, and observed very similar profiles of substrate hydrolysis (Figure S2B).

$CD39^+$, but Not $CD39^-$, T Cells Dephosphorylate pAgs

Next, we characterized isoprenoid diphosphate phosphohydrolase activity associated with expanded $CD39^+$ Vγ9Vδ2 T cells and found that these cells not only hydrolyzed ATP and ADP but also mevalonate-derived self and pathogen-associated

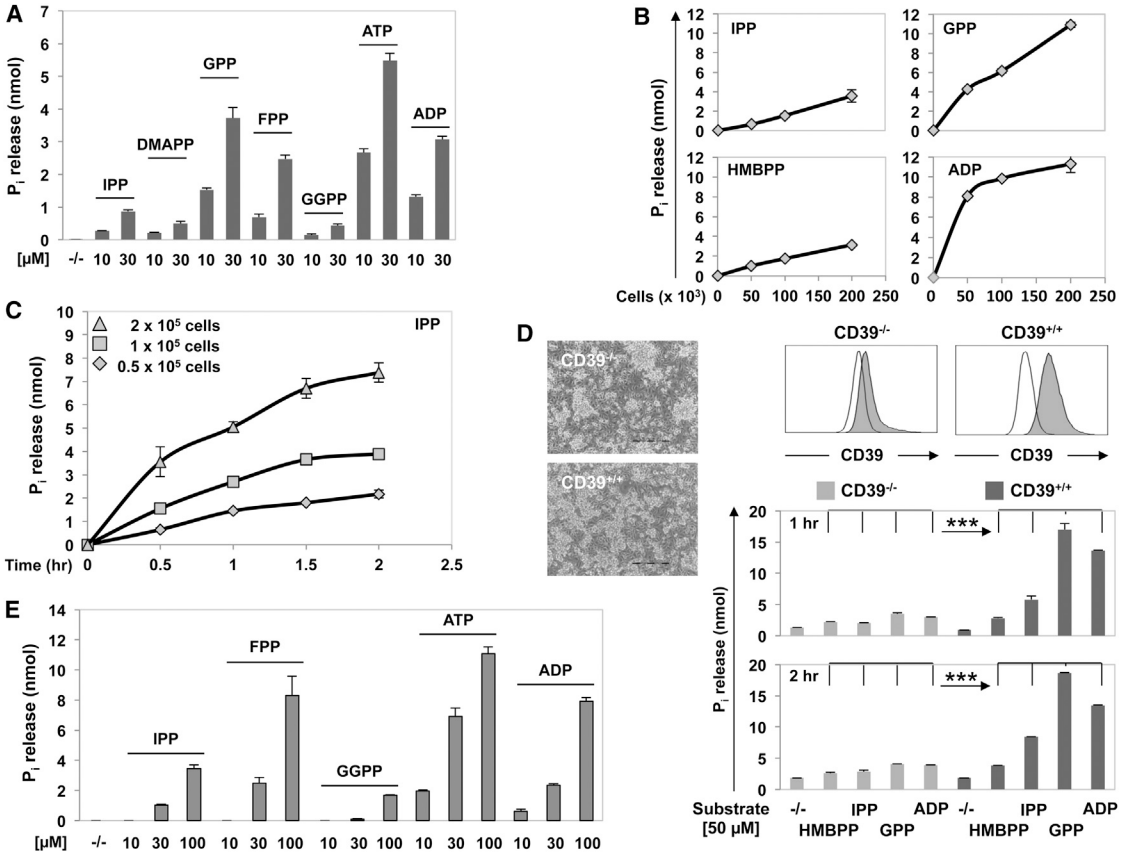

Figure 3. CD39⁺, but Not CD39⁻, Cells Degrade pAgs

(A) Expanded Vγ9Vδ2 T cells (day 10 to 12; >90% CD39⁺) were incubated for 2 hr with pAgs or ATP/ADP (10 and 30 μM = 2 and 6 nmol, respectively) in 0.2 ml of Tris-Ringer solution (pH 7.5), containing 5 mM $CaCl_2$. The amount of P_i released was determined using a colorimetric kit.

(B) Graded doses of CD39⁺ Vγ9Vδ2 T cells were incubated with pAgs or ADP (each at 50 μM = 10 nmol) for 0.5 hr in 0.2 ml of Tris-Ringer solution (pH 7.5), containing 5 mM $CaCl_2$. P_i was determined using a colorimetric kit.

(C) Graded doses of CD39⁺ Vγ9Vδ2 T cells were incubated with IPP (50 μM = 10 nmol) for 0.5 to 2 hr in 0.2 ml of Tris-Ringer solution (pH 7.5), containing 5 mM $CaCl_2$. P_i was quantified using a colorimetric kit.

(D) Wild-type ($CD39^{+/+}$) or CD39-deficient ($CD39^{-/-}$) macrophages (5×10^5 cells) grown in 24-well plates (phase contrast microscopy at 10 × 10 magnification; scale bar, 200 μm). The presence or absence of CD39 expression was determined by flow cytometry. The cells were incubated with pAgs or ADP (each at 50 μM = 10 nmol) for 1 or 2 hr in 0.2 ml of Tris-Ringer solution (pH 7.5), containing 5 mM $CaCl_2$. P_i was determined using a colorimetric kit. ***$p < 0.001$.

(E) Expanded T_{reg} cells (5×10^4 cells > 60% CD39⁺) were incubated for 2 hr with pAgs or ATP/ADP (10, 30, and 100 μM = 2, 6, and 20 nmol, respectively) in 0.2 ml of Tris-Ringer solution (pH 7.5), containing 5 mM $CaCl_2$. The amount of P_i released was determined using a colorimetric kit. The data are representative of three independent experiments and depict mean ± SEM (error bars) of triplicate measurements. See also Figure S2.

pAgs in a substrate- (Figure 3A), cell dose- (Figure 3B), and time-dependent manner (Figure 3C). Whereas GPP was again very susceptible to hydrolysis, GGPP remained relatively resistant.

To better attribute the observed isoprenoid diphosphate phosphohydrolase activity to CD39, activated Vγ9Vδ2 T cells were sorted into CD39⁺ and CD39⁻ cells and then used side by side as a source of isoprenoid diphosphate phosphohydrolase activity in the P_i release assay. CD39⁺, but not CD39⁻, Vγ9Vδ2 T cells hydrolyzed ATP and ADP as well as pAgs (Figure S2C).

