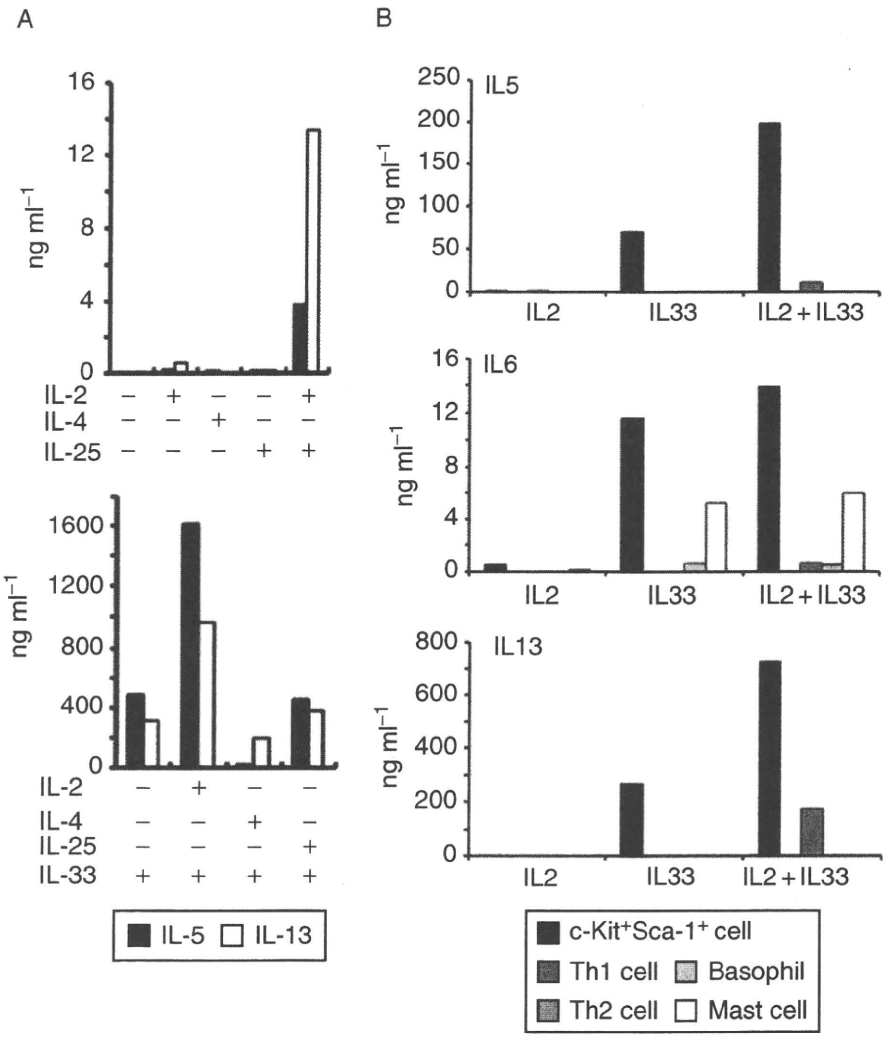

FIGURE 2.4 Th2 cytokine production from NH cells. (A) FALC-derived NH cells (5000 cells/well) were cultured with the indicated cytokines (10 ng/ml) for 5 days and culture supernatants analyzed in triplicate by ELISA. (B) Production of cytokines from various types of cells. The indicated cells (5×10^3) were stimulated with the specified cytokines for 4 days and the concentrations of IL-5, IL-6, and IL-13 in the supernatants were determined in triplicate by ELISA. Although not shown, IFNγ production was not detected in these cultures. These figures are reproduced from Moro *et al.* (2010) by the courtesy of *Nature*.

5.2. Other cells

Since our identification of NH cells, others have published papers demonstrating similar cell types. Using knock-in mice in which GFP was inserted into the IL-13 allele, Neill *et al.* reported that IL-13-producing NTNB cells (GFP^+ cells) were observed in the mesenteric lymph nodes, spleen, and small intestine of mice after administration of IL-25 or IL-33 (Neill *et al.*, 2010). They named these GFP^+ cells "nuocytes," nu being the thirteenth letter of the Greek alphabet although these cells also produce IL-5.

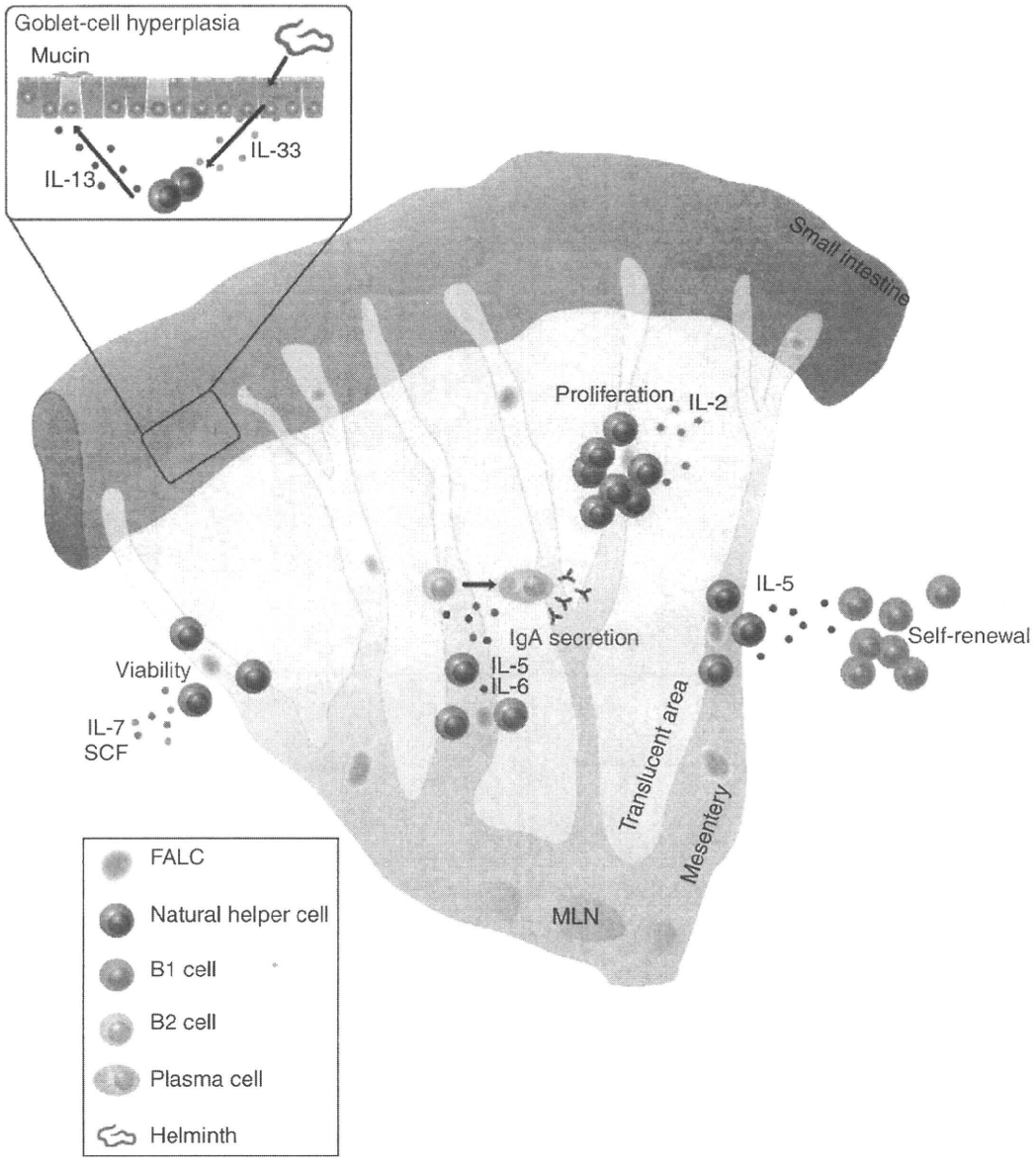

FIGURE 2.5 Schematic diagram for the role of NH cells.

As observed for NTNB cells in an earlier report (Fort *et al.*, 2001), nuocytes express MHC class II and respond to IL-25 alone, which are characteristics distinct from those of NH cells (Table 2.1). Neill *et al.* demonstrated that the adoptive transfer of wild-type nuocytes into IL-17RB deficient mice restored their ability to expel the helminth, *N. brasiliensis*. Two million per milliliter nuocytes are able to produce μg amounts of IL-5, IL-6, and IL-13 upon one-week culture with IL-7 and IL-33 (Neill *et al.*, 2010).

Using knock-in mice in which GFP is inserted into the IL-4 allele, Saenz *et al.* reported that IL-25 administration induced the expansion of IL-4-producing NTNB cells (GFP^+ cells) in gut-associated lymphoid tissues (Saenz *et al.*, 2010). These cells share cell surface markers with

TABLE 2.1 Comparison of NH cell and other innate Th2 cytokine producing cells

Markers	NH cell	Nuocyte	MPP^{type2}	Ih2 cell
c-Kit	+	+/−	+	+/−
CD45	+	+	+	+
IL-7Rα	+	lo	−/lo	?
Sca-1	+	+	+	−
Thy-1	+	+	?	+
CD34	−	−	−/lo	?
CD4	−	−	−	?
CD25	+	?[a]	?	?
CD44	+	+	?	+
CD69	+	?	−	+[b]
CD62L	−	?	−/lo	?
FcεRI	−	−	−	?
T1/ST2	+	+/−	−/lo	?
MHC class II	−	+	?[c]	?

[a] Microarray analysis showed the lack of CD25 expression on Nuocytes.
[b] Microarray analysis showed the expression of CD69 on Ih2 cells.
[c] GFP^- cells expressed MHC class II after cultivation with a combination of IL-3 and SCF.

hematopoietic stem cells or multipotent progenitor cells and were named "MPP^{type2}". In fact, this population seems to be heterogeneous and c-Kit^+GFP^+ cells differentiate into mast cells while c-Kit^+GFP^- cells have the potential to differentiate into basophils and macrophages.

Furthermore, Price *et al.* reported using both IL-4 and IL-13 reporter mice that c-Kit^+ NTNB cells capable of producing IL-5 and IL-13 are distributed in various tissues (Price *et al.*, 2010). Although the cells are IL-4-GFP^+, they do not produce IL-4 protein. The authors named these cells innate helper type 2 (Ih2) cells. It is unknown whether Ih2 cells are able to respond to IL-25 alone to produce cytokines or how much cytokines Ih2 cells produce.

At the moment, the relationship between NH cells, nuocytes, MPP^{type2}, and Ih2 cells is unclear. There are similarities and differences as summarized in Table 2.1. An important difference between NH cells and other cells mentioned above is that NH cells do not respond to IL-25 alone (Moro *et al.*, 2010; Saenz *et al.*, 2010). Future studies will uncover the relationship between these cell types.

6. PERSPECTIVES

Recent studies have shed light on the innate immune responses upon helminth infection. Invasion by helminths results in tissue destruction, leading to the secretion of alarmins including IL-25 and IL-33 from

necrotic cells. The cytokines act on innate immune cells such as NH cells to induce IL-5 and IL-13, which induce eosinophilia and goblet cell hyperplasia, respectively. Eosinophilia is important in controlling helminths in the lung stage of *N. brasiliensis* and *S. venezuelensis* and goblet cell hyperplasia in the intestine is involved in blocking the attachment of helminths during the early intestinal phase. IL-25 and IL-33 induce IL-4 from basophils, mast cells and possibly MPP^{type2} cells that support the induction of Th2 differentiation. Th2-mediated adaptive immune responses will eventually result in the expulsion and clearance of helminths.

These pictures resemble the Th1-type innate immune response before pathogen-specific Th1 cells are induced. Phagocytes, supported by NK cells, limit the growth of intracellular pathogens including protozoan parasites and viruses by providing interferon-γ, a Th1 cytokine (Korbel *et al.*, 2004). IL-22-expressing $NKp46^{+}$ NK cells deal with extracellular mucosal pathogens in the intestine as well as *M. tuberculosis* in the lung until Th1 cells are ready to work (Dhiman *et al.*, 2009; Satoh-Takayama *et al.*, 2009). From this viewpoint, it is of interest that IL-33 induces Th1 cytokines from NK cells and NKT cells (Bourgeois *et al.*, 2009; Smithgall *et al.*, 2008), implying that IL-33 induces a Th1-type innate immune response under certain circumstances.

