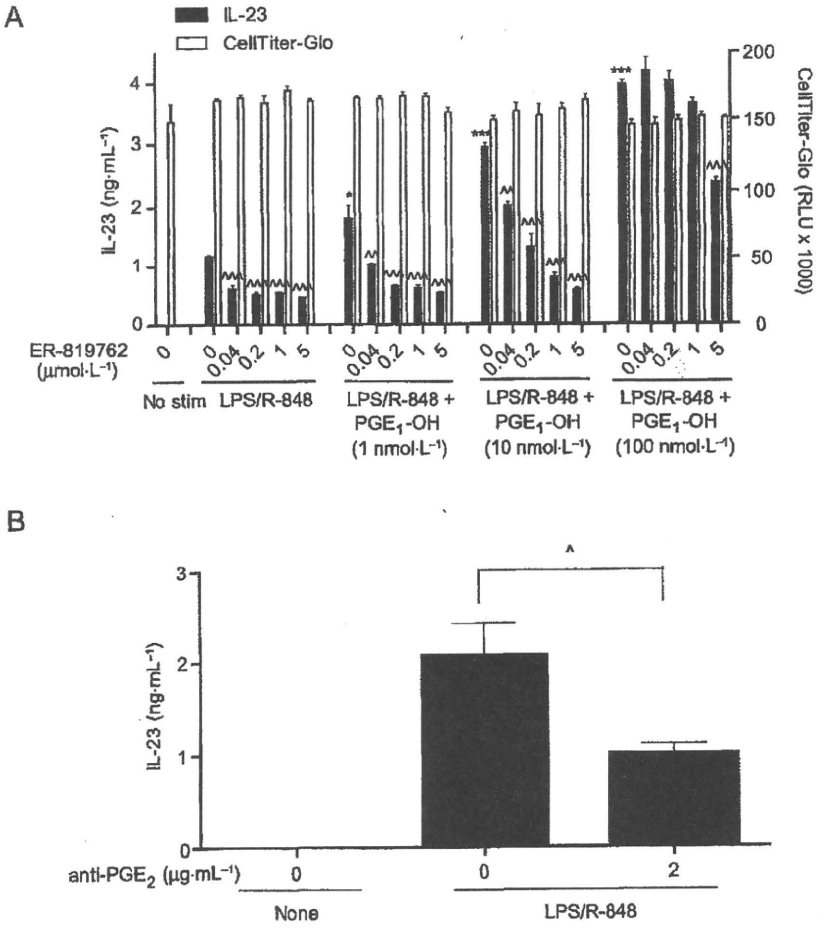


Figure 4 EP_4 receptor antagonism suppresses prostaglandin E_2 (PGE_2)-induced interleukin (IL)-23 production in human dendritic cells (DCs). (A) Human immature DCs were stimulated with 10 ng mL^{-1} lipopolysaccharide (LPS) and 2.5 μg mL^{-1} R-848 in the presence or absence of the indicated concentrations of PGE_1-OH and/or ER-819762 for 24 h. IL-23 in culture supernatants was measured by enzyme-linked immunosorbent assay and cell proliferation/viability was monitored with CellTiter-Glo. (B) Same as (A) but in the presence or absence of 2 μg mL^{-1} anti-PGE_2 antibody. All data are shown in 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 LPS/R-848-stimulated, no-PGE_1-OH, no-ER-819762 control, ^, ^^, ^^^ inhibition compared with no-ER-819762 controls within each group. These data are representative of at least two independent experiments.

Discussion

In the present study, we describe the pharmacological actions of a novel and highly selective antagonist of the EP_4 receptor, ER-819762, in models of inflammation. We show that antagonism of EP_4 receptor activation can suppress Th1 differentiation, production of IL-23 in DCs, and Th17 cell expansion *in vitro*. In addition, when tested in two mouse models of RA, ER-819762 was very effective in suppressing disease symptoms *in vivo*. A significant body of research has linked Th1 and Th17 cell development and function to autoimmune disease (Schulze-Koops and Kalden, 2001; Fouser *et al.*, 2008), and we observe in the mouse RA disease models (CIA and G6PI) that treatment with ER-819762 suppresses the ability of lymph node T cells to produce IFN-γ and IL-17 *ex vivo* in response to stimulation. We also observed reduced levels of IFN-γ and IL-17 in the serum of ER-819762-treated versus control mice in the G6PI model (Fig. 7D). However, although the suppressive effects of ER-819762 observed *in vitro* and *in vivo* are consistent, we cannot directly attribute suppression of disease in the animal models to inhibition of Th1 or Th17 development or function *in vivo*. It is possible that suppression of EP_4 receptor signalling has other unknown pharmacological effects in these models. Nevertheless, these *in vitro* and *in vivo* results show that antagonism of EP_4 receptors can suppress a broad range of pro-inflammatory responses relevant to the development of autoimmunity.

These results were initially unexpected, as earlier studies had demonstrated that PGE_2 suppresses T cell-mediated inflammation by increasing intracellular cAMP, inhibiting Th1 cytokine IFN-γ production, and inhibiting T cell activation and proliferation (Betz and Fox, 1991; Gold *et al.*, 1994; Hilkens *et al.*, 1995; Okano *et al.*, 2006). However, more recent reports have demonstrated the pro-inflammatory effects of