To address whether CD39 is the dominant pAg-degrading enzyme, we also compared bone-marrow-derived macrophages from wild-type and CD39-deficient mice (Enjyoji et al., 1999; Robson et al., 2005). $CD39^{+/+}$, but not $CD39^{-/-}$, macrophages effectively degraded IPP, GPP, and ADP (Figure 3D). In contrast, the nonself, pathogen-associated HMBPP, which is the strongest Vγ9Vδ2 T cell pAg currently known, was a relatively poor substrate of CD39 associated with murine macrophages.

CD39 is highly expressed on the surface of Foxp3⁺ T_{reg} cells (Antonioli et al., 2013; Borsellino et al., 2007). CD39⁺ T_{reg} cells, which were derived from CD4⁺CD25⁺ T cells by repetitive stimulation with anti-CD3/CD28 antibodies in the presence of high-dose IL-2 and rapamycin (Battaglia et al., 2006), showed a substrate hydrolysis profile similar to that of CD39⁺ Vγ9Vδ2 T cells (Figure 3E).

CD39 Abrogates the Vγ9Vδ2 T Cell Stimulatory Capacity of All pAgs Except GGPP

Next, we tested whether enzymatic hydrolysis affects the Vγ9Vδ2 T cell agonistic activity of various pAgs. For this

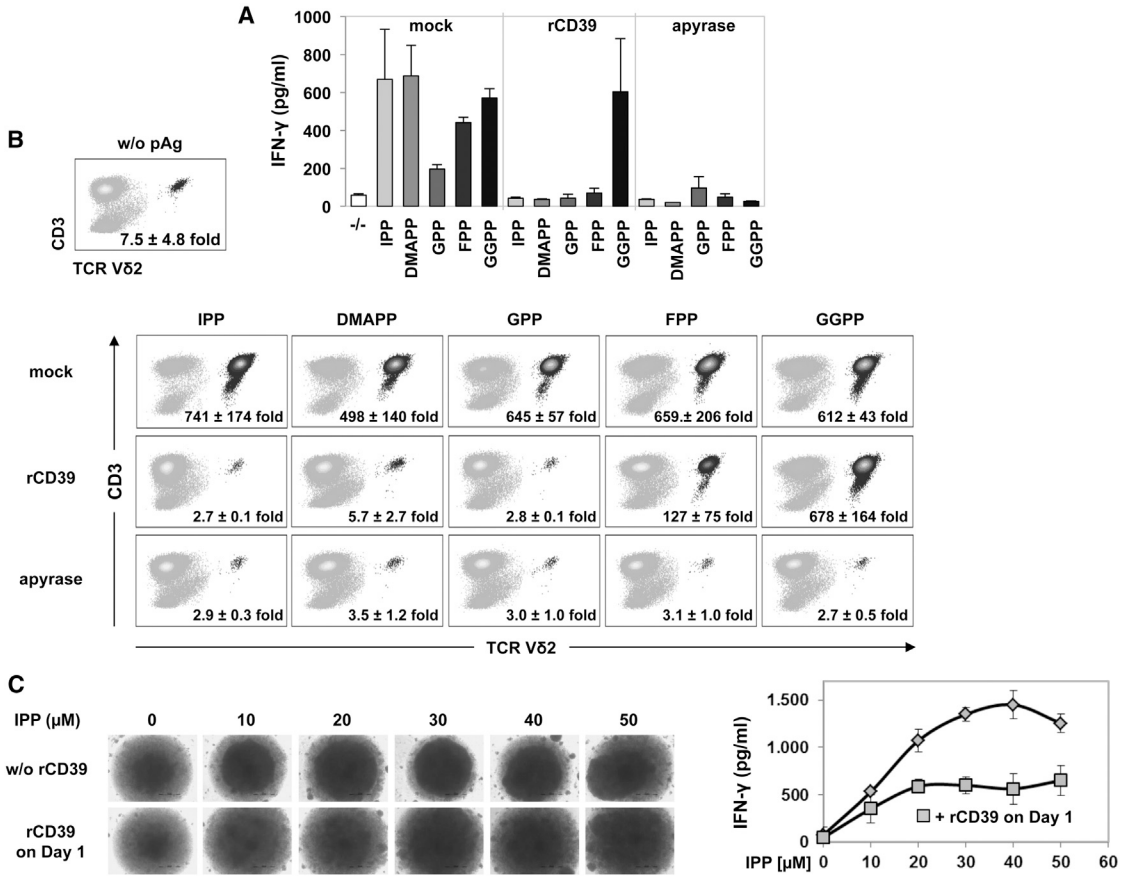

Figure 4. pAgs Exposed to CD39 Fail to Stimulate Vγ9Vδ2 T Cell Activation

(A) pAgs with or without (= mock) ATPase (rCD39, apyrase; MW, 45–51 kDa) pretreatment were subjected to centrifugal filtration. The filtrate, which contained the isoprenoids but not the ATPase, was used for stimulation of IL-2 primed PBMCs. IFN-γ was measured in culture supernatants on day 5. See also Figure S3A.

(B) IL-2 primed PBMCs were stimulated with pAgs with or without (= mock) ATPase (rCD39 or apyrase) pretreatment followed by centrifugal filtration. The filtrate, which contained the isoprenoids but not the ATPase, was used to induce Vγ9Vδ2 T cell expansion (final isoprenoid concentration, 10 μM). Absolute numbers of Vγ9Vδ2 T cells in the individual cultures were derived from total cell numbers considering Vγ9Vδ2 T cell frequency and used to quantify Vγ9Vδ2 T cell expansion. The data are representative of three independent experiments and, where applicable, depict mean ± SEM (error bars) of triplicate measurements. See also Figures S3 and S4.

(C) IL-2 primed T cells ($CD3^+$ PBMCs) were stimulated with IPP at the concentrations indicated with or without the addition of rCD39 (0.5 μg/ml) to the culture medium on day 1. On day 6, IFN-γ was measured in culture supernatants and cell aggregation was documented using an Olympus CK2 microscope (magnification, 4 × 10). Scale bar, 500 μm.

purpose, pAgs were incubated with rCD39 or with potato apyrase (Komoszyński and Wojtczak, 1996), an ATPase frequently used in purinoreceptor research to neutralize the effects of extracellular ATP (Idzko et al., 2007). Isoprenoids were separated again from the enzyme using centrifugal filtration devices (Figure S3A).