IL-5 is a critical factor in the induction of eosinophilia and IL-13 leads to goblet cell hyperplasia, both of which are involved in the pathogenesis of allergic diseases such as asthma and allergic diarrhea. It is therefore of interest and importance to elucidate the functions of innate cells producing Th2 cytokines in various allergic diseases in future studies.

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REFERENCES

Albonico, M., Crompton, D. W. T., and Savioli, L. (1999). Control strategies for human intestinal nematode infections. *Adv. Immunol.* **42,** 277–341.

Ali, S., Huber, M., Kollewe, C., Bischoff, S. C., Falk, W., and Martin, M. U. (2007). IL-1 receptor accessory protein is essential for IL-33-induced activation of T lymphocytes and mast cells. *Proc. Natl. Acad. Sci. USA* **104,** 18660–18665.

Angkasekwinai, P., Park, H., Wang, Y. H., Wang, Y. H., Chang, S. H., Corry, D. B., Liu, Y. J., Zhu, Z., and Dong, C. (2007). Interleukin 25 promotes the initiation of proallergic type 2 responses. *J. Exp. Med.* **204,** 1509–1517.

Anthony, R. M., Urban, J. F., Jr., Alem, F., Hamed, H. A., Rozo, C. T., Boucher, J. L., Van Rooijen, N., and Gause, W. C. (2006). Memory T_H2 cells induce alternatively activated macrophages to mediate protection against nematode parasites. *Nat. Med.* **12,** 955–960.

Arase, H., Arase, N., Nakagawa, K., Good, R. A., and Onoé, K. (1993). NK1.1$^+$ CD4$^+$CD8$^-$ thymocytes with specific lymphokine secretion. *Eur. J. Immunol.* **23,** 307–310.

Artis, D., Wang, M. L., Keilbaugh, S. A., He, W., Brenes, M., Swain, G. P., Knight, P. A., Donaldson, D. D., Lazar, M. A., Miller, H. R., Schad, G. A., Scott, P., *et al.* (2004). RELMb/FIZZ2 is a goblet cell-specific immune-effector molecule in the gastrointestinal tract. *Proc. Natl. Acad. Sci. USA* **101,** 13596–13600.

Beagley, K. W., Eldridge, J. H., Lee, F., Kiyono, H., Everson, M. P., Koopman, W. J., Hirano, T., Kishimoto, T., and McGhee, J. R. (1989). Interleukins and IgA synthesis. Human and murine interleukin 6 induce high rate IgA secretion in IgA-committed B cells. *J. Exp. Med.* **169,** 2133–4218.

Bourgeois, E., Van, L. P., Samson, M., Diem, S., Barra, A., Roga, S., Gombert, J. M., Schneider, E., Dy, M., Gourdy, P., Girard, J. P., and Herbelin, A. (2009). The pro-Th2 cytokine IL-33 directly interacts with invariant NKT and NK cells to induce IFN-γ production. *Eur. J. Immunol.* **39,** 1046–1055.

Camberis, M., Le Gros, G., and Urban, J., Jr. (2003). Animal model of *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus*. *Curr. Protoc. Immunol.* doi: 10.1002/0471142735.im1912s55, Chapter 19, Unit 19.12.

Capó, V., and Despommier, D. D. (1996). Clinical aspects of infection with *Trichinella* spp. *Clin. Microbiol. Rev.* **9,** 47–54.

Carrière, V., Roussel, L., Ortega, N., Lacorre, D. A., Americh, L., Aguilar, L., Bouche, G., and Girard, J. P. (2007). IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor *in vivo*. *Proc. Natl. Acad. Sci. USA* **104,** 282–287.

Cayrol, C., and Girard, J. P. (2009). The IL-1-like cytokine IL-33 is inactivated after maturation by caspase-1. *Proc. Natl. Acad. Sci. USA* **106,** 9021–9026.

Cliffe, L. J., Humphreys, N. E., Lane, T. E., Potten, C. S., Booth, C., and Grencis, R. K. (2005). Accelerated intestinal epithelial cell turnover: A new mechanism of parasite expulsion. *Science* **308,** 1463–1465.

Cohen, I., Rider, P., Carmi, Y., Braiman, A., Dotan, S., White, M. R., Voronov, E., Martin, M. U., Dinarello, C. A., and Apte, R. N. (2010). Differential release of chromatin-bound IL-1α discriminates between necrotic and apoptotic cell death by the ability to induce sterile inflammation. *Proc. Natl. Acad. Sci. USA* **107,** 2574–2579.

Cranshaw, M. L., and Leak, L. V. (1990). Milky spots of the omentum: A source of peritoneal cells in the normal and stimulated animal. *Arch. Histol. Cytol.* **53**(Suppl.), 165–177.

Dent, L. A., Daly, C. M., Mayrhofer, G., Zimmerman, T., Hallett, A., Bignold, L. P., Creaney, J., and Parsons, J. C. (1999). Interleukin-5 transgenic mice show enhanced resistance to primary infections with *Nippostrongylus brasiliensis* but not primary infections with *Toxocara canis*. *Infect. Immun.* **67,** 989–993.

Dhiman, R., Indramohan, M., Barnes, P. F., Nayak, R. C., Paidipally, P., Rao, L. V., and Vankayalapati, R. (2009). The natural cytotoxicity receptor NKp46 is dispensable for IL-22-mediated innate intestinal immune defense against *Citrobacter rodentium*. *J. Immunol.* **183,** 6639–6645.

Dixon, H., Blanchard, C., Deschoolmeester, M. L., Yuill, N. C., Christie, J. W., Rothenberg, M. E., and Else, K. J. (2006). The role of Th2 cytokines, chemokines and parasite products in eosinophil recruitment to the gastrointestinal mucosa during helminth infection. *Eur. J. Immunol.* **36,** 1753–1763.

Eisenbarth, S. C., Piggott, D. A., Huleatt, J. W., Visintin, I., Herrick, C. A., and Bottomly, K. (2002). Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J. Exp. Med.* **196,** 1645–1651.

Erickson, L. D., Foy, T. M., and Waldschmidt, T. J. (2001). Murine B1 B cells require IL-5 for optimal T cell-dependent activation. *J. Immunol.* **166,** 1531–1539.

Fairweather, D., and Cihákovά, D. (2009). Alternatively activated macrophages in infection and autoimmunity. *J. Autoimmun.* **33,** 222–230.

Fallon, P. G., Ballantyne, S. J., Mangan, N. E., Barlow, J. L., Dasvarma, A., Hewett, D. R., McIlgorm, A., Jolin, H. E., and McKenzie, A. N. (2006). Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. *J. Exp. Med.* **203,** 1105–1116.

Finkelman, F. D., Shea-Donohue, T., Morris, S. C., Gildea, L., Strait, R., Madden, K. B., Schopf, L., and Urban, J. F., Jr. (2004). Interleukin-4- and interleukin-13-mediated host protection against intestinal nematode parasites. *Immunol. Rev.* **201,** 139–155.

Fort, M. M., Cheung, J., Yen, D., Li, J., Zurawski, S. M., Lo, S., Menon, S., Clifford, T., Hunte, B., Lesley, R., Muchamuel, T., Hurst, S. D., *et al.* (2001). IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies *in vivo*. *Immunity* **15,** 985–995.

Fukao, T., Yamada, T., Tanabe, M., Terauchi, Y., Ota, T., Takayama, T., Asano, T., Takeuchi, T., Kadowaki, T., Hata, J.-i., and Koyasu, S. (2002). Selective loss of gastrointestinal mast cells and impaired immunity in PI3K-deficient mice. *Nat. Immunol.* **3,** 295–304.

Ghildyal, N., McNeil, H. P., Stechschulte, S., Austen, K. F., Silberstein, D., Gurish, M. F., Somerville, L. L., and Stevens, R. L. (1992). IL-10 induces transcription of the gene for mouse mast cell protease-1, a serine protease preferentially expressed in mucosal mast cells of *Trichinella spiralis*-infected mice. *J. Immunol.* **149,** 2123–2129.

Gurish, M. F., Humbles, A., Tao, H., Finkelstein, S., Boyce, J. A., Gerard, C., Friend, D. S., and Austen, K. F. (2002). CCR3 is required for tissue eosinophilia and larval cytotoxicity after infection with *Trichinella spiralis*. *J. Immunol.* **168,** 5730–5736.

Gurish, M. F., Bryce, P. J., Tao, H., Kisselgof, A. B., Thornton, E. M., Miller, H. R., Friend, D. S., and Oettgen, H. C. (2004). IgE enhances parasite clearance and regulates mast cell responses in mice infected with *Trichinella spiralis*. *J. Immunol.* **172,** 1139–1145.

Hammad, H., Chieppa, M., Perros, F., Willart, M. A., Germain, R. N., and Lambrecht, B. N. (2009). House dust mite allergen induces asthma via toll-like receptor 4 triggering of airway structural cells. *Nat. Med.* **15,** 410–416.

Hammad, H., Plantinga, M., Deswarte, K., Pouliot, P., Willart, M. A., Kool, M., Muskens, F., and Lambrecht, B. N. (2010). Inflammatory dendritic cells–not basophils–are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *J. Exp. Med.* **207,** 2097–2111.

Harari, Y., Russell, D. A., and Castro, G. A. (1987). Anaphylaxis-mediated epithelial Cl^- secretion and parasite rejection in rat intestine. *J. Immunol.* **138,** 1250–1255.

Hotez, P. J., and Kamath, A. (2009). Neglected tropical diseases in sub-saharan Africa: Review of their prevalence, distribution, and disease burden. *PLoS Negl. Trop. Dis.* **3,** e412. doi: 10.1371/journal.pntd.0000412.

Humphreys, N. E., Xu, D., Hepworth, M. R., Liew, F. Y., and Grencis, R. K. (2008). IL-33, a potent inducer of adaptive immunity to intestinal nematodes. *J. Immunol.* **180,** 2443–2449.

Hurst, S. D., Muchamuel, T., Gorman, D. M., Gilbert, J. M., Clifford, T., Kwan, S., Menon, S., Seymour, B., Jackson, C., Kung, T. T., Brieland, J. K., Zurawski, S. M., *et al.* (2002). New IL-17 family members promote Th1 or Th2 responses in the lung: *In vivo* function of the novel cytokine IL-25. *J. Immunol.* **169,** 443–453.

Ikeda, K., Nakajima, H., Suzuki, K., Kagami, S., Hirose, K., Suto, A., Saito, Y., and Iwamoto, I. (2003). Mast cells produce interleukin-25 upon FcεRI-mediated activation. *Blood* **101,** 3594–3596.

Ishikawa, N., Horii, Y., and Nawa, Y. (1993). Immune-mediated alteration of the terminal sugars of goblet cell mucins in the small intestine of *Nippostrongylus brasiliensis*-infected rats. *Immunology* **78,** 303–307.

Ishikawa, N., Horii, Y., and Nawa, Y. (1994). Reconstitution by bone marrow grafting of the defective protective capacity at the migratory phase but not at the intestinal phase of *Nippostrongylus brasiliensis* infection in W/W^v mice. *Parasite Immunol.* **16,** 181–186.

Ito, T., Wang, Y. H., Duramad, O., Hori, T., Delespesse, G. J., Watanabe, N., Qin, F. X., Yao, Z., Cao, W., and Liu, Y. J. (2005). TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J. Exp. Med.* **202,** 1213–1223.

Khan, A. I., Horii, Y., Tiuria, R., Sato, Y., and Nawa, Y. (1993). Mucosal mast cells and the expulsive mechanisms of mice against *Strongyloides venezuelensis*. *Int. J. Parasitol.* **23,** 551–555.

Khan, W. I., Richard, M., Akiho, H., Blennerhasset, P. A., Humphreys, N. E., Grencis, R. K., Van Snick, J., and Collins, S. M. (2003). Modulation of intestinal muscle contraction by interleukin-9 (IL-9) or IL-9 neutralization: Correlation with worm expulsion in murine nematode infections. *Infect. Immun.* **71,** 2430–2438.