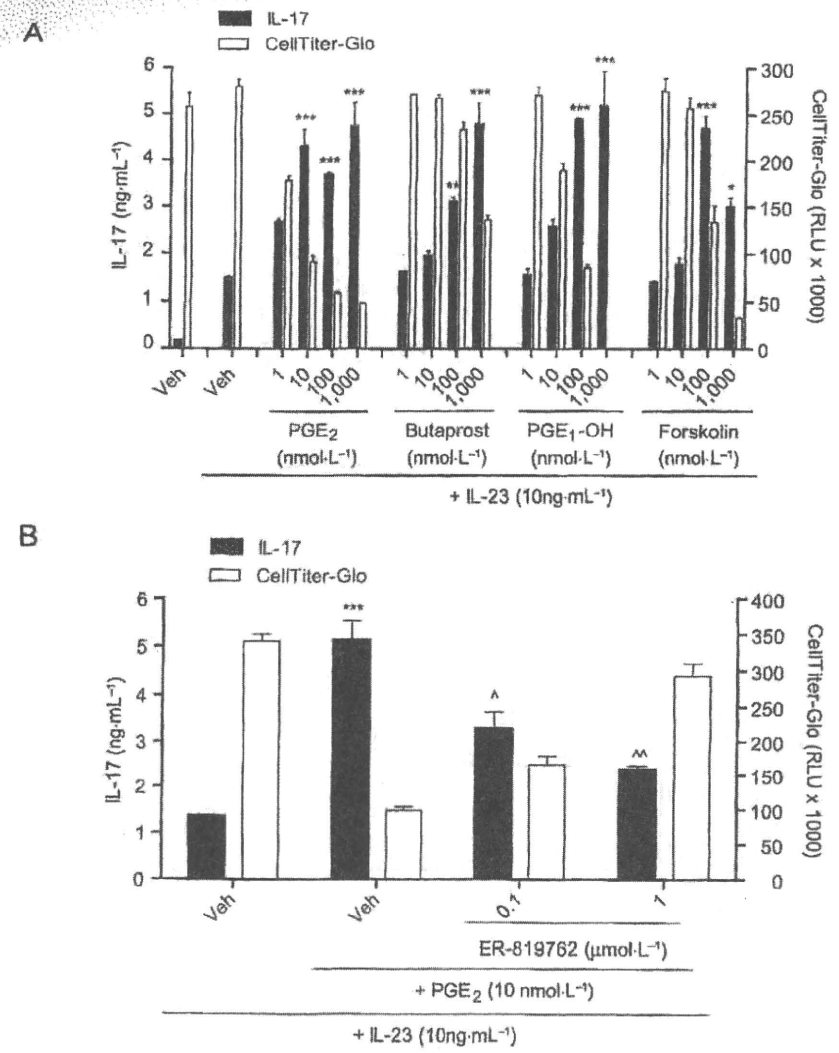


Figure 5 Prostaglandin E_2 (PGE_2)-EP_4 receptor signalling regulates Th17 cell development. (A) Total $CD4^+$ T cells isolated from mouse splenocytes were stimulated with anti-CD3/anti-CD28 plus interleukin (IL)-23 in the presence or absence of exogenous PGE_2, butaprost, PGE_1-OH, or forskolin for 3 days. IL-17 in culture supernatants was measured by enzyme-linked immunosorbent assay. (B) Same methods as in (A), except that ER-819762 was added at the indicated concentrations. (C) Total $CD4^+$ T cells were stimulated with α-TCRβ/α-CD28 ± IL-23 in the presence or absence of exogenously added PGE_2, butaprost, PGE_1-OH, or forskolin for 5 days and the percentage of Th17 cells was analysed by IL-17 intracellular staining. The horizontal broken line represents the level of IL-17 positive cells in the presence of IL-23 only. (D) Same methods as in (C), except that no PGs were added, and ER-819762 was added at the indicated concentrations. The number of Th17 cells was analysed by IL-17 intracellular staining. Upper plots show staining with control isotype-matched staining antibody, bottom plots show staining with anti-IL-17 antibody. First two columns show unstimulated and IL-23-stimulated cells. Right-hand lower two plots show IL-23-stimulated cells treated with different concentrations of ER-819762. All data are shown in 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 lipopolysaccharide/R-848-stimulated, no-PGE_1-OH, no-ER-819762 control, ^, ^^, ^^^ inhibition compared with no-ER-819762 controls within each group. These data are representative of at least two independent experiments.

PGE_2 in Th17 development (Chizzolini *et al.*, 2008; Boniface *et al.*, 2009; Napolitani *et al.*, 2009) and DC activation (Sheibanie *et al.*, 2004; Khayrullina *et al.*, 2008). As antagonism of EP_4 receptor signalling suppressed Th1 differentiation, Th17 cell expansion, and the development of pathologies in mouse CIA- and GPI-induced arthritis, we propose that the immune stimulatory activities of PGE_2 are relevant to these diseases.

Another debilitating aspect of RA is the pain associated with joint inflammation. This inflammatory pain is mediated, at least in part, by PGE_2 stimulation of EP_4 receptors (Lin *et al.*, 2006; Nakao *et al.*, 2007). Selective inhibition of EP_4 receptor signalling by several different EP_4 receptor antagonists has been shown to cause a marked reduction in joint pain, mechanical and thermal hyperalgesia and oedema in rat and in guinea pig models of pain and inflammation, often with similar efficacy to that observed with selective COX-2 inhibitors such as rofecoxib (Lin *et al.*, 2006; Nakao *et al.*, 2007; Clark *et al.*, 2008; Murase *et al.*, 2008; Jones *et al.*, 2009). Consistent with these findings, we observed that ER-819762 was