Mock-treated pAgs induced cell aggregation (Figure S3B), IFN-γ production (Figure 4A), and Vγ9Vδ2 T cell expansion (Figure 4B). Pretreatment with rCD39 abolished the stimulatory activity of all pAgs except GGPP (Figure 4; Figure S3B). Pretreatment with apyrase, which has terminal phosphatase activity, abrogated the stimulatory activity of all pAgs, including GGPP (Figures 4A and 4B). The Vγ9Vδ2 T-cell-agonistic activity of the microbial HMBPP was also abolished by rCD39 (Figure S3C). Consistent with our observations in the P_i release assay (Figure S2B), pretreatment of pAgs with murine rCD39 also abrogated their ability to stimulate cytokine production as well as Vγ9Vδ2 T cell expansion (Figure S4).

To simulate early upregulation of CD39, we added rCD39 on day 1 to T cell cultures that had been stimulated with graded doses of IPP. As a consequence, cell aggregation and IFN-γ production were clearly attenuated (Figure 4C). Collectively, the data demonstrate that, with the exception of the relatively resistant GGPP, all mevalonate-derived pAgs as well as the pathogen-derived HMBPP are subject to CD39-mediated hydrolysis and that hydrolytic degradation abrogates their activity as Vγ9Vδ2 T cell agonists.

GGPP Regulates ATP and pAg Breakdown

Given that pAgs can be substrates of CD39, they may compete with ATP. Specifically, GGPP may act as a hardly hydrolyzable competitor of ATP for CD39-mediated hydrolysis. Indeed,

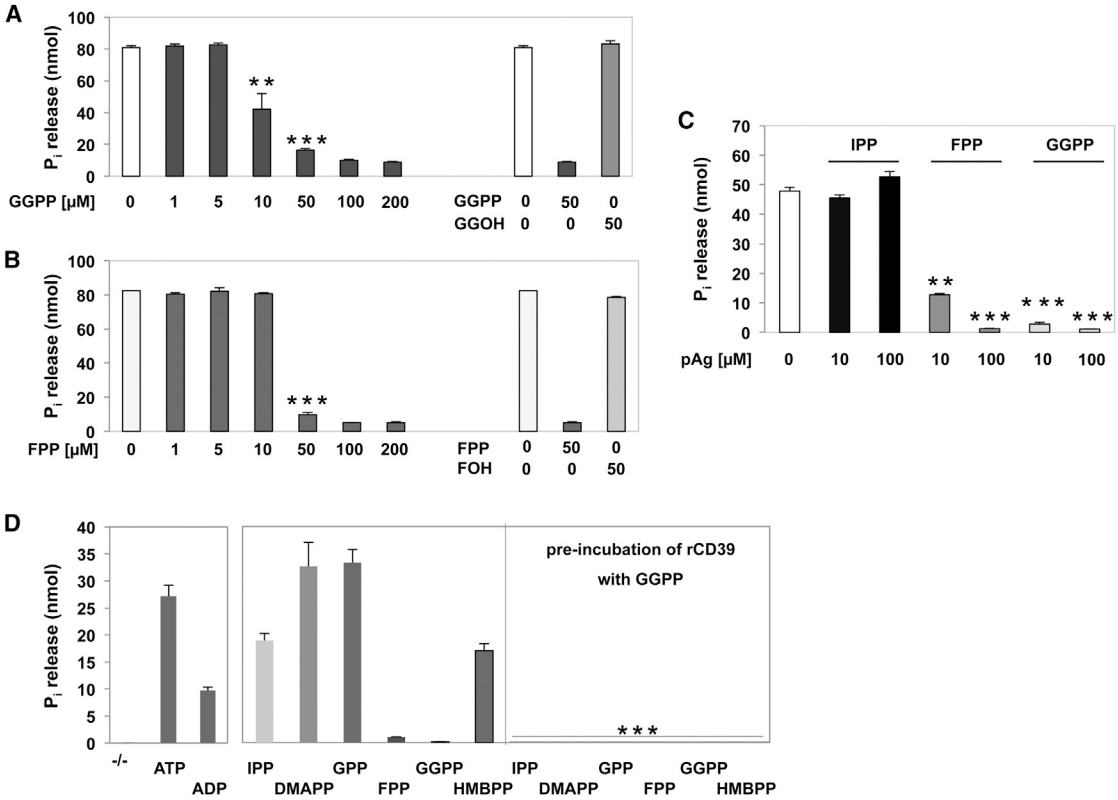

Figure 5. FPP and GGPP Prevent CD39-Mediated ATP and pAg Degradation

(A) Hydrolysis of ATP (200 μM) by rCD39 (0.5 μg/ml) was performed for 30 min at 37°C in 0.2 ml of 25 mM Tris, 5 mM $CaCl_2$ (pH 7.5) in the absence or presence of increasing concentrations of GGPP (control GGOH), and inorganic phosphate (P_i) released during CD39-mediated hydrolysis was measured using a colorimetric P_i assay kit. **$p < 0.01$, ***$p < 0.001$.

(B) Hydrolysis of ATP (200 μM in 0.2 ml) by rCD39 (0.5 μg/ml) was performed in the absence or presence of increasing concentrations of FPP (control FOH), and P_i production was measured using a colorimetric P_i assay kit. ***$p < 0.001$; see also Figure S2C.

(C) Hydrolysis of ATP (200 μM) by murine rCD39 (0.5 μg/ml) was performed in a final volume of 0.2 ml in the absence or presence of IPP, FPP, or GGPP (each at 50 μM), and P_i released during CD39-mediated hydrolysis was measured using a colorimetric Pi assay kit. **$p < 0.05$, ***$p < 0.001$.

(D) Hydrolysis of self and pathogen-associated pAgs (all at 100 μM) mediated by rCD39 (0.5 μg/ml) was performed in a final volume of 0.2 ml with or without a 15-min pre-incubation of rCD39 with GGPP (50 μM). ***$p < 0.001$.