Kim, M. R., Manoukian, R., Yeh, R., Silbiger, S. M., Danilenko, D. M., Scully, S., Sun, J., DeRose, M. L., Stolina, M., Chang, D., Van, G. Y., Clarkin, K., *et al.* (2002). Transgenic overexpression of human IL-17E results in eosinophilia, B-lymphocyte hyperplasia, and altered antibody production. *Blood* **100,** 2330–2340.

Knight, P. A., Wright, S. H., Brown, J. K., Huang, X., Sheppard, D., and Miller, H. R. (2002). Enteric expression of the integrin $\alpha_v\beta_6$ is essential for nematode-induced mucosal mast cell hyperplasia and expression of the granule chymase, mouse mast cell protease-1. *Am. J. Pathol.* **161,** 771–779.

Knight, P. A., Brown, J. K., and Pemberton, A. D. (2008). Innate immune response mechanisms in the intestinal epithelium: Potential roles for mast cells and goblet cells in the expulsion of adult *Trichinella spiralis*. *Parasitology* **135,** 655–670.

Korbel, D. S., Finney, O. C., and Riley, E. M. (2004). Natural killer cells and innate immunity to protozoan pathogens. *Int. J. Parasitol.* **34,** 1517–1528.

Korenaga, M., Hitoshi, Y., Takatsu, K., and Tada, I. (1994). Regulatory effect of anti-interleukin-5 monoclonal antibody on intestinal worm burden in a primary infection with *Strongyloides venezuelensis* in mice. *Int. J. Parasitol.* **24,** 951–957.

Little, M. C., Bell, L. V., Cliffe, L. J., and Else, K. J. (2005). The characterization of intraepithelial lymphocytes, lamina propria leukocytes, and isolated lymphoid follicles in the large intestine of mice infected with the intestinal nematode parasite *Trichuris muris*. *J. Immunol.* **175,** 6713–6722.

Liu, Y. J. (2006). Thymic stromal lymphopoietin: Master switch for allergic inflammation. *J. Exp. Med.* **203,** 269–273.

Lüthi, A. U., Cullen, S. P., McNeela, E. A., Duriez, P. J., Afonina, I. S., Sheridan, C., Brumatti, G., Taylor, R. C., Kersse, K., Vandenabeele, P., Lavelle, E. C., and Martin, S. J. (2009). Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases. *Immunity* **31,** 84–98.

Mahida, Y. R. (2003). Host–parasite interactions in rodent nematode infections. *J. Helminthol.* **77,** 125–131.

Martin, F., and Kearney, J. F. (2000). B-cell subsets and the mature preimmune repertoire. Marginal zone and B1 B cells as part of a "natural immune memory". *Immunol. Rev.* **175,** 70–79.

Maruyama, H., Yabu, Y., Yoshida, A., Nawa, Y., and Ohta, N. (2000). A role of mast cell glycosaminoglycans for the immunological expulsion of intestinal nematode, *Strongyloides venezuelensis*. *J. Immunol.* **164,** 3749–3754.

McDermott, J. R., Bartram, R. E., Knight, P. A., Miller, H. R., Garrod, D. R., and Grencis, R. K. (2003). Mast cells disrupt epithelial barrier function during enteric nematode infection. *Proc. Natl. Acad. Sci. USA* **100,** 7761–7766.

McKenzie, G. J., Bancroft, A., Grencis, R. K., and McKenzie, A. N. (1998). A distinct role for interleukin-13 in Th2-cell-mediated immune responses. *Curr. Biol.* **8,** 339–342.

Min, B., Prout, M., Hu-Li, J., Zhu, J., Jankovic, D., Morgan, E. S., Urban, J. F., Jr., Dvorak, A. M., Finkelman, F. D., LeGros, G., and Paul, W. E. (2004). Basophils produce IL-4 and accumulate in tissues after infection with a Th2-inducing parasite. *J. Exp. Med.* **200,** 507–517.

Morimoto, M., Morimoto, M., Whitmire, J., Xiao, S., Anthony, R. M., Mirakami, H., Star, R. A., Urban, J. F., Jr., and Gause, W. C. (2004). Peripheral CD4 T cells rapidly accumulate at the host:parasite interface during an inflammatory Th2 memory response. *J. Immunol.* **172,** 2424–2430.

Moro, K., Yamada, T., Tanabe, M., Takeuchi, T., Ikawa, T., Kawamoto, H., Furusawa, J.-I., Ohtani, M., Fujii, H., and Koyasu, S. (2010). Innate production of Th2 cytokines by adipose tissue-associated c-Kit^{+}Sca-1^{+} lymphoid cells. *Nature* **463,** 540–544.

Moussion, C., Ortega, N., and Girard, J. P. (2008). The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: A novel 'alarmin'? *PLoS ONE* **3,** e3331. doi: 10.1371/journal.pone.0003331.

Murata, Y., Shimamura, T., and Hamuro, J. (2002). The polarization of T_h1/T_h2 balance is dependent on the intracellular thiol redox status of macrophages due to the distinctive cytokine production. *Int. Immunol.* **14,** 201–212.

Murrell, K. D. (1985). *Trichinella spiralis*: Acquired immunity in swine. *Exp. Parasitol.* **59,** 347–354.

Negrão-Corrêa, D. (2001). Importance of immunoglobulin E (IgE) in the protective mechanism against gastrointestinal nematode infection: Looking at the intestinal mucosae. *Rev. Inst. Med. Trop. São Paulo* **43,** 291–299.

Neill, D. R., Wong, S. H., Bellosi, A., Flynn, R. J., Daly, M., Langford, T. K., Bucks, C., Kane, C. M., Fallon, P. G., Pannell, R., Jolin, H. E., and McKenzie, A. N. (2010). Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* **464,** 1367–1370.

Nishikawa, S., Honda, K., Vieira, P., and Yoshida, H. (2003). Organogenesis of peripheral lymphoid organs. *Immunol. Rev.* **195,** 72–80.

Novak, N., Koch, S., Allam, J. P., and Bieber, T. (2010). Dendritic cells: Bridging innate and adaptive immunity in atopic dermatitis. *J. Allergy Clin. Immunol.* **125,** 50–59.

Ohnmacht, C., Schwartz, C., Panzer, M., Schiedewitz, I., Naumann, R., and Voehringer, D. (2010). Basophils orchestrate chronic allergic dermatitis and protective immunity against helminths. *Immunity* **33,** 364–374.

Owyang, A. M., Zaph, C., Wilson, E. H., Guild, K. J., McClanahan, T., Miller, H. R., Cua, D. J., Goldschmidt, M., Hunter, C. A., Kastelein, R. A., and Artis, D. (2006). Interleukin 25 regulates type 2 cytokine-dependent immunity and limits chronic inflammation in the gastrointestinal tract. *J. Exp. Med.* **203,** 843–849.

Pan, G., French, D., Mao, W., Maruoka, M., Risser, P., Lee, J., Foster, J., Aggarwal, S., Nicholes, K., Guillet, S., Schow, P., and Gurney, A. L. (2001). Forced expression of murine IL-17E induces growth retardation, jaundice, a Th2-biased response, and multiorgan inflammation in mice. *J. Immunol.* **167,** 6559–6567.

Patel, N., Kreider, T., Urban, J. F., Jr., and Gause, W. C. (2009). Characterisation of effector mechanisms at the host:parasite interface during the immune response to tissue-dwelling intestinal nematode parasites. *Int. J. Parasitol.* **39,** 13–21.

Perrigoue, J. G., Saenz, S. A., Siracusa, M. C., Allenspach, E. J., Taylor, B. C., Giacomin, P. R., Nair, M. G., Du, Y., Zaph, C., van Rooijen, N., Comeau, M. R., Pearce, E. J., *et al.* (2009). MHC class II-dependent basophil-CD4^{+} T cell interactions promote T_H2 cytokine-dependent immunity. *Nat. Immunol.* **10,** 697–705.

Phythian-Adams, A. T., Cook, P. C., Lundie, R. J., Jones, L. H., Smith, K. A., Barr, T. A., Hochweller, K., Anderton, S. M., Hämmerling, G. J., Maizels, R. M., and MacDonald, A. S.

(2010) CD11c depletion severely disrupts Th2 induction and development *in vivo*. *J. Exp. Med.* **207,** 2089–2096.

Piccinni, M. P., Macchia, D., Parronchi, P., Giudizi, M. G., Bani, D., Alterini, R., Grossi, A., Ricci, M., Maggi, E., and Romagnani, S. (1991). Human bone marrow non-B, non-T cells produce interleukin 4 in response to cross-linkage of Fcε and Fcγ receptors. *Proc. Natl. Acad. Sci. USA* **88,** 8656–8660.

Plaut, M., Pierce, J. H., Watson, C. J., Hanley-Hyde, J., Nordan, R. P., and Paul, W. E. (1989). Mast cell lines produce lymphokines in response to cross-linkage of FcεRI or to calcium ionophores. *Nature* **339,** 64–67.

Price, A. E., Liang, H. E., Sullivan, B. M., Reinhardt, R. L., Eisley, C. J., Erle, D. J., and Locksley, R. M. (2010). (2010). Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc. Natl. Acad. Sci. USA* **107,** 11489–11494.

Rangel-Moreno, J., Moyron-Quiroz, J. E., Carragher, D. M., Kusser, K., Hartson, L., Moquin, A., and Randall, T. D. (2009). Omental milky spots develop in the absence of lymphoid tissue-inducer cells and support B and T cell responses to peritoneal antigens. *Immunity* **30,** 731–743.

Richard, M., Grencis, R. K., Humphreys, N. E., Renauld, J. C., and Van Snick, J. (2000). Anti-IL-9 vaccination prevents worm expulsion and blood eosinophilia in *Trichuris muris*-infected mice. *Proc. Natl. Acad. Sci. USA* **97,** 767–772.

Robinson, M., Wahid, F., Behnke, J. M., and Gilbert, F. S. (1989). Immunological relationships during primary infection with *Heligmosomoides polygyrus* (*Nematospiroides dubius*): Dose-dependent expulsion of adult worms. *Parasitology* **98,** 115–124.

Saenz, S. A., Siracusa, M. C., Perrigoue, J. G., Spencer, S. P., Urban, J. F., Jr., Tocker, J. E., Budelsky, A. L., Kleinschek, M. A., Kastelein, R. A., Kambayashi, T., Bhandoola, A., and Artis, D. (2010). IL25 elicits a multipotent progenitor cell population that promotes T_H2 cytokine responses. *Nature* **464,** 1362–1366.

Sanada, S., Hakuno, D., Higgins, L. J., Schreiter, E. R., McKenzie, A. N., and Lee, R. T. (2007). IL-33 and ST2 comprise a critical biomechanically induced and cardioprotective signaling system. *J. Clin. Invest.* **117,** 1538–1549.

Satoh-Takayama, N., Dumoutier, L., Lesjean-Pottier, S., Ribeiro, V. S., Mandelboim, O., Renauld, J. C., Vosshenrich, C. A., and Di Santo, J. P. (2009). IL-22 produced by human NK cells inhibits growth of *Mycobacterium tuberculosis* by enhancing phagolysosomal fusion. *J. Immunol.* **183,** 6579–6587.

Savioli, L. (2009). A letter from the director. WHO report. http://www.who.int/neglected_-diseases/director/en/index.html.

Scaffidi, P., Misteli, T., and Bianchi, M. E. (2002). Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* **418,** 191–195.

Schmitz, J., Owyang, A., Oldham, E., Song, Y., Murphy, E., McClanahan, T. K., Zurawski, G., Moshrefi, M., Qin, J., Li, X., Gorman, D. M., Bazan, J. F., *et al.* (2005). IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokine. *Immunity* **23,** 479–490.

Seder, R. A., Paul, W. E., Ben-Sasson, S. Z., LeGros, G. S., Kagey-Sobotka, A., Finkelman, F. D., Pierce, J. H., and Plaut, M. (1991). Production of interleukin-4 and other cytokines following stimulation of mast cell lines and *in vivo* mast cells/basophils. *Int. Arch. Allergy Appl. Immunol.* **94,** 137–140.