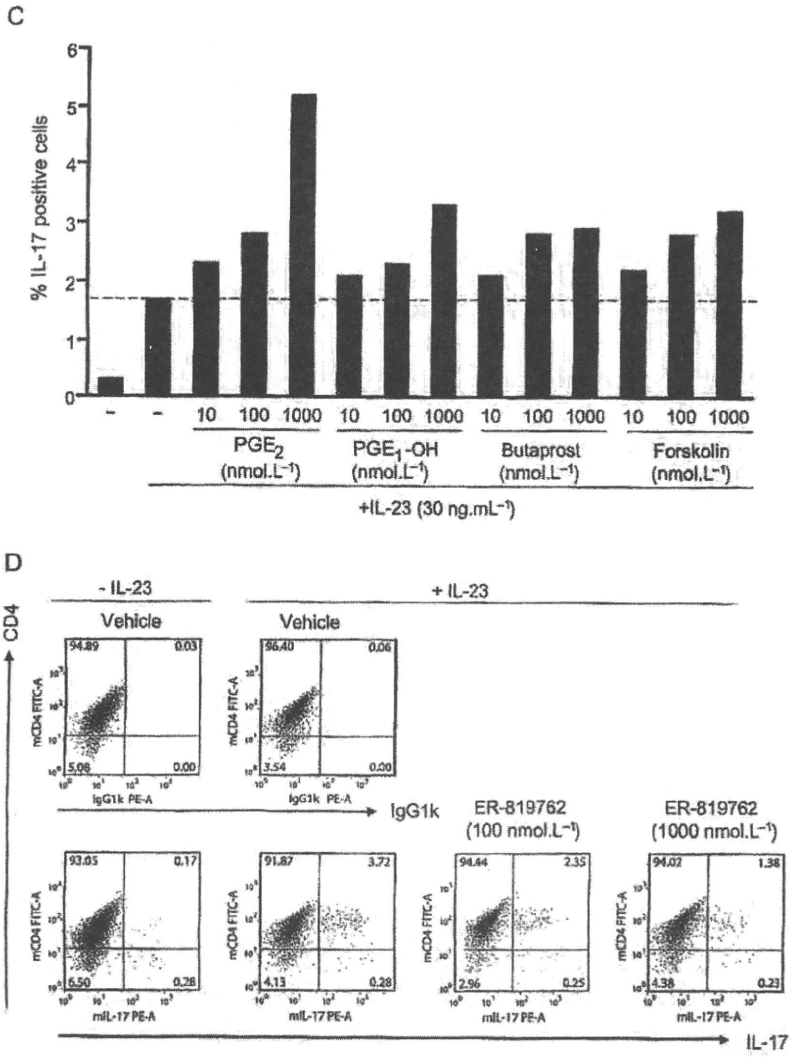


Figure 5 *Continued.*

effective in relieving inflammatory pain in a rat model of inflammatory pain induced by CFA injection into the paws. The analgesic effect of ER-819762 could be associated with reduced peripheral sensitization by suppression of PGE_2-mediated action on the peripheral terminals of nociceptor sensory neurons (Lin *et al.*, 2006). Alternatively, inhibition of IFN-γ and TNF-α by ER-819762 can also have an analgesic effect, because these cytokines have been shown to induce hypernociception (Verri *et al.*, 2006). Thus, an EP_4 receptor antagonist may have multiple benefits in relieving both the symptoms and modifying the disease mechanisms leading to RA.

EP_4 receptors have been reported to signal by at least two pathways (Regan, 2003): (i) activation of adenylate cyclase via the G_s protein to increase cAMP, and (ii) activation of PI3K via a G protein-independent signalling process. The suppression of T-cell activation by PGE_2 and other cAMP-elevating agents was proposed to be mediated by the activation of PKA, activation of C-terminal src kinase (Csk) and repression of leukocyte-specific protein tyrosine kinase (Lck)-dependent signalling through the T cell receptor (Mustelin and Tasken, 2003; Chemnitz *et al.*, 2006). In this study, however, we show that PGE_2 utilizes the PI3K pathway to promote Th1 differentiation (Fig. 3). Our data also suggest that the cAMP signalling pathway may promote Th17 expansion (Fig. 5), although our results are not definitive.

Recently, Chizzolini *et al.* (2008), Boniface *et al.* (2009) and Napolitani *et al.* (2009) have reported that PGE_2 can enhance the expansion and/or production of Th17 cells via cAMP signalling, and that this is accompanied by enhanced expression of IL-23R, IL-1R1, RORγt, the chemokine CCL20 and its receptor CCR6. Boniface *et al.* (2009) suggested that EP_2 receptors may be more important than EP_4 receptors for Th17 cell development and/or expansion, at least in human cells. We also observed enhanced IL-17 production and modest expansion of Th17 cells by incubation with the EP_2 receptor-selective agonist butaprost, but our data show that antagonism of EP_4 receptors is sufficient to suppress