GGPP effectively inhibited ATP hydrolysis by rCD39 (Figure 5A). At 10 μM, GGPP already inhibited the hydrolysis of ATP (200 μM) by 50% and at 50 μM by 90%. Although somewhat less effective, FPP also inhibited ATP breakdown (Figure 5B). In contrast, farnesol (FOH) and geranylgeraniol (GGOH), the lipophilic alcohol precursors to FPP and GGPP (Crick et al., 1997), respectively, which lack the diphosphate group, did not inhibit at all (Figures 5A and 5B). FPP and GGPP also prevented ATP hydrolysis mediated by murine rCD39, and again GGPP was more potent than FPP (Figure 5C). GGPP inhibited not only ATP hydrolysis but also the hydrolytic degradation of pAgs (Figure 5D). GGPP may thus increase the stability of ATP and pAgs.

CD39 hydrolyzes both nucleotides and pAgs. By way of analogy, CD73 might therefore be able to degrade the monophosphorylated pAg that results from CD39-mediated dephosphorylation of the isoprenoid diphosphate. However, rCD73 hydrolyzed only AMP but not IP (Figure S2D). Moreover, IP failed to inhibit rCD73-mediated hydrolysis of AMP.

GGPP Promotes IL-15 Driven Differentiation of CCL2-Producing Macrophage-like Cells

The relative resistance of GGPP against CD39-mediated degradation as well as its ability to regulate ATP breakdown suggested that this pAg serves additional purposes and that it may participate in the regulation of other cell types. A blockade of CD39 on monocytes has been shown to increase the levels of extracellular ATP, leading to purinergic signaling and macrophage activation (Cohen et al., 2013; Piccini et al., 2008; Sakaki et al., 2013). In addition, GGPP has been shown to induce cellular differentiation by increasing the expression of peroxisome proliferator activated receptor-γ (PPARγ) (Weivoda and Hohl, 2012), which has also been implicated in dendritic cell (DC) development from monocytes (Szatmari et al., 2004).

We therefore tested GGPP effects on DCs differentiated from monocytes in the presence of IL-15 (Harris, 2011; Mohamadzadeh et al., 2001), a cytokine known to function as a danger signal during tissue destruction (Jabri and Abadie, 2015). Whereas granulocyte/macrophage colony-stimulating factor (GM-CSF)

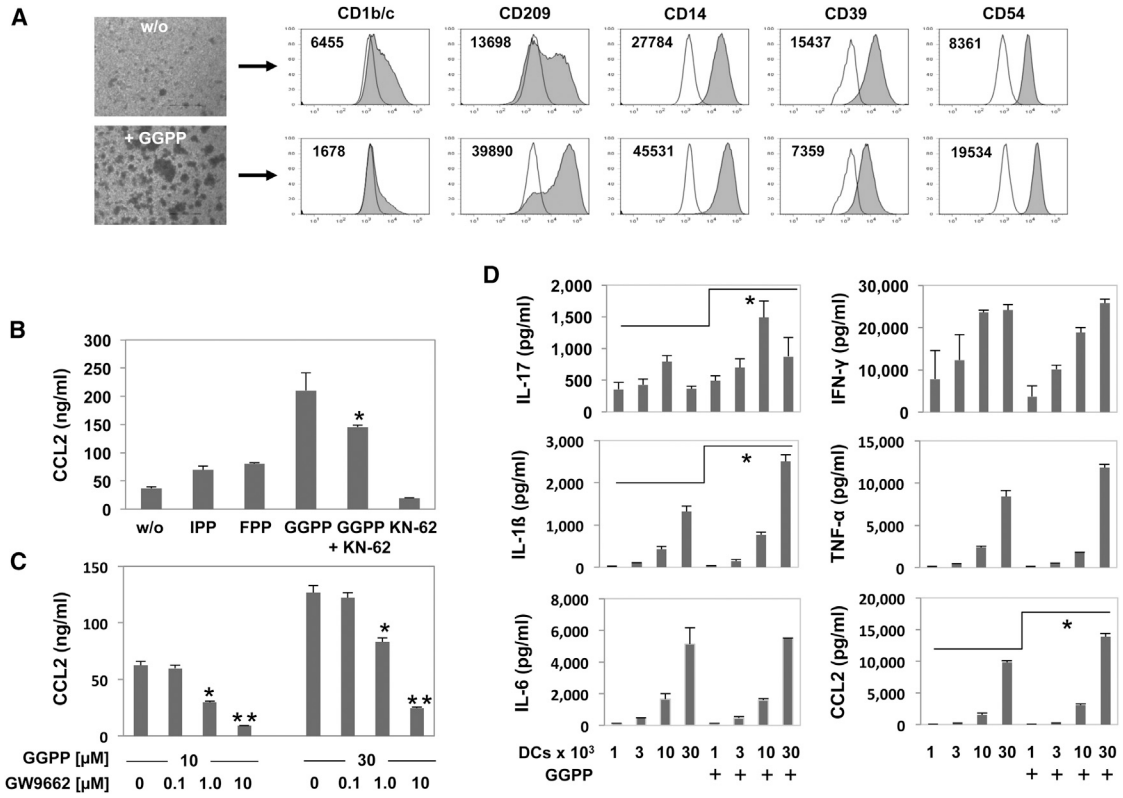

Figure 6. GGPP Promotes Differentiation of Macrophage-like Cells with Increased T Cell-Stimulatory Potential

(A) $CD14^+$ monocytes (1.5×10^6/ml) were cultured in the presence of GM-CSF (800 U/ml) and IL-15 (25 ng/ml) in 96-well plates for 3 days. Cultures were performed ± GGPP at 30 μM and DCs were subsequently stained for the surface markers indicated (MFI, mean fluorescence intensity; open histogram, isotype controls). DC aggregation was documented by phase contrast microscopy. See also Figure S3B.

(B) CCL2 was measured in DC culture supernatants. $P2X_7$ receptor signaling was inhibited using the non-competitive antagonist KN-62 (50 nM; IC_{50}, 15 nM). $^*p < 0.05$.

(C) CCL2 was measured in DC culture supernatants and the role of PPARγ was examined using the selective antagonist GW9662. $^*p < 0.05$, $^{**}p < 0.01$.