Shan, L., Redhu, N. S., Saleh, A., Halayko, A. J., Chakir, J., and Gounni, A. S. (2010). Thymic stromal lymphopoietin receptor-mediated IL-6 and CC/CXC chemokines expression in human airway smooth muscle cells: Role of MAPKs (ERK1/2, p38, and JNK) and STAT3 pathways. *J. Immunol.* **184,** 7134–7143.

Shortman, K., and Heath, W. R. (2010). The $CD8^+$ dendritic cell subset. *Immunol. Rev.* **234,** 18–31.

Smithgall, M. D., Comeau, M. R., Yoon, B. R., Kaufman, D., Armitage, R., and Smith, D. E. (2008). IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. *Int. Immunol.* **20,** 1019–1030.

Sokol, C. L., Barton, G. M., Farr, A. G., and Medzhitov, R. (2008). A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nat. Immunol.* **9,** 310–318.

Sokol, C. L., Chu, N. Q., Yu, S., Nish, S. A., Laufer, T. M., and Medzhitov, R. (2009). Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response. *Nat. Immunol.* **10,** 713–720.

Sonoda, E., Matsumoto, R., Hitoshi, Y., Ishii, T., Sugimoto, M., Araki, S., Tominaga, A., Yamaguchi, N., and Takatsu, K. (1989). Transforming growth factor β induces IgA production and acts additively with interleukin 5 for IgA production. *J. Exp. Med.* **170,** 1415–1420.

Soumelis, V., Reche, P. A., Kanzler, H., Yuan, W., Edward, G., Homey, B., Gilliet, M., Ho, S., Antonenko, S., Lauerma, A., Smith, K., Gorman, D., *et al.* (2002). Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat. Immunol.* **3,** 673–678.

Steinman, R. M., and Hemmi, H. (2006). Dendritic cells: Translating innate to adaptive immunity. *Curr. Top. Microbiol. Immunol.* **311,** 17–58.

Suzukawa, M., Iikura, M., Koketsu, R., Nagase, H., Tamura, C., Komiya, A., Nakae, S., Matsushima, K., Ohta, K., Yamamoto, K., and Yamaguchi, M. (2008). An IL-1 cytokine member, IL-33, induces human basophil activation via its ST2 receptor. *J. Immunol.* **181,** 5981–5989.

Terashima, A., Watarai, H., Inoue, S., Sekine, E., Nakagawa, R., Hase, K., Iwamura, C., Nakajima, H., Nakayama, T., and Taniguchi, M. (2008). A novel subset of mouse NKT cells bearing the IL-17 receptor B responds to IL-25 and contributes to airway hyperreactivity. *J. Exp. Med.* **205,** 2727–2733.

Urban, J. F., Jr., Steenhard, N. R., Solano-Aguilar, G. I., Dawson, H. D., Iweala, O. I., Nagler, C. R., Noland, G. S., Kumar, N., Anthony, R. M., Shea-Donohue, T., Weinstock, J., and Gause, W. C. (2007). Infection with parasitic nematodes confounds vaccination efficacy. *Vet. Parasitol.* **148,** 14–20.

Vadlamudi, R. S., Chi, D. S., and Krishnaswamy, G. (2006). Intestinal strongyloidiasis and hyperinfection syndrome. *Clin. Mol. Allergy* **4,** 8. doi: 10.1186/1476-7961-4-8.

Vermillion, D. L., and Collins, S. M. (1988). Increased responsiveness of jejunal longitudinal muscle in *Trichinella*-infected rats. *Am. J. Physiol.* **254,** G124–G129.

Voehringer, D., Reese, T. A., Huang, X., Shinkai, K., and Locksley, R. M. (2006). Type 2 immunity is controlled by IL-4/IL-13 expression in hematopoietic non-eosinophil cells of the innate immune system. *J. Exp. Med.* **203,** 1435–1446.

Willyard, C. (2009). Large trial to examine parasites' influence on global killers. *Nat. Med.* **15,** 1097.

Wood, I. S., Wang, B., and Trayhurn, P. (2009). IL-33, a recently identified interleukin-1 gene family member, is expressed in human adipocytes. *Biochem. Biophys. Res. Commun.* **384,** 105–109.

Xu, D., Chan, W. L., Leung, B. P., Huang, F., Wheeler, R., Piedrafita, D., Robinson, J. H., and Liew, F. Y. (1998). Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells. *J. Exp. Med.* **187,** 787–794.

Yamasaki, S., Ishikawa, E., Sakuma, M., Hara, H., Ogata, K., and Saito, T. (2008). Mincle is an ITAM-coupled activating receptor that senses damaged cells. *Nat. Immunol.* **9,** 1179–1188.

Ying, S., O'Connor, B., Ratoff, J., Meng, Q., Mallett, K., Cousins, D., Robinson, D., Zhang, G., Zhao, J., Lee, T. H., and Corrigan, C. (2005). Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity. *J. Immunol.* **174,** 8183–8189.

Yoshimoto, T., and Paul, W. E. (1994). $CD4^{pos}$, $NK1.1^{pos}$ T cells promptly produce interleukin 4 in response to in vivo challenge with anti-CD3. *J. Exp. Med.* **179,** 1285–1289.

Yoshimoto, T., Yasuda, K., Tanaka, H., Nakahira, M., Imai, Y., Fujimori, Y., and Nakanishi, K. (2009). Basophils contribute to T_H2-IgE responses in vivo via IL-4 production and presentation of peptide-MHC class II complexes to $CD4^+$ T cells. *Nat. Immunol.* **10,** 706–712.

Zaph, C., Troy, A. E., Taylor, B. C., Berman-Booty, L. D., Guild, K. J., Du, Y., Yost, E. A., Gruber, A. D., May, M. J., Greten, F. R., Eckmann, L., Karin, M., *et al.* (2007). Epithelial-cell-intrinsic IKK-β expression regulates intestinal immune homeostasis. *Nature* **446,** 552–556.

Zhang, D. H., Cohn, L., Ray, P., Bottomly, K., and Ray, A. (1997). Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. *J. Biol. Chem.* **272,** 21597–21603.

Zheng, W., and Flavell, R. A. (1997). The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* **89,** 587–596.

Zhu, J., Yamane, H., and Paul, W. E. (2010). Differentiation of effector CD4 T cell populations. *Annu. Rev. Immunol.* **28,** 445–489.

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RESEARCH PAPER

A novel antagonist of the prostaglandin E_2 EP_4 receptor inhibits Th1 differentiation and Th17 expansion and is orally active in arthritis models

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Background and purpose: Rheumatoid arthritis (RA) is an autoimmune disorder involving subsets of activated T cells, in particular T helper (Th) 1 and Th17 cells, which infiltrate and damage tissues and induce inflammation. Prostaglandin E_2 (PGE_2) enhances the Th17 response, exacerbates collagen-induced arthritis (CIA) and promotes inflammatory pain. The current study investigated whether selective antagonism of the PGE_2 EP_4 receptor would suppress Th1/Th17 cell development and inflammatory arthritis in animal models of RA.

Experimental approach: Effects of PGE_2 and a novel EP_4 receptor antagonist ER-819762 on Th1 differentiation, interleukin-23 (IL-23) production by dendritic cells (DCs), and Th17 development were assessed *in vitro*. The effect of ER-819762 was evaluated in CIA and glucose-6-phosphate isomerase (GPI)-induced arthritis models. In addition, the effects of ER-819762 on pain were evaluated in a model of chronic inflammatory pain induced by complete Freund's adjuvant (CFA) in the rat.

Key results: Stimulation of the EP_4 receptor enhanced Th1 differentiation via phosphatidylinositol 3 kinase signalling, selectively promoted Th17 cell expansion, and induced IL-23 secretion by activated DCs, effects suppressed by ER-819762 or anti-PGE_2 antibody. Oral administration of ER-19762 suppressed Th1 and Th17 cytokine production, suppressed disease in collagen- and GPI-induced arthritis in mice, and suppressed CFA-induced inflammatory pain in rats.

Conclusion and Implications: PGE_2 stimulates EP_4 receptors to promote Th1 differentiation and Th17 expansion and is critically involved in development of arthritis in two animal models. Selective suppression of EP_4 receptor signalling may have therapeutic value in RA both by modifying inflammatory arthritis and by relieving pain.

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Keywords: PGE_2; EP_4 receptor; EP_4 antagonist; Th1; Th17; IFN-γ; IL-17; IL-23; CIA; GPI-induced arthritis model

Abbreviations: BSA, bovine serum albumin; CIA, collagen-induced arthritis; CFA, complete Freund's adjuvant; CRE, cAMP response elements; ER-819762, (S)-1′-(3,5-dimethylbenzyl)-2-ethyl-7,9-dimethoxy-10-methyl-5,10-dihydrospiro[benzo[e]imidazo[1,5-a]azepine-1,4′-piperidin]-3(2H)-one; FBS, fetal bovine serum; GPCR, G protein-coupled receptors; GPI, glucose-6-phosphate isomerase; IBMX, isobutylmethylxanthine; imDC, immature human dendritic cell; PGE_2, prostaglandin E_2; PI3K, phosphatidylinositol 3-kinase; PLAP, placental-like alkaline phosphatase; RA, rheumatoid arthritis; Th1, T helper 1; Th17, T helper 17; TLR, Toll-like receptor

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disorder that is estimated to affect up to 1% of the population worldwide (Williams, 2006). Although not life-threatening, RA is a painful and debilitating disease that progressively limits the ability of patients to carry on normal lives. The factors that trigger this disease are not well understood but are believed to include both genetic and environmental components. Over the last decade, novel discoveries into the regulation of the immune system have permitted a better understanding of the development of autoimmunity. Upon encountering antigen, T helper (Th) cells differentiate into several subtypes, depending on various factors such as the cytokines produced, the consequent activation of intracellular signalling pathways,

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and the expression of transcription factors. Among these T cell subtypes, Th1 and Th17 cells have been shown to be critically involved in the development of autoimmunity (Schulze-Koops and Kalden, 2001; Fouser *et al.*, 2008). While the molecular mechanisms controlling the differentiation and expansion of these T cell subsets have begun to be elucidated, specific agents to suppress the function of these critical T cell subtypes are only in early-stage development.

Prostaglandin E_2 (PGE_2) is an arachidonic acid metabolite that acts as a potent biological mediator, exerting its effects via activation of membrane G protein-coupled receptors (GPCRs). There are four receptor subtypes ($EP_{1,\ 2,\ 3}$ and $_4$; nomenclature follows Alexander *et al.*, 2009) which selectively bind PGE_2 and mediate its effects: Activation of EP_1 receptors leads to the influx of calcium. Activation of EP_3 receptors can induce a variety of signalling events depending on the particular EP_3 splicing variant being expressed, with inhibition of adenylate cyclase activity via Gi being the most common effect. EP_2 and EP_4 receptors induce Gs-mediated activation of adenylate cyclase and a subsequent increase in intracellular cyclic AMP (cAMP). In addition, EP_4 receptors activate the phosphatidylinositol 3-kinase (PI3K) signalling pathway (Fujino *et al.*, 2003). PGE_2 is known to play important roles in mediating many inflammatory responses and is often found at increased concentrations under a variety of inflammatory conditions (Hata and Breyer, 2004). Many reports suggest that PGE_2, via the induction of intracellular cAMP, can suppress pro-inflammatory T cell function, including T cell receptor signalling and consequent production of interleukin (IL)-2 (Mustelin and Tasken, 2003; Chemnitz *et al.*, 2006). PGE_2 has also been implicated in T-cell differentiation and is reported to inhibit Th1 but not Th2 cytokines via the induction of intracellular cAMP (Betz and Fox, 1991; Gold *et al.*, 1994; Hilkens *et al.*, 1995; Okano *et al.*, 2006). However, other reports indicate a pro-inflammatory role for PGE_2. PGE_2 can induce production of IL-23 from dendritic cells (DCs), which promotes the differentiation of pro-inflammatory Th17 cells (Sheibanie *et al.*, 2004; Khayrullina *et al.*, 2008). Recent reports also suggest that PGE_2 can synergize with IL-23 to promote expansion of human Th17 cells and enhance IL-17 production (Chizzolini *et al.*, 2008; Boniface *et al.*, 2009; Napolitani *et al.*, 2009). Furthermore, PGE_2 has been shown to exacerbate symptoms in mouse models of arthritis (Sheibanie *et al.*, 2007a) and inflammatory bowel disease (Sheibanie *et al.*, 2007b), and the blockade of EP_2 and EP_4 receptor signalling in a mouse model of arthritis can alleviate the severity of the disease (Mccoy *et al.*, 2002; Honda *et al.*, 2006).