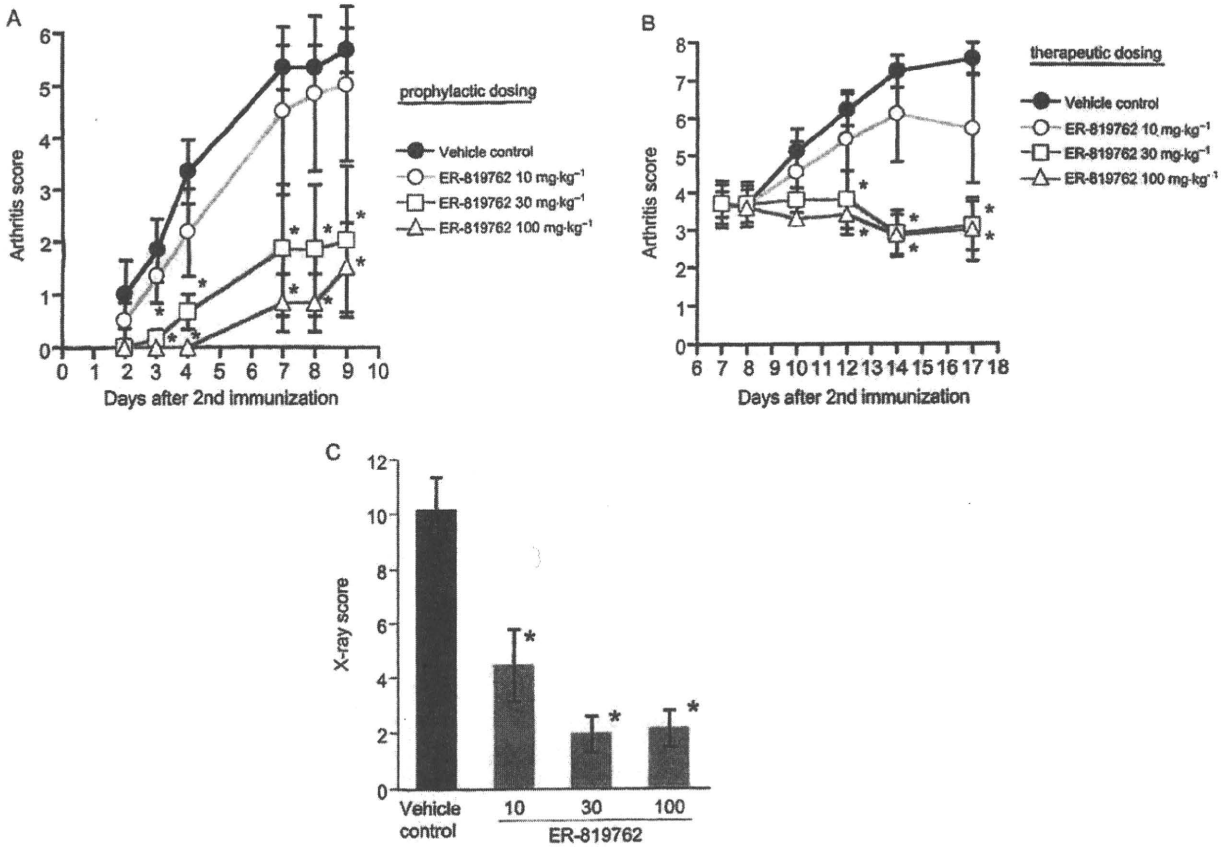
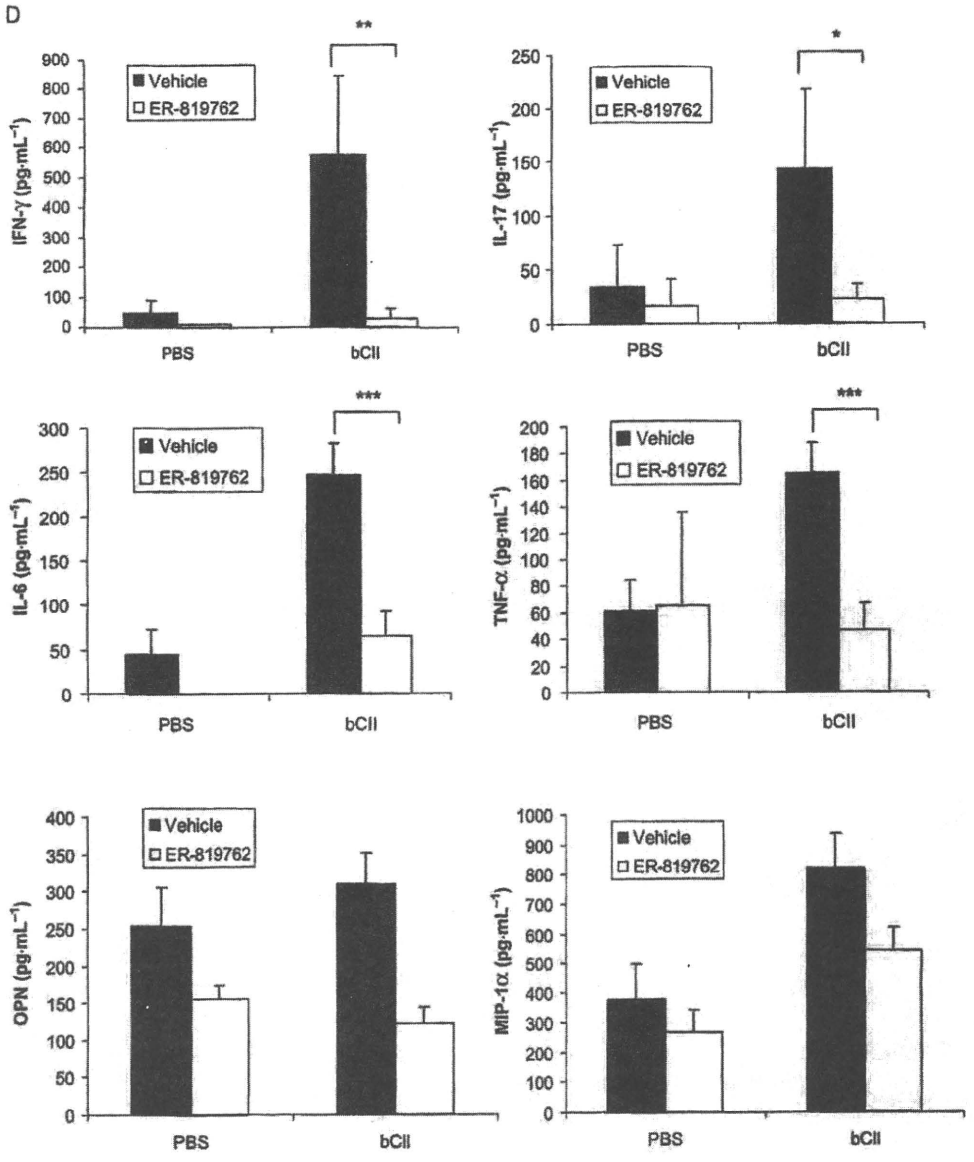


Figure 6 EP_4 receptor antagonism suppresses disease and Th1/Th17 cytokines in collagen-induced arthritis in mice. (A) DBA/1 mice were immunized with bovine type II collagen (bCII)/complete Freund's adjuvant (CFA) (primary immunization) and boosted with bCII in incomplete Freund's adjuvant at day 21 (second immunization) to induce arthritis as described in *Methods*. ER-819762 was orally administered daily from day 20 after primary immunization but before the onset of disease, and arthritis scores were monitored over the course of the study as described in *Methods*. (B) Same methods as in (A), but ER-819762 was administered after induction of disease on day 7 after second immunization. (C) Radiological analysis of inflamed paws at the end of the therapeutic collagen-induced arthritis study shown in 6B. The X-ray score is defined in *Methods*. (D) *Ex vivo* cytokine analysis. Mice were immunized with bCII/CFA or vehicle, similar to (A), except that ER-819762 was administered from the day of primary immunization (day 0). Lymph node cells were purified at day 15 and cultured in the presence of bCII (50 μg mL^{-1}) or phosphate-buffered saline for 72 h, and cytokine production was analysed. Statistical analysis was performed by Dunnett-type multiple comparison test compared with vehicle control (A–C) or paired *t*-test (D). Levels of significance: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. These data are representative of at least two independent experiments.

PGE_2-mediated Th17 expansion and IL-17 production in mouse cells. Our data also showed that PGE_2 did not promote Th17 differentiation *per se*, as we did not see an increase in Th17 cell frequency following PGE_2 stimulation of purified naïve $CD4^+$ T cells in the presence of TGF-β and IL-6. Rather, we observed an increase in Th17 cells when total $CD4^+$ T cells were stimulated with PGE_2 in the presence of IL-23, indicating the expansion of pre-differentiated Th17 cells. Napolitani *et al.* (2009) suggested that PGE_2 acts by inhibiting expansion of $CCR6^-$ T cells rather than increasing the proliferation of $CCR6^+$ Th17 cells, independent of IL-23. In agreement with this report, we also observed enhanced IL-17 production by PGE_2 in the absence of IL-23 co-stimulation (data not shown). In addition, we showed that EP_4 receptor stimulation can enhance IL-23 production by activated human DCs and that this activity can be inhibited by a selective EP_4 receptor antagonist or anti-PGE_2 antibody in the presence or absence of exogenously added PGE_2. Sheibanie *et al.* (2007a) have also recently reported that PGE_2 exacerbates disease in the CIA mouse by enhancing DC IL-6 and IL-23 production, the latter of which maintains Th17 cell survival and proliferation and consequently promotes IL-17 production. Collectively, these results support the idea that PGE_2 stimulation of EP_4 receptors promotes Th17 cell expansion at two stages by: (i) enhancing IL-23 production by DCs, and (ii) directly acting on memory T cells to promote IL-17 production and Th17 cell expansion (Sheibanie *et al.*, 2004; Chizzolini *et al.*, 2008; Khayrullina *et al.*, 2008; Boniface *et al.*, 2009).