(D) Graded doses of IL15-DCs differentiated in the absence or presence of GGPP (30 μM) were cocultured with 3×10^5 T cells ($CD3^+$ cells) in round-bottom 96-well plates for 4 days. Cytokine levels in culture supernatants were assessed using the corresponding BD CBA Flex Set. The dataset is representative of three experiments with three different donors. $^*p < 0.05$; see also Figure S6.

is known to stimulate the development of $CD1b^+$ antigen-presenting cells (Kasinrerk et al., 1993), IL-15 favors the differentiation of DC-SIGN$^+$ macrophages with enhanced phagocytic and cytotoxic potential (Krutzik et al., 2005; Tel et al., 2014). In DCs generated with GM-CSF and IL-15 (IL15-DCs), GGPP induced CD1b/c downregulation and DC-SIGN (CD209) as well as CD14 upregulation (Figure 6A), indicative of macrophage differentiation. Furthermore, CD39 itself was consistently downregulated (Figures 6A, 6B, and S5A), while ICAM-1 (CD54), which interacts with LFA-1 (CD11a/CD18) to costimulate T cell activation (Van Seventer et al., 1990), was upregulated by GGPP (Figure 6A). Although GGPP-induced phenotypic changes were less pronounced in DCs generated with GM-CSF and IL-4 (IL4-DCs; not shown), CD39 was also significantly downregulated in these cells (Figure S5A).

Multiplexed bead-based cytokine analysis of culture supernatants revealed high levels of CCL2 in GGPP-induced macrophage-like cells. While the potent non-competitive $P2X_7$ receptor antagonist KN-62 only partially inhibited GGPP-induced CCL2 production (Figure 6B), the selective PPARγ antagonist GW9662 effectively suppressed CCL2 production in a dose-dependent manner (Figure 6C). GGPP also induced the production of CCL2 in IL4-DCs, although less effectively, but with a similar inhibition pattern caused by KN-62 and GW9662, respectively (Figures S5C and S5D). Collectively, these data suggested that GGPP may act by increasing ATP-mediated purinergic signaling and by triggering PPARγ-mediated effects.

GGPP-Imprinted DCs Stimulate Activation of CD161$^+$ Effector T Cells

Both IL15-DCs (Figure 6D) and IL4-DCs (Figure S6) effectively induced activation of IL-2 primed T cells. As little as 10^3 DCs were sufficient to induce substantial IFN-γ and IL-17 production, and increasing DC numbers increased the levels of not only IFN-γ and IL-17 but also IL-1β, TNF-α, IL-6, and CCL2. At 3×10^4 DCs, IL-17, but not IFN-γ, production started to decline. Strikingly, during T cell activation, GGPP-differentiated DCs produced higher levels of IL-1β, which is known to be critical

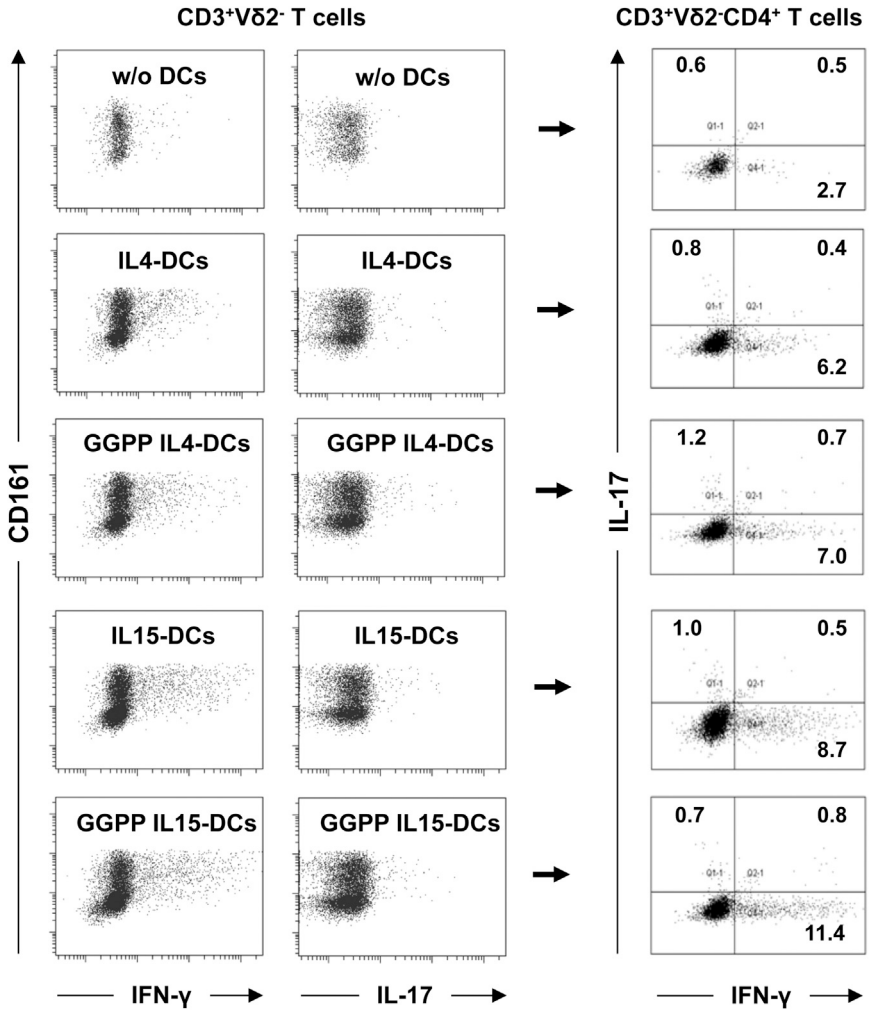

Figure 7. DCs Imprinted by GGPP Preferentially Activate CD161+ T Cells

IL4-DCs (10^4) or IL15-DCs (10^4) differentiated in the absence or presence of GGPP (30 μM) were washed extensively and then cocultured with T cells (3×10^5) in round-bottom 96-well plates for 18 hr. Brefeldin A was added during the last 4 hr. Cells were stained for surface CD3, CD4, CD161, and Vδ2 TCR as well as for intracellular IFN-γ and IL-17. Among $CD3^+$ cells, $V\delta2^-$ cells were gated and selectively analyzed for CD161 and cytokine expression (left). Among $CD3^+V\delta2^-$ cells, $CD4^+$ cells were gated and selectively analyzed for CD161 and cytokine expression (right). The data are representative of three experiments with three different donors. See also Figure S7.

for the differentiation of IL-17 producing T cells (Acosta-Rodriguez et al., 2007), and consequently, GGPP-differentiated DCs indeed induced higher levels of IL-17 (Figure 6D; Figure S6).