Here, we show that PGE_2 stimulation of the EP_4 receptor can promote Th1 differentiation, IL-23 production in DCs, and Th17 cell expansion. These effects can be suppressed by a novel EP_4 receptor antagonist ER-819762 ((S)-1'-(3,5-dimethylbenzyl)-2-ethyl-7,9-dimethoxy-10-methyl-5,10-dihydrospiro[benzo[e]imidazo[1,5-a]azepine-1,4'-piperidin]-3(2H)-one) (Spyvee *et al.*, 2009) or an anti-PGE_2 antibody. We also show that oral administration of ER-819762 to DBA/1 mice can effectively suppress disease in collagen-induced arthritis (CIA) or glucose-6-phosphate isomerase (GPI)-induced arthritis models. ER-819762 was also effective in treating chronic inflammatory pain in a rat model. These results suggest that PGE_2 signalling via the EP_4 receptor exerts a pro-inflammatory effect *in vivo* and is physiologically relevant to the pathology of inflammatory arthritis. EP_4 receptors might therefore be an attractive drug target for the treatment of RA, with the potential not only to relieve pain and symptoms but also to modify the underlying aetiology of the disease

Methods

Animals

All animal studies were performed with the approval of the Animal Ethics Committee at Eisai according to Laboratory Animal Welfare guidelines. BALB/c and DO11.10 mice were purchased from Jackson Laboratory. C57BL/6 and DBA/1 mice and F344 rats were purchased from Charles River Laboratories. Mice and rats for each strain were group-housed under controlled conditions with a constant temperature (23 ± 3°C) and humidity (55 ± 5%), a 12-h light/dark cycle and *ad libitum* access to water and standard pelleted food.

Radioligand EP_4 receptor binding assay

The radioligand EP_4 receptor binding assay was performed using ChemiScreen recombinant human EP_4 receptor membrane preparations from Millipore, according to the manufacturer's instructions. Briefly, membranes prepared from Chem-1 cells overexpressing human EP_4 receptor cDNA (Millipore) were mixed with 1.8 nmol·L^{-1} [^{3}H]-PGE_2 and 5 μmol·L^{-1} unlabelled PGE_2 in the presence or absence of various concentrations of ER-819762 in binding buffer [50 mmol·L^{-1} HEPES, pH 7.4, 5 mmol·L^{-1} $MgCl_2$, 1 mmol·L^{-1} $CaCl_2$, 0.2% bovine serum albumin (BSA)] in a nonbinding 96-well plate, and incubated for 1–2 h at room temperature. Prior to filtration, a GF/C 96-well filter plate was coated with 0.33% polyethyleneimine for 30 min, then washed with 50 mmol·L^{-1} HEPES, pH 7.4, 0.5% BSA. Binding reactions were transferred to the filter plate, and washed three times with wash buffer (1 mL per well per wash). The plate was dried and radioactivity counted. Binding of ER-819762 to other related prostanoid receptors was performed by MDS Pharma Services (Bothell, WA, USA) using a similar radiolabelled ligand displacement method.

Cell-based GPCR assays

SE302 is a clone of the human embryonic kidney 293 (HEK/293) cell line containing a reporter driven by cAMP response elements (CRE) in its promoter, and producing secreted placental-like alkaline phosphatase (PLAP). HEK/293 cells express endogenous EP_4 receptors and show induction of PLAP in response to PGE_2 and EP_4 receptor agonists, but not $EP_{1,\ 2}$ or $_3$ receptor agonists (Supplementary Fig. 2). Cells were maintained in DMEM/F12 (50:50) (MediaTech, Inc., Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS; Tissue Culture Biologicals) plus penicillin/streptomycin. When used for assays, cells were plated in a 96-well plate at 2 × 10^4 cells/100 μL per well in serum-free assay medium (DMEM/F12 supplemented with 0.1% BSA plus penicillin/

streptomycin) and incubated for 4–6 h. Cells were then stimulated with 3 ng mL^{-1} PGE_2 in the presence or absence of various concentrations of ER-819762 overnight, and PLAP activity was measured by mixing 15 μL of culture supernatants with 75 μL of Lumi-phos (Lumigen, Inc.) and 60 μL of assay buffer containing 8 mmol·L^{-1} $MgSO_4$ in 0.1 mol·L^{-1} carbonate-bicarbonate buffer pH11 in a new 96-well black plate and incubated for 2 h at room temperature. Luminescence was read with an Envision 2102 Multilabel reader. Characterization of compound selectivity was performed by Millipore GPCR Profiler Service, which assays intracellular calcium mobilization in cells expressing individual GPCRs and the promiscuous $G\alpha_{15}$ protein. Endogenous EP_2 receptor activity in U2-OS cells was assayed using the EPIC Resonant Waveguide Biosensor system (Corning).

In vitro *T-cell assays*

Naive $CD4^+$ T cells were purified from spleens of either BALB/c or DO11.10 mice by antibody-coated magnetic beads as described by the manufacturer (Robosep; StemCell Technologies). For BALB/c mice, 1×10^5 $CD4^+$ T cells were cultured for 3–6 days in a 96-well plate in 100 μL complete RPMI medium (CellGro) containing 10% regular FBS under: (i) neutral conditions (1 μg mL^{-1} plate-bound anti-CD3 + 1 μg mL^{-1} soluble anti-CD28 + 10 ng mL^{-1} mouse IL-2), (ii) Th1-promoting conditions (neutral + 5 ng mL^{-1} mouse IL-12 + 10 μg mL^{-1} anti-IL-4 antibody) or (iii) Th2-promoting conditions [neutral + 10 ng mL^{-1} of mouse IL-4 + 10 μg mL^{-1} anti-interferon (IFN)-γ antibody]. In experiments where exogenous PGE_2 or EP_4 receptor agonists were added to the culture, charcoal-stripped FBS (Hyclone) was used, which has reduced amounts of lipids. IFN-γ or IL-4 in culture supernatants were quantified by enzyme-linked immunosorbent assay (ELISA). Cell proliferation was assayed with either Alamar Blue or CellTiter-Glo reagents according to the manufacturers' instructions. For DO11.10 mice, mitomycin C-treated splenocytes from BALB/c mice were used as antigen-presenting cells and co-cultured with naive $CD4^+$ T cells in a 5:1 ratio (5×10^5 mitomycin C-treated splenocytes in 100 μL medium + 1×10^5 CD4 T cells in 100 μL medium). These cultures were stimulated with an ovalbumin peptide 323-339 (OVA peptide; 0.3 ng mL^{-1}) under neutral, Th1- or Th2-promoting conditions, as described above. EP_4 receptor agonists and antagonists, other cAMP-inducing agents, inhibitors of PI3K or protein kinase A (PKA) or anti-PGE_2 antibody were added during Th cell differentiation.

In order to study the effect of EP_4 receptor agonists, antagonists and cAMP-inducing agents on IL-17 production, total $CD4^+$ T cells isolated from spleens of C57BL/6 mice were activated with plate-bound anti-CD3 (2 μg mL^{-1}) plus soluble anti-CD28 (2 μg mL^{-1}) in the presence or absence of IL-23 (10 ng mL^{-1}) and presence or absence of EP_4 receptor agonist/antagonists or other agents at the indicated concentrations for 3–5 days. Culture supernatants were analysed by IL-17 ELISA, and cell proliferation was measured with CellTiter-Glo.

IL-23-induced Th17 expansion

$CD4^+$ T cells were isolated from C57BL/6 mice and activated with antibody against T cell receptor β chain (1 μg mL^{-1} plate-bound) and anti-CD28 (2 μg mL^{-1} soluble) with or without IL-23 (30 ng mL^{-1}) for 5 days in complete RPMI medium containing 10% normal FBS. IL-17-producing cells were analysed by IL-17 intracellular staining. Briefly, cells were stimulated for 5 h with phorbol 12-myristate 13-acetate (50 ng mL^{-1}), ionomycin (500 ng mL^{-1}) and Golgistop (1 μL mL^{-1}), stained with anti-CD4 antibody, fixed and permeabilized (Cytofix/Cytoperm) and stained with anti-IL-17 antibody (all from BD Biosciences) and then analysed by flow cytometry.

In vitro *human monocytes-derived DC assay*

Human peripheral blood monocytes (PBMC) were isolated by Ficoll gradient from heparinized venous blood of healthy, drug-free volunteers, following written informed consent. $CD14^+$ cells were purified from human PBMC using Miltenyi CD14 microbeads according to the manufacturer's instructions, and differentiated with human GM-CSF (500 U mL^{-1}) + human IL-4 (500 U mL^{-1}) in complete RPMI medium containing 10% charcoal-stripped FBS for 8 days. Detached immature DCs (imDCs) were stimulated with lipopolysaccharide (LPS) (*Escherichia coli* 0111:B4; 10 ng mL^{-1}) and the Toll-like receptor (TLR)7 ligand, R-848 (2.5 μg mL^{-1}) with or without the addition of EP_4 receptor agonist/antagonists or anti-PGE_2 at the concentrations indicated for 24 h. Concentrations of IL-23 in culture supernatants were measured by ELISA (eBioscience).

Collagen-induced arthritis model

Male DBA/1 mice were immunized by injection at the base of the tail with 0.1 mL emulsion containing 150 μg bovine type II collagen (bCII) emulsified in complete Freund's adjuvant (CFA). Three weeks after the first immunization, all mice were boosted with bCII emulsified in Freund's incomplete adjuvant. ER-819762 was given orally daily at a dose of 10, 30 or 100 mg·kg^{-1} from day 20 after primary immunization but before disease onset (prophylactic evaluation) or after the disease induction (therapeutic evaluation). The severity of arthritic symptoms in the paws of each mouse was graded, in a double-blind manner, according to Williams (2004). At the end of the experiments, knee and ankle joints were used for radiography analysis. X-ray score was defined as the total score of a combination of osteopenia, bone erosion and new bone formation as follows: 0 – no change; 1 – slight change, 2 – moderate change; 3 – severe change (Kop *et al.*, 2006). Each treatment group consists of six to eight mice.

GPI-induced arthritis model

Male DBA/1 mice were immunized by injecting at the base of the tail 0.15 mL of emulsion containing 300 μg recombinant human GPI-glutathione-S-transferase fusion protein (hGPI) in CFA. ER-819762 was given orally daily at a dose of 10 or 30 mg·kg^{-1} from day 6 after primary immunization but before disease onset (prophylactic evaluation) or after the disease induction (therapeutic evaluation). Each treatment group consisted of six to eight mice. Arthritic animals were clinically assessed by an arthritis scoring system as follow (Iwanami *et al.*, 2008): 0 = no evidence of inflammation, 1 = subtle

inflammation or localized oedema, 2 = easily identified swelling but localized to dorsal or ventral surface of paws, and score 3 = swelling on all aspects of paws. Serum samples were collected at the end of the study and analysed for cytokine levels by ELISA. To analyse popliteal lymph node cells, emulsified GPI was injected into the foot pad of DBA/1 mice and ER-819762 was orally administered once daily at 30 $mg \cdot kg^{-1}$ from the day of immunization. Lymph nodes were removed 6 days later, and cells were stimulated with 10 $\mu g\ mL^{-1}$ recombinant GPI and GolgiStop (BD) for 12 h (Iwanami *et al.*, 2008). IL-17- and IFN-γ-producing cells were analysed as described above.

CFA-induced hyperalgesia

Complete Freund's adjuvant, consisting of 100 μg of *Mycobacterium tuberculosis* H37 RA (Difco, Detroit, MI, USA) in 100 μL of liquid paraffin (Wako Pure Chemicals), was injected into the right hind footpad of 7-week-old male F344 rats. Three days after CFA injection, rats exhibited a lame walking reaction consisting of a three-legged gait. ER-819762 or indomethacin was orally administered at day 3 and the inhibitory effect on the lame walking reaction was evaluated, without knowledge of treatment, after 2–3 h of dosing (Higuchi *et al.*, 1986). Each treatment group consisted of seven rats.