We observed that while low concentrations of PGE_2 promoted IFN-γ production under Th1-differentiation conditions, production started to decrease at higher concentrations of PGE_2 or PGE_1-OH without loss of cell viability (Figs 2A and 3A). Similar results were seen with higher concentrations of butaprost (data not shown). Thus, PGE_2 appears to have a bimodal effect on immune stimulation; promoting inflammation at lower concentrations while attenuating inflammation



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at higher concentrations, possibly in concert with other factors that contribute to PI3K and/or cAMP signalling. This bimodal action may explain why PGE_2 exerts a pro-inflammatory effect in some systems and anti-inflammatory in others. For example, Betz and Fox (1991) have reported that PGE_2 can inhibit the production of Th1 cytokines, which is contrary to our results; however, these researchers used micromolar concentrations of PGE_2 for many of their experiments. There are also a number of potential sources of PGE_2 in cell culture systems that could contribute to higher PGE_2 levels. For example, our data suggest that autocrine production of PGE_2 can significantly contribute to Th1 differentiation, Th17 expansion and IL-23 production by DCs. We also observed that normal FBS, but not charcoal-stripped or PGE_2-immunodepleted serum stimulated EP_4 receptors in HEK/293 cells (Supplementary Fig. 1). Thus, there may be significant basal stimulation of EP_2 and EP_4 receptors in many cell culture systems, in which case further addition of exogenous PGE_2 could reduce inflammation.

COX inhibitors have also shown some efficacy in animal models of RA (Ochi *et al.*, 2003). Our results suggest that among the downstream effectors of the COX pathway, EP_4 receptors may play a particularly important role in the pathology of RA. In our own experiments, we saw only limited efficacy of the COX inhibitor indomethacin in suppressing arthritis in the mouse CIA model, and higher dosing was limited by toxicity (data not shown). Thus, a selective antagonist(s) of one or more critical downstream prostaglandin receptors may be more effective than broad inhibition of COX activity. Prostaglandins play a variety of

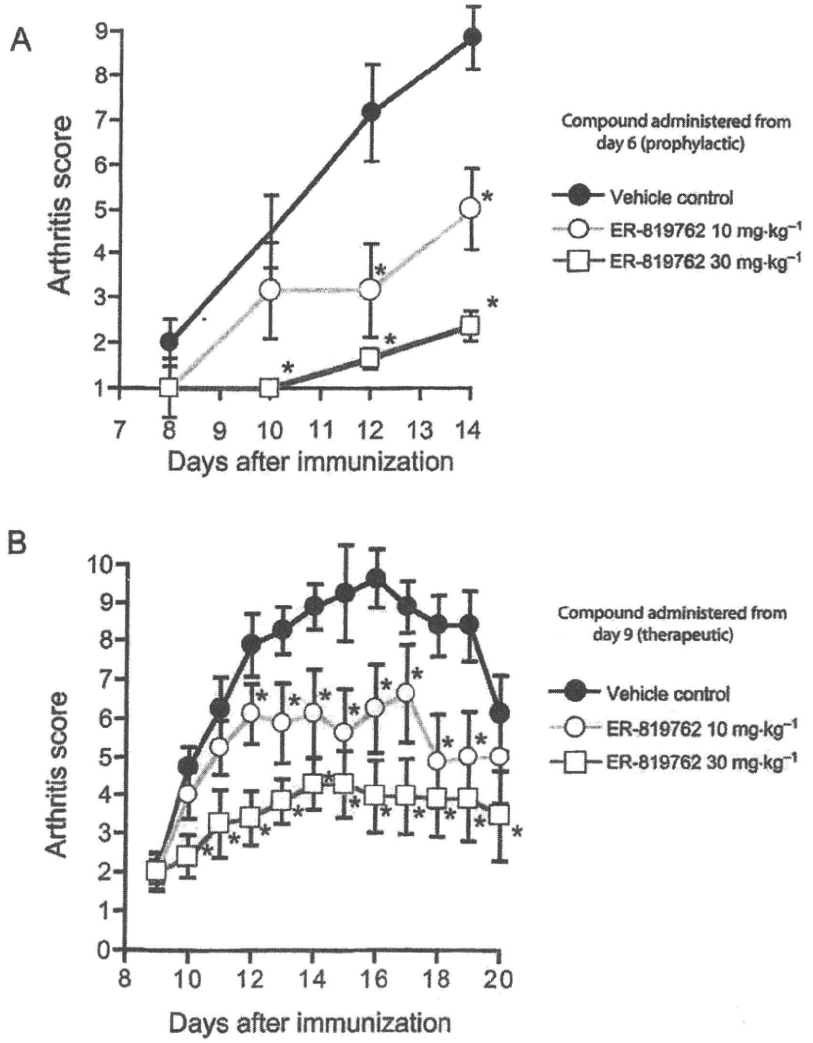
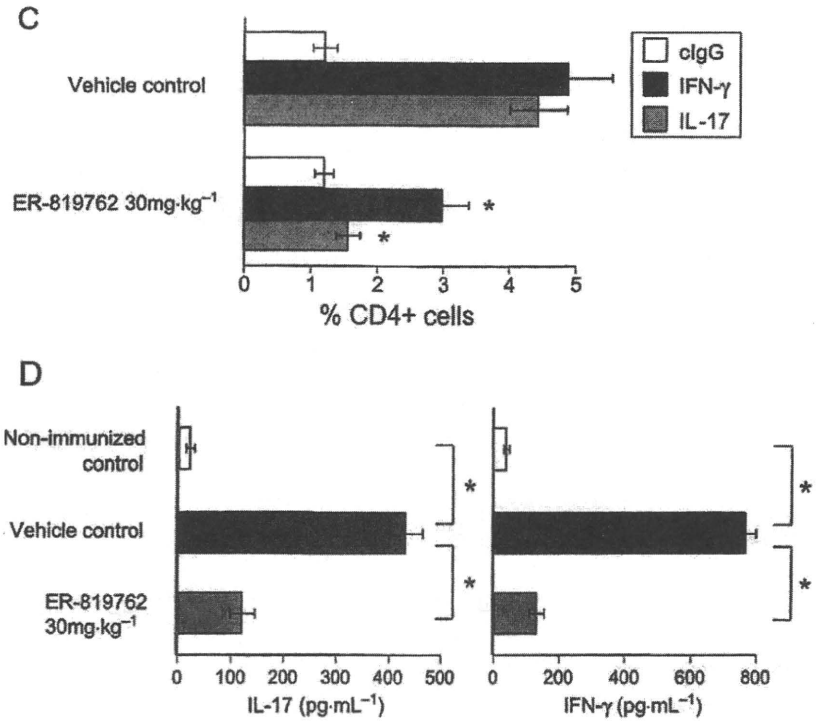