Antibody-mediated neutralization of IL-1β, IL-12, or IL-18 partially inhibited IFN-γ and IL-17 production, and simultaneous neutralization of these cytokines almost completely abolished the inflammatory response (Figure S7A). In contrast, anti-IL-15 antibody had almost no effect on IFN-γ production and relatively little effect on IL-17 production. The release of IL-1β could be inhibited by caspase-1 inhibitor Ac-Tyr-Val-Ala-Asp-2,6-dimethylbenzoyloxymethyl-ketone (YVAD) (Figure S7B), indicating that IL-1β bioactivity depended on caspase-1-mediated processing of the IL-1β pro-form (Lalor et al., 2011). Cell-to-cell contact was absolutely required for IFN-γ and IL-17 production (Figure S7C) as well as for the production of TNF-α, IL-1β, and IL-6 (not shown), but not for CCL2 production (Figure S7C).

CD161-expressing unconventional T cells comprising both αβ and γδ T cells have recently been reported to perform such TCR-independent, but cell-to-cell contact, responses to DC-derived cytokines (Fergusson et al., 2014; Godfrey et al., 2015; Harris, 2011). To examine the contribution of these cells, we performed intracellular cytokine stainings. Vγ9Vδ2 T cells, which made up less than 2% of all T cells and homogenously expressed CD161, contributed to the IFN-γ response (Figure S7D). However, the contribution of $CD4^+CD161^+$ T cells was more prominent (Figure 7).

DISCUSSION

The present study points toward a previously unrecognized role of human CD39. In addition to its well-known function as an ecto-ATPase, CD39 exhibited isoprenoid diphosphate phosphohydrolase activity and could thus inactivate self pAgs derived from the potentially oncogenic mevalonate pathway (Thurnher and Gruenbacher, 2015; Thurnher et al., 2012) as well as the potent pathogen-associated pAg HMBPP (Chien et al., 2014). Both ATP and isoprenoids are evolutionarily conserved molecules, and isoprenoids are even among the oldest known biomolecules (Brocks et al., 1999), raising the intriguing question of who was first to serve as a substrate of CD39.

With regard to differences in the velocities of CD39-mediated hydrolysis of nucleotides and pAgs, respectively, it is important to consider that P2Rs and TCRs operate on different scales of time. The fast P2XRs may act within milliseconds because ligand binding is directly linked to channel opening (Khakh, 2001). P2YRs are somewhat slower and trigger upon activation second-messenger cascades that amplify and prolong the duration of the signal over hundreds of milliseconds or even seconds (Khakh, 2001). By contrast, TCR-mediated T cell activation requires sustained signaling over several hours (Rachmilewitz and Lanzavecchia, 2002). Thus, in order to control magnitude and duration of P2R- or TCR-induced responses, respectively, CD39-mediated breakdown of nucleotides and pAgs may occur with different kinetics.

Our finding that CD39 expression was not upregulated before day 2 to 3, when IFN-γ production already declines, is consistent

with its regulatory role. CD39 obviously serves to degrade pAgs in the late phase of the response and may thus contribute to the homeostatic maintenance of Vγ9Vδ2 T cells. This view is supported by a previous report showing that long-lasting exposure to supraphysiologic IPP levels produced by chronic lymphocytic leukemia cells, especially in the absence of adequate IL-2 costimulation, may lead to functional exhaustion and loss of Vγ9Vδ2 T cells (Coscia et al., 2012). Since ATP release and purinergic signaling have also been implicated in T cell lineage choice and shaping of the peripheral γδ T cell compartment (Frascoli et al., 2012), the isoprenoid diphosphate phosphohydrolase activity of CD39 might therefore also play a role in the regulation of Vγ9Vδ2 T cell development.

One important function of CD39 during interaction of antigen-presenting cells and T cells is to rapidly degrade ATP in order to prevent purinoreceptor desensitization (Mizumoto et al., 2002). Along the same line, pAg degradation by CD39 that is associated with either antigen-presenting cells or T cells may also serve to prevent Vγ9Vδ2 TCR desensitization.

The phosphohydrolase activity of CD39 has been reported to depend on the cellular activation status. T_{reg} cells, for instance, displayed increased CD39 activity only upon TCR-mediated activation, while CD39 was found to be inactive on resting cells (Borsellino et al., 2007). Although CD39 is constitutively expressed on T_{reg} cells (Antonioli et al., 2013), CD39 expression is also well known to be associated with effector function (Dombrowski et al., 1998). Our observation that $CD39^+$ Vγ9Vδ2 T cells expanded with pAg and IL-2 expressed markers of innate-like effector cells such as CD56 and CD161 but lacked molecules associated with T_{reg} cells such as Foxp3, CD73, CD25, and CD152 indicated that these cells are effector and not regulatory T cells. Specifically, absence of CD73 means that $CD39^+$ Vγ9Vδ2 T cells cannot convert AMP generated by CD39 into immunosuppressive adenosine. Conversely, the presence of CD161, which has also been identified as a top favorable pan-cancer prognostic gene (Gentles et al., 2015), clearly defines the effector phenotype of $CD39^+$ Vγ9Vδ2 T cells (Fergusson et al., 2014).

Although the intra- versus extracellular distribution of isoprenoid diphosphates in various tissues has hardly been examined, it is well established that accumulation of both exogenous and endogenous isoprenoid diphosphates can lead to Vγ9Vδ2 T cell activation (Gober et al., 2003; Tanaka et al., 1995). In contrast to αβ T cells, which recognize peptide antigens in the context of major histocompatibility complex (MHC) molecules, it has been unknown how pAgs are presented to the Vγ9Vδ2 TCR. Quite recently, BTN3A1 has been identified as the elusive cell surface molecule that can directly bind pAg for presentation to the γδ TCR (Vavassori et al., 2013).