Ex vivo lymph node studies

Male DBA/1 mice were immunized with bCII emulsified in CFA, as described above. ER-819762 was orally administered daily at a dose of 30 $mg \cdot kg^{-1}$ from the day of immunization. Suspensions of single cells were prepared from draining lymph nodes from mice, 15 days after immunization. Cells were plated in a 96-well plate at 4×10^5 cells per 200 μL per well in complete RPMI medium and stimulated with either bCII (50 $\mu g\ mL^{-1}$) or phosphate-buffered saline for 72 h. Cytokine production in culture supernatants was analysed by ELISA, and cell proliferation was measured by CellTiter-Glo.

Data analyses

All values shown in the text and figures are mean ± SD. Nonlinear regression analysis of the data and calculation of IC_{50} values were performed using Prism 4 (GraphPad Software, San Diego, CA, USA). Statistical analysis was performed by Dunnett-type multiple comparison test or paired *t*-test; a value of $P < 0.05$ (two-sided) was considered statistically significant.

Materials

ER-819762 ((S)-1'-(3,5-dimethylbenzyl)-2-ethyl-7,9-dimethoxy -10-methyl-5,10-dihydrospiro [benzo[e]imidazo [1,5-a]azepine-1,4'-piperidin]-3(2H)-one) was synthesized at Eisai Research Institute of Boston Inc. Indomethacin was purchased from Cayman Chemicals. For *in vitro* testing, ER-819762 was dissolved in 100% dimethyl sulphoxide (DMSO) and the final concentration of DMSO in the assay was 0.1%. For *in vivo* evaluation, ER-819762 or indomethacin was suspended in 0.5% (w/v) methylcellulose and orally administered at 10 $mL \cdot kg^{-1}$ per mouse or 5 $mL \cdot kg^{-1}$ per rat.

Mouse IL-2, IL-12, IL-23 and human GM-CSF were purchased from R&D Systems. Human IL-4 and GM-CSF were from Peprotec. Anti-CD3 (clone 145-2C11), anti-CD28 (clone 37.51), anti-IL-4 (clone 11B11), anti-IFN-γ (clone XMG12) and PE-anti-mouse IL-17 (clone TC11-18H10) were purchased from BD Pharmingen. Anti-TCR (clone H57-597) was purchased from eBioscience. OVA peptide, mitomycin C, H-89, KT5721, LY-294002 and isobutylmethylxanthine (IBMX) were purchased from Sigma. PGE_2, PGE_1-alcohol (PGE_1–OH), butaprost, forskolin and anti-PGE_2 IgG1 monoclonal antibody (clone 2B5) were purchased from Cayman Chemicals. LPS and R-848 were from InVivoGen. $CD14^+$ cell isolation kits were from MiltenyiBiotec. $CD4^+$ T cell isolation kits were from MiltenyiBiotec or StemCell Technologies. IFN-γ ELISA kits were from Pierce; IL-4 ELISA kits are from R&D Systems; IL-23 ELISA kits were from eBioscience. Intracellular IL-17 staining reagents were from BD Biosciences. Alamar Blue reagents were from Biosource International. CellTiter-Glo reagents were from Promega. CFA for mouse RA models was an emulsion form prepared by Difco (Michigan) and CFA for the lame walking model was desiccated powder from Difco, which we suspended in liquid paraffin (Wako Chemicals).

Results

Identification of selective EP_4 receptor antagonists

In the course of screening for drugs unrelated to the prostanoid receptors, we discovered a series of compounds that could suppress the expression of a stably transfected cytomegalovirus (CMV) promoter in HEK/293 cells. The CMV promoter is known to be modulated by cAMP signalling (Hunninghake *et al.*, 1989), and we found that these compounds inhibited the induction of CMV promoter activity by a factor present in FBS. The inducing factor in FBS was identified as PGE_2 and induction of cAMP was found to be mediated solely by the endogenous EP_4 receptor in HEK/293 cells (Supplementary Figs 1 and 2). A representative of this series of compounds, ER-819762 (structure shown in Fig. 1D), displaced PGE_2 binding to human EP_4 receptors (IC_{50} value of 70 ± 11 $nmol \cdot L^{-1}$; Fig. 1A), but did not displace ligand binding to several related human prostanoid GPCRs, including EP_2, DP, CRTH2 and TP receptors, and the leukotriene GPCRs LTB_4, $CysLT_1$ and $CysLT_2$ receptors (Fig. 1C). ER-819762 also suppressed human EP_4 receptor-mediated cell signalling as measured in a cAMP-dependent reporter assay (IC_{50} value of 59 ± 6 $nmol \cdot L^{-1}$) (Fig. 1B). In a larger cell signalling panel of 107 GPCRs, ER-819762 (1 $\mu mol \cdot L^{-1}$) was highly selective for EP_4 receptors, exhibiting no agonism or antagonism for any other receptor, including the related PGE_2 EP_1, EP_2 and EP_3 receptors (Table 1).

EP_4 receptor antagonism suppresses in vitro *Th1 differentiation*

As PGE_2 has been reported to modulate T cell differentiation and function, we tested the effect of ER-819762 in Th1 and Th2 differentiation assays. Th1 differentiation was induced by activating naïve $CD4^+$ T cells with anti-CD3 and anti-CD28 antibodies in 10% charcoal-stripped FBS in the presence of

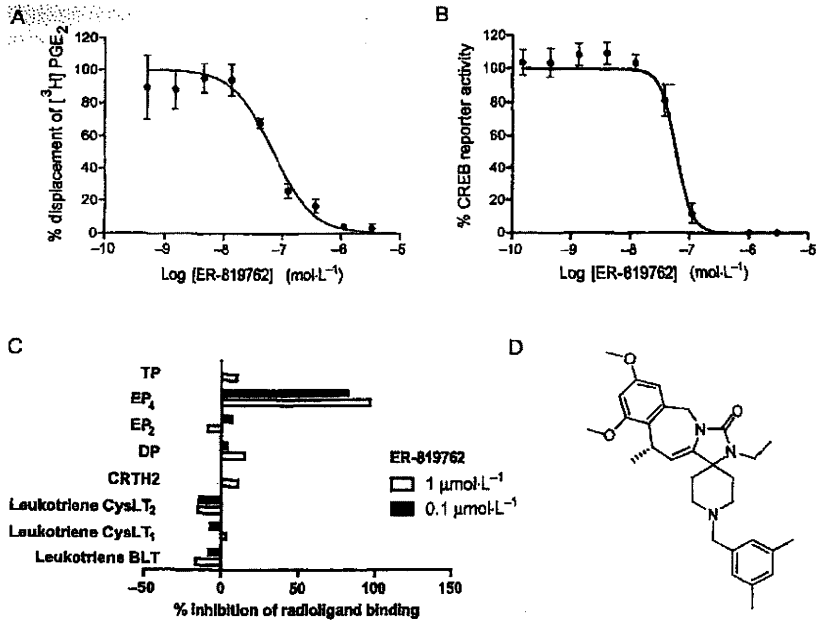

Figure 1 Activity and structure of ER-819762. (A) Competitive displacement of radiolabelled prostaglandin E_2 (PGE_2) from cell membranes overexpressing EP_4 receptors (Millipore ChemiScreen). (B) Inhibition of PGE_2-induced cAMP response element–placental-like alkaline phosphatase reporter activity in human embryonic kidney cells, which express endogenous EP_4 receptors (Supplementary Figure 2). Data are representative of mean ± SD derived from three independent experiments. (C) Competitive displacement of radiolabelled ligands from cell membranes overexpressing various prostanoid and leukotriene receptors by 0.1 and 1 μmol·L^{-1} ER-819762 (data from MDS Pharma, Bothel, WA, USA). (D) Chemical structure of ER-819762: (S)-1'-(3,5-dimethylbenzyl)-2-ethyl-7,9-dimethoxy-10-methyl-5,10 dihydrospiro[benzo[e]imidazo[1,5-a]azepine-1,4'-piperidin]-3(2H)-one.

IL-2, IL-12 and anti-IL-4 antibody. Th2 differentiation was induced by IL-4 and anti-IFN-γ antibody. Addition of PGE_2, butaprost (an EP_2 receptor agonist) and prostaglandin E_1 alcohol (PGE_1-OH; an EP_3/EP_4 receptor agonist) significantly enhanced the differentiation of naïve CD4$^+$ T cells into Th1 cells (Fig. 2A). ER-819762 suppressed PGE_2- and PGE_1-OH-induced IFN-γ production by Th1-differentiating cells in a concentration-dependent manner (Fig. 2B), but had no effect on cellular ATP levels (CellTiter-Glo, Promega), an indicator of cell metabolic activity. Figure 2B also shows that ER-819762 inhibited IFN-γ in the absence of added prostaglandins, suggesting that the PGE_2 produced by the T cells themselves acts in an autocrine manner to promote Th1 differentiation. ER-819762 had no effect on butaprost-stimulated IFN-γ production at up to 1 μmol·L^{-1} (Supplementary Fig. 3). Th1 and Th2 differentiation were also induced by co-culturing naïve CD4$^+$ T cells isolated from DO11.10 mice with mitomycin C-treated splenocytes and activating with the OVA peptide under neutral, Th1- or Th2-polarizing conditions in normal 10% FBS as described in *Methods*. In this experiment, IFN-γ production was suppressed by either ER-819762 or a neutralizing monoclonal anti-PGE_2 antibody (clone 2B5; Fig. 2C), and these effects were non-additive. We also observed no effect of ER-819762 on Th2 differentiation at up to 10 μmol·L^{-1} (Fig. 2D).

Although Th1 differentiation was enhanced by PGE_2, as measured by increased IFN-γ production (Fig. 2), neither forskolin, an activator of adenylate cyclase, nor IBMX, a phosphodiesterase inhibitor, caused a statistically significant enhancement in IFN-γ production (Fig. 3A), suggesting that the promotion of Th1 differentiation by PGE_2 was not due to cAMP signalling. Moreover, PGE_2-stimulated Th1 differentiation was not suppressed by the PKA inhibitors H-89 (1 μmol·L^{-1}) or was only weakly suppressed by a structurally unrelated PKA inhibitor KT-5720 (10 μmol·L^{-1}), but was strongly suppressed by the PI3K inhibitor LY294002 (2 μmol·L^{-1}), as well as by ER-819762 (Fig. 3B). Higher concentrations of H-89 (10 μmol·L^{-1}) were toxic (data not shown). These results suggest that the PI3K pathway, but not the PKA-cAMP signalling pathway functioning downstream of EP_4 receptors is primarily responsible for PGE_2-enhanced Th1 differentiation. Butaprost also induced Th1 differentiation (Fig. 2A), raising the possibility that EP_2 receptors may signal via PI3K in addition to PKA-cAMP.