Figure 7 EP_4 receptor antagonism suppresses disease and Th1/Th17 cytokines in glucose-6-phosphate isomerase (GPI)-induced arthritis in mice. (A) DBA/1 mice were immunized with GPI/complete Freund's adjuvant to induce arthritis as described in *Methods*. ER-819762 was orally administered daily from day 6 after immunization, but before the onset of disease. Clinical scores were monitored over the course of the study. (B) Same methods as in (A), but ER-819762 was administered after disease induction (day 9). (C) Same methods as in (A), but ER-819762 was administered from the day of immunization. Popliteal lymph node cells were removed from mice at day 6 and re-stimulated with GPI in culture. interleukin (IL)-17- and interferon (IFN)-γ-producing cells were quantified by intracellular staining and flow cytometry. Experiments with isotype control IgG are shown as cIgG. (D) Serum was collected at the end of the GPI study shown in (A), and analysed by IL-17 and IFN-γ enzyme-linked immunosorbent assay. Statistical analysis was performed by Dunnett-type multiple comparison test compared with vehicle control (A and B) or paired *t*-test (C and D). Levels of significance: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. These data are representative of at least two independent experiments.

roles in modulating inflammation and can exert both anti- and pro-inflammatory effects. For example, one proposed explanation for why aspirin and other COX inhibitors are ineffective in treating allergic inflammation is that PGD_2 produced downstream of the COX enzymes stimulates the DP receptor, which promotes allergic inflammation, while PGE_2 stimulates the EP_3 receptor, which suppresses allergic inflammation (Kunikata *et al.*, 2005). In addition, the more targeted approach of antagonizing EP_4 receptors might suppress inflammation without the side-effects associated with some non-steroidal anti-inflammatory drugs and COX inhibitors, including increased gastrointestinal and cardiovascular risks. Consistent with this, Takeuchi *et al.* (2007) showed that the EP_4 receptor antagonist CJ-042794 did not produce any damaging effects in the gastrointestinal mucosa of control or adjuvant-induced arthritic rats, whereas indomethacin caused gross lesions. More importantly, we found that ER-819762 not only could prevent, but could suppress established disease in the CIA and GPI-induced arthritis models. Bone destruction in CIA was also significantly reduced by ER-819762. The effects of ER-819762 in suppressing bone destruction may be due in part to suppression of osteoclastogenesis promoted by IL-17 and PGE_2. IL-17 stimulates osteoblasts to synthesize PGE_2



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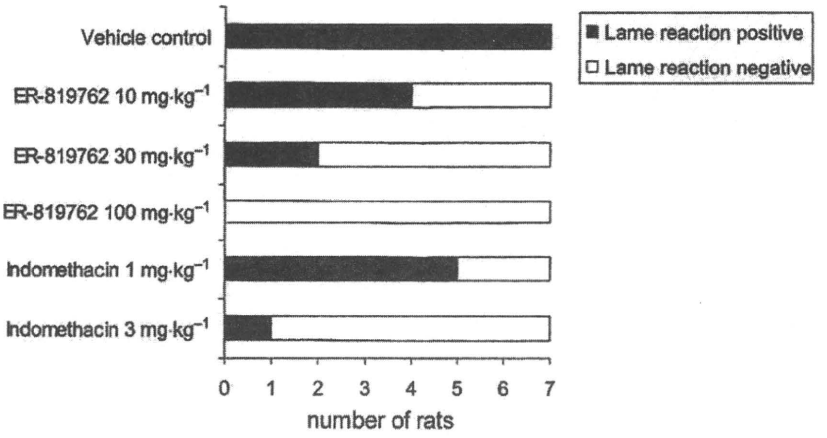

Figure 8 The analgesic effect of ER-819762 on the lame walk response in complete Freund's adjuvant (CFA) injected rats. CFA was injected into the right hind footpad of each rat (seven rats per treatment group). Three days after CFA injection, the rats exhibited a lame walking reaction as described in *Methods*. Compounds were given orally 3 days after CFA and the lame reaction was evaluated at 3 h after drug administration. These data are representative of at least two independent experiments.

and express receptor activator of NF-κB (RANK), which induces osteoclastogenesis (Kuligowska and Odrowaz-Sypniewska, 2004). We have observed that RANK-ligand mRNA levels in arthritic joints were lower in mice treated with an ER-819762 analogue in both the CIA and GPI-induced arthritis mouse models (unpublished results). Previous studies have also reported that anti-TNF-α therapy was effective in the GPI-induced arthritis model (Matsumoto *et al.*, 2008), but had little effect treating disease in the CIA model (Joosten *et al.*, 1996; Williams *et al.*, 2000). In contrast, ER-819762 was effective in both models, suggesting that an EP_4 receptor antagonist strategy may be beneficial to RA patients, including those who are insensitive to anti-TNF therapy.