Alternatively, pAgs accumulating in tumor cells, infected cells, or in cells exposed to N-BPs may bind to a basic pocket within the intracellular B30.2 domain of BTN3A1 (Sandstrom et al., 2014). pAg binding to B30.2 may change the conformation of the extracellular IgV domain of BTN3A1, leading to Vγ9Vδ2 TCR engagement (Harly et al., 2012). The B30.2 domain of BTN3A1 may thus act as a sensor for increased intracellular pAg levels and alert Vγ9Vδ2 T cells via conformational changes of the extracellular IgV domain. In this context, it will be important to examine whether Vγ9Vδ2 T-cell-stimulating cytokines such as IL-15 and IL-18 (Gruenbacher et al., 2014) affect mevalonate metabolism and may increase intracellular levels of isoprenoid diphosphates, thus promoting Vγ9Vδ2 T cell activation.

Similar to ATP, pAgs may also be released into the extracellular space. pAg release may occur during cell lysis or by more specific mechanisms. Specific export of pAgs could be mediated, for example, by ATP binding cassette transporter proteins, which are also responsible for the export of cholesterol, a lipid synthesized from isoprenoid diphosphates (Thurnher and Gruenbacher, 2015). By membrane insertion via the hydrophobic isoprenoid chain, these molecules may also participate in membrane traffic and may thus reach the plasma membrane or may be released from cells in the form of exosomes. Controlled or lytic release of isoprenoid-derived pAgs into the extracellular space can lead to direct binding of pAgs to BTN3A1 on healthy neighboring tissue, inevitably causing collateral damage. As an ecto-enzyme with isoprenoid diphosphate phosphohydrolase activity, CD39 expressed by Vγ9Vδ2 T cells, T_{reg} cells, or other cells may inactivate secreted pAgs to avoid such side effects and ensure the specific elimination of the dysregulated cells. Consistent with such a view, anti-BTN3A1 (CD277) antibody was the most potent stimulus of CD39 upregulation in our study.

We observed differences in the susceptibility of the various isoprenoid diphosphates to CD39-mediated hydrolysis as well as differences in substrate specificity between recombinant and cell-associated CD39. Mevalonate-derived FPP (C_{15}) and GGPP (C_{20}) serve as lipid donors in protein prenylation, a post-translational modification facilitating membrane attachment of the prenylated protein. The failure of rCD39, which lacks the two transmembrane domains, to hydrolyze FPP and GGPP may indicate that membrane anchoring of both the enzyme and the $C_{15/20}$ substrate is required for effective pAg hydrolysis (Grinthal and Guidotti, 2006).

Pathogen-associated HMBPP displayed lower susceptibility to CD39-mediated breakdown when compared to mevalonate-derived self pAgs. It may indeed be this lower susceptibility of HMBPP to CD39-mediated degradation that contributes to its high potency in stimulating Vγ9Vδ2 T cell activation. Mevalonate-derived GPP, which is an intermediate during FPP synthase-catalyzed conversion of DMAPP into FPP, appeared to be highly susceptible to CD39-mediated hydrolysis. In contrast, GGPP, a lipid donor in protein prenylation (Thurnher and Gruenbacher, 2015), turned out to be relatively resistant to CD39-mediated breakdown, indicating that this metabolite may serve additional purposes. In the present study, we identified GGPP as a regulator of CD39 activity and expression, which may thus increase stability of other pAgs as well as of ATP and enhance TCR and purinoreceptor signaling. We have previously reported that GGPP can also exhibit antioxidant effects and protect immune cells from oxidative damage (Gruenbacher et al., 2014). Bai et al. (2015) recently reported that CD39 expression depends on NADH oxidase-mediated generation of reactive oxygen species (ROS). Therefore, GGPP-mediated suppression of CD39 expression was most likely due to the prevention of ROS effects.

Yet another effect of GGPP that we observed in our study was the stimulation of high CCL2 production in DCs differentiated in the presence of IL-15. Moreover, GGPP-imprinted IL15-DCs

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acquired a macrophage-like phenotype. By metabolizing GGPP, these macrophage-like cells may contribute to the final disposal of CD39-resistant GGPP. Both IL-15 and CCL2 are known to be produced in response to tissue damage (Jabri and Abadie, 2015; Matsushima et al., 1989), which inevitably results in the appearance of ATP and possibly also isoprenoid diphosphates in the extracellular space. Resistance against *Mycobacterium tuberculosis*, which is known to involve Vγ9Vδ2 T cells (Shen et al., 2002), also requires CCL2 effects mediated by its receptor CCR2, since $CCR2^{-/-}$ mice exhibited an early defect in macrophage recruitment to the lung and a later defect in recruitment of DCs and T cells to the lung (Peters et al., 2001).

Our further analyses led to the identification of a TCR-independent response of $CD161^{+}$ T cells comprising αβ and γδ T cells that was almost exclusively mediated by DC-derived IL-1β, IL-12, and IL-18. CD161 (*KLRB1*) has recently been reported to be a top favorable pan-cancer prognostic gene (Gentles et al., 2015) and a marker of enhanced innate immune characteristics in diverse T cell subsets (Fergusson et al., 2014). The pan-cancer prognostic significance of CD161 strongly indicates the immunotherapeutic potential of the family of unconventional T cells and therefore highly recommends the use of pAg-pulsed DCs, which may be able to link the early response of Vγ9Vδ2 T cells with the subsequent response of other $CD161^{+}$ unconventional T cells.

The fact that murine CD39 exhibited similar isoprenoid diphosphate phosphohydrolase activity suggested that this particular function of CD39 is not exclusively related to Vγ9Vδ2 T cell biology, since this γδ T cell subset does not exist in mice (Karunakaran et al., 2014). CD39-mediated degradation of mevalonate-derived isoprenoid diphosphates may also serve to regulate their function as cell-intrinsic or cell-extrinsic metabolic cues. In transcellular lipid metabolism that has previously been reported to occur during eicosanoid biosynthesis (Capra et al., 2015), a lipid intermediate is synthesized and released by one cell type and then incorporated and further metabolized by another cell type. In accordance with such a concept, extracellular isoprenoid diphosphates have previously been shown to enter cells and contribute, for instance, to protein prenylation (Dunn et al., 2006; Freed-Pastor et al., 2012; Thurnher and Gruenbacher, 2015). The ability of CD39 to degrade extracellular isoprenoid diphosphates may be crucial, since "over-prenylation" may lead to malignant transformation (Clendening et al., 2010; Freed-Pastor et al., 2012).