EP_4 receptor antagonism suppresses IL-23 secretion in human monocyte-derived DCs

It was recently reported that PGE_2 can promote Th17 cell differentiation in mice by inducing DCs to preferentially produce IL-23 (Sheibanie *et al.*, 2007a; Khayrullina *et al.*, 2008). Similarly, receptors that mobilize cAMP have been reported to enhance IL-23 secretion by human DCs (Schnurr *et al.*, 2005). We therefore examined the role of EP_4 receptor signalling in immature human dendritic cells (imDCs). imDCs were generated from CD14$^+$ monocytes by differentiation with GM-CSF plus IL-4 and assayed for IL-23 production in media containing charcoal-stripped FBS. IL-23 production

Table 1 Selectivity of ER-819762 against 107 G protein-coupled receptors (GPCRs)

GPCR Target	% inhibition by ER-819762 (1 μmol·L⁻¹)	GPCR Target	% inhibition by ER-819762 (1 μmol·L⁻¹)	GPCR Target	% inhibition by ER-819762 (1 μmol·L⁻¹)
M1 (CHRM1)	1.1% ± 1.9%	CCK2 (CCKBR)	−8.1% ± 4.0%	NTR1	−3.7% ± 1.1%
M2 (CHRM2)	−10.5% ± 0.8%	CRF1 (CRHR1)	−16.2% ± 0.4%	FPR1	−6.6% ± 1.1%
M3 (CHRM3)	−3.2% ± 0.4%	CRF2 (CRHR2)	−9.5% ± 0.1%	FPR2/FPRL1	−7.3% ± 1.9%
M5 (CHRM5)	−4.7% ± 4.0%	D1	−8.1% ± 1.5%	NOP/ORL1	−11.2% ± 2.4%
A1 (ADORA1)	4.9% ± 6.0%	D2	0.6% ± 6.6%	δ (OPRD1)	−11.3% ± 1.0%
A3 (ADORA3)	−0.8% ± 1.4%	D5	25.2% ± 9.5%	κ (OPRK1)	8.3% ± 11.7%
A2A (ADORA2A)	13.4% ± 4.5%	ETA (EDNRA)	4.2% ± 2.4%	OX2 (HCRTR2)	−1.3% ± 0.3%
A2B (ADORA2B)	−1.4% ± 4.2%	GPR40	−3.1% ± 3.1%	PTH1 (PTHR1)	−4.7% ± 5.3%
α1A (ADRA1A)	−2.2% ± 0.1%	GPR43	5.1% ± 2.5%	PAF (PTAFR)	1.6% ± 2.3%
α2A (ADRA2A)	−13.2% ± 5.7%	GABBAb1	−9.7% ± 0.5%	PK1/GPR73	−0.4% ± 5.3%
β1 (ADRB1)	−12.1% ± 3.9%	GAL1 (GALR1)	22.9% ± 1.9%	PRP/GPR10	6.7% ± 0.7%
β2 (ADRB2)	−4.2% ± 3.4%	GCGR	−4.5% ± 2.0%	PTGDR (DP)	−13.7% ± 1.8%
C3aR	8.5% ± 0.3%	mGlu1	0.6% ± 8.2%	PTGER1 (EP1)	−4.6% ± 0.1%
C5aR	−10.2% ± 0.1%	GnRH	−12.3% ± 1.1%	PTGER2 (EP2)	(See legend)
ChemR23	3.5% ± 2.4%	H1 (HRH1)	−4.6% ± 1.0%	PTGER3 (EP3)	−2.4% ± 0.0%
AT1	−2.7% ± 1.7%	H2 (HRH2)	−4.8% ± 0.9%	PTGFR (FP)	0.1% ± 5.1%
BB2 (GRPR)	8.1% ± 0.3%	NK1 (TACR1)	−7.7% ± 6.2%	**PTGER4 (EP4)**	**90.3% ± 0.7%**
BB1	5.2% ± 4.6%	NK2	0.3% ± 2.6%	PTGIR (IP)	(See legend)
B2 (BDKRB2)	5.7% ± 1.3%	H3 (HRH3)	2.9% ± 4.1%	TBXAR2 (TP)	−9.8% ± 5.1%
AMY1	12.0% ± 0.9%	GPR54	−11.2% ± 4.4%	PAR1	−5.1% ± 9.3%
CGRP1	−7.6% ± 8.0%	BLT1 (LTB4R1)	−3.3% ± 2.9%	PAR2	−6.1% ± 8.0%
CaS	−12.6% ± 0.2%	CysLT1	−4.4% ± 4.0%	5-HT1A (HTR1A)	0.9% ± 4.5%
CB1	0.0% ± 2.5%	CysLT2	−0.7% ± 3.9%	5-HT1B	6.9% ± 15.6%
CXCR1	−5.1% ± 3.0%	S1P2 (EDG5)	−16.2% ± 0.9%	5-HT2B (HTR2B)	2.2% ± 2.9%
CXCR2	−1.7% ± 2.5%	S1P3 (EDG3)	−5.9% ± 0.6%	5-HT2C (HTR2C)	11.4% ± 3.8%
CXCR3	−14.6% ± 7.8%	LPA1 (EDG2)	−11.9% ± 5.9%	SST3	−8.9% ± 11.3%
CXCR4	−6.1% ± 2.4%	LPA3 (EDG7)	−0.2% ± 1.0%	SST4	−12.4% ± 1.8%
CCR1	−3.5% ± 1.2%	MRGX2	−8.6% ± 1.3%	GPR68(OGR1)	0.0% ± 0.0%
CCR2b	−6.7% ± 4.7%	MCHR1	−0.2% ± 0.5%	TRH	−6.9% ± 2.9%
CCR4	−10.6% ± 5.8%	MCHR2	−10.2% ± 4.0%	V1A	−0.6% ± 1.0%
CCR6	−7.5% ± 4.5%	MC4R	−3.6% ± 10.5%	V1B (AVPR1B)	−5.0% ± 1.5%
CCR7	9.6% ± 5.5%	Motilin	−12.6% ± 3.3%	V2 (AVPR2)	−17.8% ± 0.2%
CCR8	−13.2% ± 16.2%	NMUR1	−12.2% ± 4.6%	OT (OXTR)	6.5% ± 9.0%
CCR9	−15.1% ± 0.4%	Y1 (NPY1R)	15.8% ± 7.0%	PAC1 long	−7.5% ± 2.3%
CCR10	6.0% ± 2.5%	Y2 (NPY2R)	−17.7% ± 1.9%	VPAC1 (VIPR1)	−10.5% ± 1.0%
CRTH2	−2.8% ± 3.5%			VPAC2 (VIPR2)	−12.0% ± 1.9%

ER-819762 was assayed by Millipore GPCR Profiler Service, which monitors calcium flux in cells expressing the specific GPCR and promiscuous GPCR-coupling $G\alpha_{15/16}$ proteins. Both agonism and antagonism of the GPCRs listed above were examined, except for EP_2 and IP receptors. No significant agonism (>15%) was found for any GPCR (data not shown), and only antagonism of EP_4 receptors was found. ER-819762 was not able to displace binding of radiolabelled PGE_2 to EP_2 receptors (Fig. 1C, MDS Pharma Profiling Service. We also used the human U2-OS cell line, which endogenously expresses EP_2 and IP but not EP_4, receptors, to assay agonism and antagonism by ER-819762 using the Corning EPIC Resonate Waveguide Biosensor system. We observed no antagonism of butaprost- (EP_2 receptors) or iloprost- (IP receptors) induced signalling by 10 μM ER-819762, and no signalling induced by compound alone in this cell line (data not shown). Bold texts indicate positive results.

could be induced in imDCs by stimulation with LPS, a ligand for the TLR4, and co-stimulation with the TLR7 ligand R-848 (Fig. 4A), but not by LPS or R-848 alone (data not shown). We found that the $EP_3/_4$ receptor agonist PGE_1-OH enhanced LPS/R-848-induced IL-23 production in imDCs and that this response was antagonized by ER-819762 (Fig. 4A). These experiments were performed using charcoal-stripped 10% FBS, yet we observed that IL-23 production was partially suppressed by either ER-819762 (Fig. 4A) or by anti-PGE_2 antibody (Fig. 4B) in the absence of added prostaglandins. This suggests that IL-23 production in these activated DCs involves endogenously produced PGE_2.

EP_4 receptors antagonism suppresses IL-17 production and inhibits IL-23-induced Th17 expansion in activated $CD4^+$ T cells in vitro

We next determined whether EP_4 receptor stimulation might influence the development or function of Th17 cells, which play a critical role in inflammation and autoimmune diseases (Fouser *et al.*, 2008). First we tested the effect of EP_4 receptor stimulation on IL-17 production, a typical cytokine of Th17 cells, in activated $CD4^+$ T cells. Total $CD4^+$ T cells were isolated from mouse splenocytes, which include both naïve and memory T cells, and pre-existing Th17 cells were stimulated for 3–5 days using IL-23 and either anti-TCRβ or anti-CD3/anti-CD28 antibodies. Addition of PGE_2, butaprost or PGE_1-OH (Fig. 5A) suppressed overall T cell proliferation, yet enhanced IL-17 production. Both of these PGE_2-induced effects were reversed by 0.1 or 1.0 μmol·L⁻¹ ER-819762 (Fig. 5B). Further analysis by flow cytometry showed that PGE_2 stimulation increased the percentage of IL-17-producing cells within this population (Fig. 5C), and that this increase was suppressed by ER-819762 (data not shown). ER-819762 (Fig. 5D) or anti-PGE_2 antibody (Supplementary Fig. 4) could also suppress IL-23-induced Th17 expansion in the absence of exogenously added PGE_2. However, some PGE_2 could be present in the media, because we used normal 10% FBS and

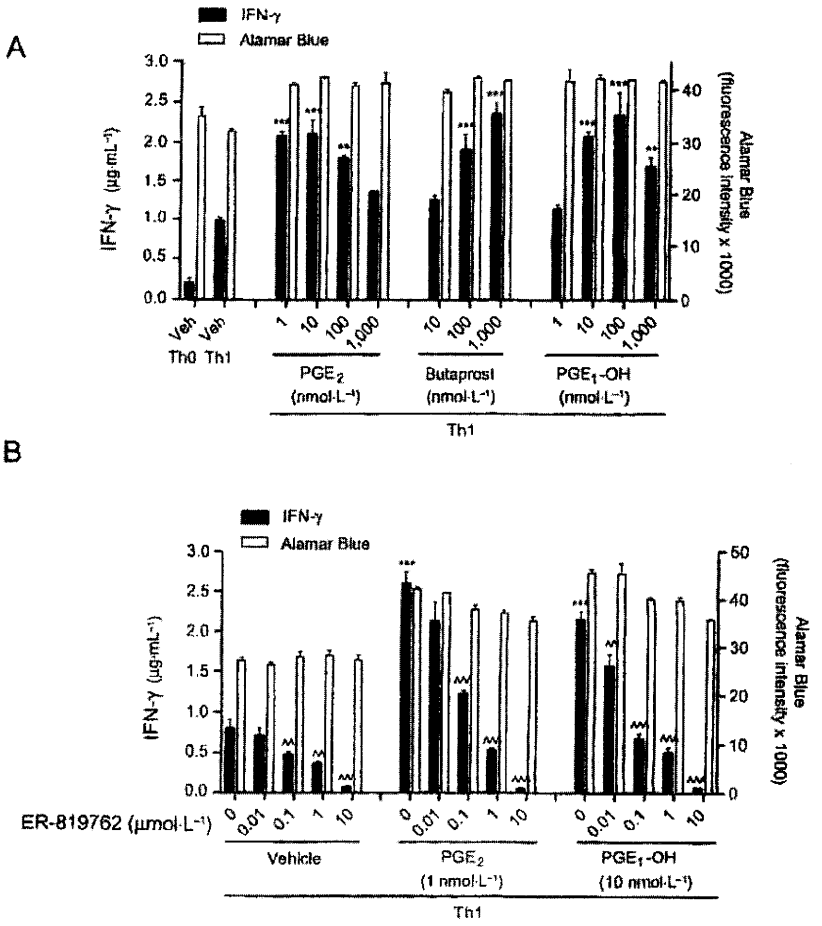

Figure 2 EP_4 receptor antagonism selectively suppresses prostaglandin E_2 (PGE_2)-induced Th1 differentiation *in vitro*. (A) Naive $CD4^+$ T cells from BALB/c mice were stimulated with α-CD3/α-CD28 under Th1-promoting conditions (see *Methods*) in media supplemented with 10% charcoal-stripped fetal bovine serum (FBS), together with PGE_2, butaprost, or PGE_1-OH at the indicated concentrations. After 3 days, interferon (IFN)-γ production was analysed by enzyme-linked immunosorbent assay (ELISA) and cell proliferation/viability was monitored by AlamarBlue. (B) was the same as (A), except that cells were cultured in the presence or absence of the indicated concentrations of PGE_2, PGE_1-OH and/or ER-819762. (C) Naive $CD4^+$ T cells isolated from DO11.10 mice were stimulated with ovalbumin (OVA)/antigen-presenting cells (APCs) under Th1-promoting conditions in 10% normal FBS in the presence or absence of ER-819762 and/or anti-PGE_2 for 3 days. IFN-γ production in culture supernatants was analysed by ELISA and cell proliferation/viability was monitored by AlamarBlue assay. (D) Naive $CD4^+$ T cells isolated from DO11.10 mice were stimulated with OVA/APCs under Th2-promoting conditions in normal 10% FBS in the presence or absence of ER-819762 for 6 days. Cells were collected at day 6 and re-stimulated with α-CD3 overnight. Cytokine [interleukin (IL)-4, left; IL-10, right] production in culture supernatants was analysed by ELISA. All data are shown as means ± SD ($n = 3$). Statistical analysis was performed by Dunnett-type multiple comparison test. *, ^ indicate $P < 0.05$; **, ^^ indicate $P < 0.01$ and ***, ^^^ indicate $P < 0.001$ levels of significance. *, **, *** Induction compared with Th1, vehicle, no ER-819762 control. ^, ^^, ^^^ inhibition compared with untreated controls within each group. These data are representative of at least two independent experiments.

not charcoal-stripped FBS for this experiment. These results suggest that PGE_2 signalling via EP_4 receptors results in an increase in IL-17 production and/or in the fraction of IL-17-producing cells in the population while suppressing non-Th17 cell proliferation. These results also indicate that PGE_2 produced by the T cells themselves and/or present in serum is involved in IL-17 production or/and Th17 cell expansion. Stimulation of these cells in the presence of PGE_2 or PGE_1-OH enhanced expression of IL-23 receptors, the retinoic acid receptor-related orphan receptor γ-T and IL-17A mRNA (Supplementary Fig. 5A). PGE_2-induced up-regulation of IL-23 receptors and IL-17A mRNA was suppressed by ER-819762 or anti-PGE_2 antibodies (Supplementary Fig. 5B and data not shown). IL-17 production and the percentage of IL-17-producing cells were also increased by other cAMP-inducing agents including butaprost or forskolin (Fig. 5A and C), suggesting a possible role for cAMP signalling in this process. No significant enhancement of PGE_2 on Th17 cell differentiation was observed when naïve $CD4^+$ T cells were activated in the presence of transforming growth factor (TGF)-β + IL-6, and no inhibitory effect of ER-819762 was observed in this system (data not shown).