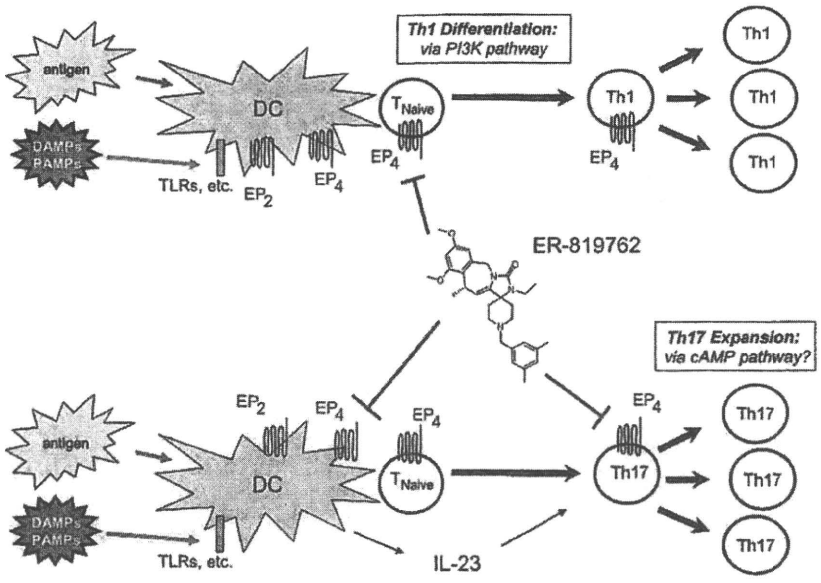

Figure 9 Multiple effects of ER-819762 on pro-inflammatory responses. Blue lines indicate the multiple steps at which ER-819762 was observed to exert an immunosuppressive effect in our studies. During infection or under conditions of chronic autoimmune inflammation, exogenous pathogen-associated molecular pattern stimuli (PAMPs) and/or endogenous danger-associated molecular pattern stimuli (DAMPs) drive immune cell activation in conjunction with antigen. In the case of Toll-like receptors, this signalling synergizes with the prostaglandin E_2 (PGE_2)-activated EP_4 receptor signalling pathway to enhance IL-23 production by dendritic cells (DCs). EP_4 receptor signalling in naïve T cells promotes their differentiation into Th1 effector cells via the phosphatidylinositol 3-kinase (PI3K) pathway, whereas EP_4 receptor signalling promotes the expansion of Th17 effector cells via the cyclic AMP pathway. ER-819762 blocks EP_4 receptor-enhanced Th1 differentiation and suppresses Th17 function both indirectly, by reducing DC IL-23 production and, as a consequence, Th17 survival, and directly by suppressing EP_4 receptor-enhanced Th17 expansion and/or IL-17 production. However, it is unknown if these actions of the EP_4 receptor antagonist can completely account for suppression of disease in the animal models, and other mechanisms are possible in addition.

These results and methodologies have been shared earlier with colleagues in another laboratory, and they have recently confirmed that PGE_2-EP_4 receptor signalling promotes Th1 cell differentiation, IL-23 production by DCs and Th17 cell expansion (Yao *et al.*, 2009). This group also tested an EP_4 receptor antagonist with a very different molecular structure from ER-819762, supporting the idea that the anti-inflammatory effects of ER-819762 are indeed due to EP_4 receptor antagonism and not due to action on another, unidentified target of the compound.

In summary, we show that an antagonist of EP_4 receptors, ER-819762, can suppress inflammation at multiple stages, as summarized in Fig. 9, as well as moderating inflammatory pain. Our results suggest that selective antagonism of EP_4 receptors could have therapeutic benefit in modifying both the underlying pathology of RA and alleviating pain, thus providing potential total management for RA patients.

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Conflict of Interest

All authors were employed by Eisai Inc. (USA) or Eisai Co., Ltd. (Japan) at the time of these studies. The authors have no further conflicting financial interests.

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Supporting information

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

Figure S1 Modulation of CMV promoter activity by ER-819762. Our initial drug screen utilized the cytomegalovirus immediate-early (CMV) promoter driving expression of a protein unrelated to the prostanoid receptors in HEK/293 cells. After we observed down-regulation of protein expression by ER-819762, we examined activity of the CMV promoter by stably transfecting a plasmid containing the Renilla luciferase reporter driven by the CMV promoter (pRL-CMV; Promega) into HEK/293 cells. Cells were cultured overnight in DMEM media supplemented with 0.1% fatty-acid free bovine serum albumin (Sigma A0281), and *Renilla* luciferase activity was assayed the next day (Promega Renilla Luciferase assay kit). Addition of 10% fetal bovine serum (FBS) to the cultures up-regulated CMV activity, and this induction was suppressed by 1 $\mu mol \cdot L^{-1}$ ER-819762. Pre-treatment of FBS by incubation with activated charcoal (CSFBS), which removes a variety of lipids, abolished induction of the CMV promoter. Activity could be restored by addition of prostaglandin E_2 (PGE_2) to CS-FBS, and this activity was inhibited by ER-819762. Addition of the cAMP-inducing agent forskolin (FSK) could also induce CMV activity, but this induction was not suppressed by ER-819762. FBS that had been immunodepleted using anti-PGE_2 antibodies (ΔP-FBS: Cayman Chemicals, clone 2B5) was not able to induce CMV activity, indicating that the CMV-inducing activity present in FBS is PGE_2 or a PGE_2-related molecule.

Figure S2 PGE_2 induction of cAMP signalling in HEK/293 cells is mediated by EP_4. HEK/293 cells were stably transfected with a vector containing response elements for the CREB transcription factor driving expression of a secreted alkaline phosphatase reporter (CRE-PLAP). This reporter construct can be up-regulated by stimuli that induce intracellular cAMP, as shown here for forskolin. We also stimulated these cells with PGE_2 ($EP_{1,\ 2,\ 3,}$ and $_4$ agonist), butaprost (EP_2 agonist) or PGE_1-OH (EP_3 and $_4$ agonist). We observed induction of PLAP activity in response to forskolin, PGE_2 or PGE_1-OH, but not to butaprost. ER-819762 could suppress induction by PGE_2 or PGE_1-OH (data not shown). We also saw no induction of CRE-PLAP by up to 100 $nmol \cdot L^{-1}$ sulprostone, an agonist of EP_3 and EP_1 (data not shown). These data indicate that of the four PGE_2 receptors, only EP_4 is able to induce cAMP signalling in HEK/293 cells.

Figure S3 Effect of ER-819762 on Th1 cell differentiation. Naive $CD4^+$ T cells from BALB/c mice were stimulated with α-CD3/α-CD28 under Th1-promoting conditions in the presence or absence of exogenous PGE_2, butaprost, PGE_1-OH plus increasing amount of ER-819762 for 3 days. IFN-γ production (solid bars) was analyzed by ELISA and cell proliferation/viability (open bars) was monitored by AlamarBlue assay.

Figure S4 Effect of anti-PGE_2 antibody on IL-23 mediated Th17 cell expansion. Total $CD4^+$ T cells were stimulated with α-TCRβ/α-CD28 ± 30 $ng \cdot mL^{-1}$ IL-23 in the presence or absence of α-PGE_2 antibody for 5 days. No exogenous PGE_2 was added in these experiments. The number of IL-17 cells was analyzed by IL-17 intracellular staining and showed that treatment with α-PGE_2 antibody results in a striking decrease in the proportion of IL-17-producing cells induced by IL-23.