Yet another ecto-ATPase has previously been implicated in Vγ9Vδ2 T cell activation. An entity related to the mitochondrial F1-ATPase and expressed on the tumor cell surface has been demonstrated to promote tumor recognition by Vγ9Vδ2 T cells (Scotet et al., 2005). F1-ATPase was therefore proposed to be one of several presentation structures for pAgs (Mookerjee-Basu et al., 2010).

In addition to its well-known function as an ecto-ATPase, the present study identifies CD39 as an isoprenoid diphosphate phosphohydrolase that may dephosphorylate self and pathogen-associated pAgs and thus abrogate Vγ9Vδ2 T cell responses. Only GGPP acted as a CD39 regulator and facilitated macrophage-mediated effector T cell responses in an innate-like manner.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies

Isoprenoids obtained from Echelon Biosciences or from Isoprenoids were analyzed by nuclear magnetic resonance (NMR) and were at least 95% pure. Recombinant human and murine CD39/ENTPD1 (molecular weight [MW], ~50 kDa) as well as human CD73 were from R&D. ATP, ADP, potato apyrase, KN-62, and GW9662 were obtained from Sigma-Aldrich. Zoledronate was from Novartis Pharmaceuticals. Pamidronate (Pamidronat Dinatrium Mayne) was obtained from Mayne Pharma. YVAD was purchased from Alexis Biochemicals. Recombinant human cytokines were obtained as follows: GM-CSF (LEUKINE), Berlex Laboratories; IL-2, Novartis; IL-4 (CELL GRO), CellGenix; IL-15, PeproTech; and IL-18, Medical and Biological Laboratories (MBL). Murine M-CSF was from Miltenyi. Monoclonal antibodies used in this study are listed in the Supplemental Experimental Procedures.

Enzymatic Assays

pAgs (IP, IPP, IPPP, DMAPP, GPP, FPP, GGPP, HMBPP) or nucleotides (ATP, ADP, AMP) were incubated at the concentrations indicated for 0.5 to 2 hr at 37°C with rCD39 (0.5 μg/ml), rCD73 (0.5 μg/ml), or potato apyrase (10 U/ml) in a final volume of 0.2 ml of 25 mM Tris buffer (pH 7.5), supplemented with 5 mM $CaCl_2$. When CD39 activity associated with intact cells was measured, Tris-Ringer (1:3) was used as buffer solution. P_i released during enzyme-mediated hydrolysis of pAgs and ATP/ADP or CD73-mediated hydrolysis of AMP was measured using the colorimetric phosphate assays kit (Abcam). The phosphate ion forms chromogenic complex with malachite green and ammonium molybdate (650 nm). The lower limit of detection was ~0.1 nmol; control values obtained with either recombinant or cell-associated enzymes or substrate alone were subtracted.

Centrifugal Filtration of Isoprenoid-Derived pAgs

To study the functional consequences of pAg hydrolysis, Amicon centrifugal filter devices with a cutoff (nominal molecular weight limit [NMWL]) of 3 kDa (Merck Millipore: UFC500324) were used to separate the enzyme from the low molecular weight isoprenoids (15 min at 14,000 × *g*). The filtrate containing the isoprenoid, but not the ATPase, was used for Vγ9Vδ2 T cell stimulation.

Human and Murine Cell Culture

All donors (n = 12) gave written informed consent in accordance with the Declaration of Helsinki to the use of their residual buffy coats for research purposes, with approval from the University Hospital of Innsbruck Review Board. Vγ9Vδ2 T cell frequency in the study population was 1.92% ± 0.86 (range, 0.9%–4.0%). All cell cultures were performed in complete RPMI 1640 supplemented with 10% fetal bovine serum (FBS). $CD3^{+}$ PBMCs were isolated using the human Pan T cell isolation Kit (Miltenyi), and 1.5×10^{6} cells/ml were stimulated with IL-2 (100 U/ml) either alone or in combination with pAgs (10 to 30 μM). Whereas pAgs were added as an initial single dose, fresh IL-2 was added every 2 to 3 days.

$CD4^{+}CD25^{+}$ T cells were isolated using the human regulatory T cell isolation kit (Miltenyi), and T_{reg} cultures were established by repetitive stimulation with CD3/CD28 microbeads (T cell activation/expansion kit, Miltenyi) in the presence of IL-2 (1,000 U/ml) and rapamycin (100 nM) (Battaglia et al., 2006). T_{reg} cell identity and CD39 expression were confirmed by flow cytometry.

In the mixed leukocyte reaction (MLR), $CD3^{+}$ cells prepared with the Pan T cell isolation kit from Miltenyi were stimulated with graded doses of DCs in the presence of IL-2 (100 U/ml). After co-culture, cytokines were quantified in culture supernatants or stained intracellularly. Human DCs were generated from monocytes prepared with CD14 microbeads from Miltenyi using GM-CSF (800 U/ml) in combination with IL-15 (25 ng/ml) or IL-4 (500 U/ml) for 3 to 4 days.

To generate murine macrophages, bone marrow cells from murine tibias and femurs of wild-type or CD39 (−/−) mice (Enjyoji et al., 1999; Robson et al., 2005) were passed through a nylon mesh to remove debris, and 1×10^{6} cells were seeded in 24-well plates in 1 ml in complete RPMI 1640 supplemented with 10% FBS and 50 ng/ml murine M-CSF. After 3 days, 1 ml of medium containing all supplements was added. For flow cytometric analysis, cells were seeded on ultra-low attachment surfaces (Corning). All experiments were

performed according to institutional guidelines of the animal ethics committee from the city of Freiburg.

Statistical Analyses

Experiments were always set up as triplicates and were repeated at least two times. Group comparisons were performed using Student's t test for paired samples. A p value equal to or less than 0.05 was considered statistically significant. Microsoft Excel and SPSS software (SPSS) were used for calculations.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.06.009.

AUTHOR CONTRIBUTIONS

G.G., M.I., O.N., and M.T. planned and designed experiments and analyzed results. G.G., H.G., and A.R. performed experiments. M.T. wrote the manuscript with review and comments by all authors and provided overall guidance.

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