EP_4 receptor antagonist suppresses inflammatory arthritis

As Th1 and Th17 cells have been implicated in the pathogenesis of autoimmune diseases such as RA (Schulze-Koops and

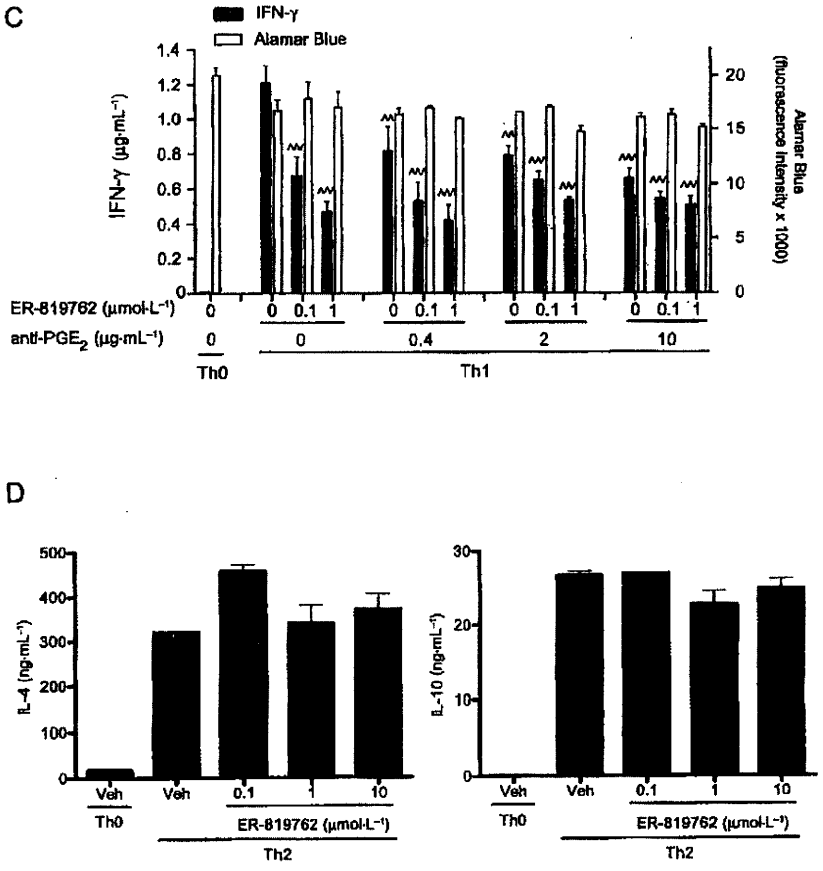

Figure 2 *Continued.*

Kalden, 2001; Fouser *et al.*, 2008), we tested whether the selective EP_4 receptor antagonist ER-819762 might influence disease in two mouse models of RA. We first tested the effect of ER-819762 in the murine CIA model, an animal model widely used for assessing therapeutic agents for treatment of RA. When given orally prior to the onset of disease, ER-819762 dose-dependently suppressed the clinical signs of arthritis and delayed disease onset (Fig. 6A). ER-819762 also significantly suppressed disease progression when it was administered subsequent to the onset of disease (Fig. 6B). Furthermore, ER-819762 retarded bone erosion in the CIA model, as demonstrated by radiological evaluation (Fig. 6C).

Matsumoto *et al.* (2008) have suggested that the GPI-induced arthritis model is more predictive of clinical efficacy than the CIA model, as the therapeutic effects of anti-tumour necrosis factor (TNF), anti-IL-6 and CTLA-4/Ig fusion in the GPI-induced arthritis model are similar to those seen in human patients treated with the equivalent biological agents. In the GPI-induced arthritis model in DBA/1 mice, oral administration of ER-819762 significantly reduced arthritis severity and delayed disease onset when administered prior to the onset of disease (Fig. 7A). ER-819762 also significantly suppressed disease progression when administered after the establishment of arthritis (Fig. 7B). Collectively, these results demonstrate that ER-819762 not only prevents the development of inflammatory arthritis, but is effective against the established disease by reducing both inflammation and joint destruction in animal models of RA.

Suppression of Th1 and Th17 responses in vivo

To determine whether suppression of arthritis by ER-819762 is associated with reduced Th1 and Th17 responses, we performed *ex vivo* challenge of cells obtained from mice with CIA. Cultured lymph node cells obtained from CIA mice were challenged *ex vivo* with collagen and cytokine production was determined. Cells from mice that had been treated with ER-819762 had significantly lower production of IFN-γ (>90%), IL-17 (>80%), TNF-α (>60%), IL-6 (>70%), osteopontin (>55%) and the chemokine CCL3 (MIP-1α; >35%) compared with lymph node cells from vehicle-treated mice (Fig. 6D). Overall lymph node cell proliferation was suppressed in ER-819762-treated mice to levels similar to those in non-immunized mice (data not shown).

We next examined the frequency of Th1 and Th17 cells found in the lymph nodes of GPI-induced arthritic mice. Popliteal lymph nodes were obtained from naïve, vehicle- or ER-819762-treated animals at 6 days post immunization and analysed by intracellular IFN-γ and IL-17 staining. The percentage of IL-17- and IFN-γ-producing $CD4^+$ T cells was

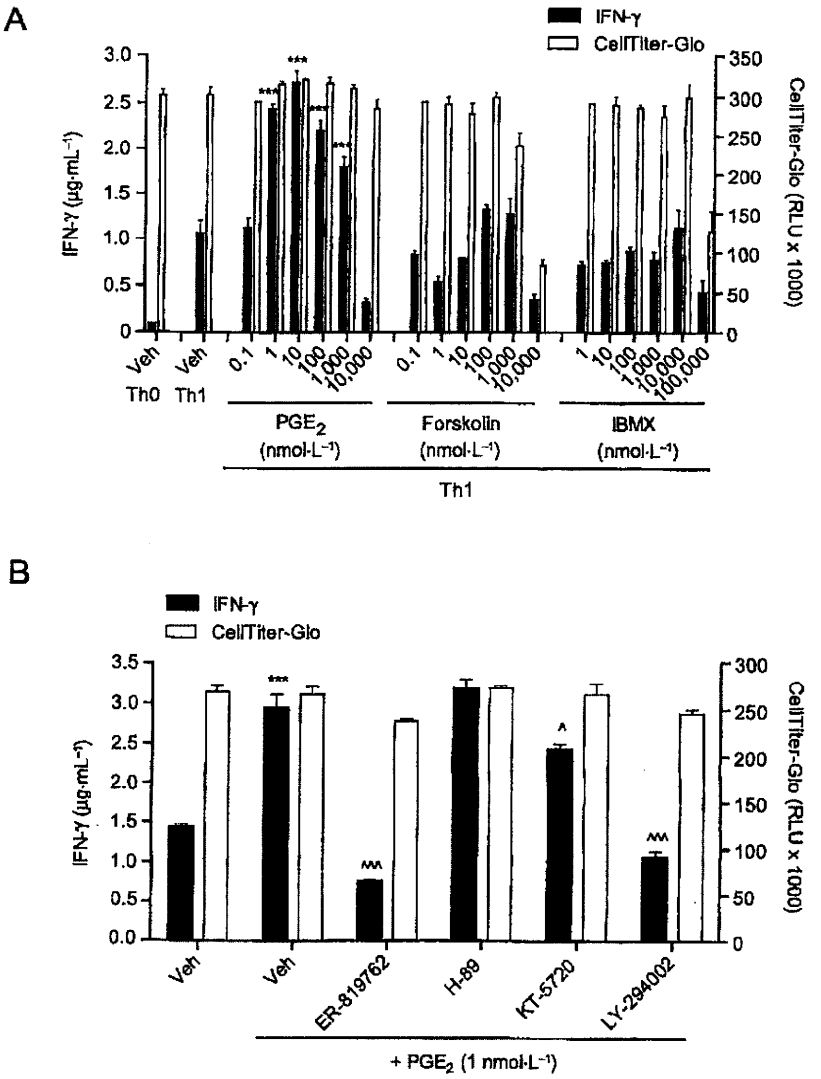

Figure 3 Enhancement of Th1 differentiation by EP_4 receptor stimulation requires phosphatidylinositol 3-kinase (PI3K) signalling. (A) The effect of cyclic AMP-inducing agents on Th1 differentiation was examined using the same methods as in Figure 2A. Cell proliferation/viability was monitored with CellTiter-Glo. (B) The effect of PKA and PI3K inhibitors on Th1 differentiation was examined as in (A). Inhibitor concentrations were as follows: 1 μmol·L^{-1} H-89, 10 μmol·L^{-1} KT-5720, 2 μmol·L^{-1} LY-294002 and 1 μmol·L^{-1} ER-819762. All data are shown as means ± SD (n = 3). Statistical analysis was performed by Dunnett-type multiple comparison test. *, ^ indicate $P < 0.05$; **, ^^ indicate $P < 0.01$ and ***, ^^^ indicate $P < 0.001$ levels of significance. *, **, *** induction compared with Th1, vehicle, no ER-819762 control. ^, ^^, ^^^ inhibition compared with untreated controls within each group. These data are representative of at least two independent experiments.

significantly lower in ER-819762-treated mice compared with vehicle-treated controls (Fig. 7C). The serum levels of IL-17 and IFN-γ were also significantly decreased in ER-819762-treated animals (Fig. 7D). Collectively these results indicate that EP_4 receptor-mediated PGE_2 signalling is important *in vivo* for the Th1/Th17 response and the development of inflammatory arthritis.

Suppression of CFA-induced hyperalgesia in rat

PGE_2 has been reported to be a key mediator of peripheral inflammatory pain, and evidence from studies using EP_4 receptor specific antagonists and small inhibitory RNAs implicate EP_4 receptors in this process (Lin *et al.*, 2006; Nakao *et al.*, 2007; Clark *et al.*, 2008). We therefore examined if ER-819762 might modulate the inflammatory pain response in a CFA-induced chronic inflammatory pain model in the rat. In this model, rats develop a lame walk reaction, characterized by a three-legged gait, 3 days after CFA injection. In the vehicle control group, 7 of 7 rats exhibited a positive lame walk reaction, while in the group treated with 100 mg·kg^{-1} ER-819762, 0 of 7 rats exhibited a positive lame walk reaction (Fig. 8). As a positive control, rats were also treated with the cyclooxygenase (COX) inhibitor, indomethacin and we observed significant suppression of lame walking at doses of 1 and 3 mg·kg^{-1} (Fig. 8).