Figure S5 PGE_2 treatment induces mRNA expression of IL-23R, RORγt and IL-17A during Th17 cell development. Total $CD4^+$ T cells were stimulated with α-TCRβ/α-CD28 ± 30 $ng \cdot mL^{-1}$ IL-23 in the presence or absence of 10 $nmol \cdot L^{-1}$ PGE_2 or 100 $nmol \cdot L^{-1}$ PGE_1-OH (a) or in the presence or absence of 1 $\mu mol \cdot L^{-1}$ ER-819762 or 10 $\mu g \cdot mL^{-1}$ α-PGE_2 Ab (b) for 5 days. Total RNA was isolated and analysed by real-time PCR for the expression of IL-23R, RORγt and IL-17A mRNA.

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Laser microdissection-based analysis of cytokine balance in the kidneys of patients with lupus nephritis

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Summary

To determine the cytokine balance in patients with lupus nephritis (LN), we analysed kidney-infiltrating T cells. Renal biopsy samples from 15 systemic lupus erythematosus (SLE) patients were used. In accordance with the classification of International Society of Nephrology/Renal Pathology Society, they were categorized into Class III, Class III+V (Class III-predominant group, $n = 4$), Class IV, Class IV+V (Class IV-predominant group, $n = 7$) and Class V ($n = 4$) groups. The single-cell samples of both the glomelular and interstitial infiltrating cells were captured by laser-microdissection. The glomerular and interstitial infiltrating T cells produced interleukin (IL)-2, IL-4, IL-10, IL-13 and IL-17 cytokines in the Class III-predominant, Class IV-predominant and Class V groups. Interferon-gamma was detected only in the glomeruli of the Class III-predominant and Class V group samples. The expression level of IL-17 was correlated closely with clinical parameters such as haematuria, blood urea nitrogen level, SLE Disease Activity Index scores in both glomeruli and interstitium, urine protein level in glomeruli and serum creatinine and creatinine clearance levels in interstitium. This suggests that the glomerular infiltrating T cells might act as T helper type 1 (Th1), Th2 and Th17 cells while the interstitial infiltrating T cells, act as Th2 and Th17 cells in the Class III-predominant and Class V groups. In contrast, both the glomerular and interstitial infiltrating T cells might act as Th2 and Th17 cells in the Class IV-predominant group. The cytokine balances may be dependent upon the classification of renal pathology, and IL-17 might play a critical role in SLE development.

Keywords: laser-microdissection, lupus nephritis, SLEDAI, Th17

Introduction

Systemic lupus erythematosus (SLE) is a multi-system autoimmune disease characterized by various clinical manifestations. T cell-derived cytokine production plays a determinant role in SLE development. Previous studies have reported that an imbalance in cytokine production between T helper type 1 (Th1) and Th2 T cells (predominance of Th2 cytokine) in the peripheral blood of SLE patients is associated with the pathogenesis of the disease [1–3]. In contrast, Akahoshi *et al.* [4] demonstrated that a substantial predominance of Th1-type response took place in the peripheral blood samples of lupus nephritis (LN) patients categorized in WHO Class IV. Not only T cells in the peripheral blood, but also the balance in cytokine production between Th1 and Th2 cells in the kidney has drawn a great deal of attention. Masutani *et al.* [5] analysed the expression levels of interferon (IFN)-γ and interleukin (IL)-4 on intrarenal T cells as well as those in the peripheral blood samples from SLE patients with diffuse proliferative LN by immunohistochemistry, demonstrating the predominance of Th1 type response. They suggested that the Th1 : Th2 ratio in the peripheral blood might directly reflect the local histopathological findings. However, Murata *et al.* [6] indicated that the kidney-infiltrating T cells could produce Th2 type cytokines such as IL-4 and IL-10 through reverse transcription–polymerase chain reaction (RT–PCR), and made an assumption that this discrepancy might arise from a difference in sensitivity between the methods used in detection of cytokines. The expression level of IL-13, one of the Th2 type cytokines, was reported to be higher in the serum from the rheumatoid arthritis (RA), SLE, Sjögren's syndrome and

systemic sclerosis patient groups than that in the normal healthy control group [7]. Morimoto *et al.* [8] also showed elevated expression level of IL-13 in SLE patients. Recently, it has been reported that naive murine $CD4^+$ T helper cells can be induced to differentiate into Th1, Th2, Th17 and regulatory phenotypes [9]. IL-17 is a proinflammatory cytokine, as possibly known from the pathological conditions of various inflammatory diseases in both humans and mice [9]. We have reported previously that both IL-13 and IL-17 were produced in the murine LN (MRL/lpr mice) cells; however, we did not analyse them at a single-cell level [10]. The laser microdissection (LMD) technique has been adopted recently to obtain tissue samples exclusively from specific regions of interest. This new technique has been used successfully in various fields, including oncology [11], endocrinology [12], gastroenterology [13], rheumatology [14–16] and nephrology [10,17–19]. With this technique, attempts to analyse single-cell gene expression were made [13,16,20]. In our study, we analysed the single-cell expression levels of cytokines, including IL-13 and IL-17, by infiltrating T cells in the kidneys of LN patients.

Patients and methods

Renal biopsy samples were obtained from 15 SLE patients, two minor glomerular abnormalities (MGA) patients (female, 16 years old; male, 14 years old) and one minimal change nephrotic syndrome (MCNS) patient (male, 14 years old), and used in our experiments. In accordance with the classification criteria defined by International Society of Nephrology/Renal Pathology Society (ISN/RPS) [21,22], renal pathologies were diagnosed as: Class III, three cases; Class III+V, one case; Class IV, two cases; Class IV+V, five cases; and Class V, four cases. To ensure consistency with the World Health Organization (WHO) classification criteria, a further membranous lesion (Class V) may be added to Class III or Class IV in ISN/RPS. They were categorized as Class III-predominant group (Class III-predominant group included patients with both Class III and Class III+V, $n = 4$) and Class IV-predominant group (including patients with both Class IV and Class IV+V, $n = 7$). The patients, who had underwent renal biopsy before 2004, had already been classified in accordance with the WHO classification criteria [23] at the time of biopsy, but in this study were re-evaluated by nephrologists in accordance with the ISN/RPS classification criteria. The SLE Disease Activity Index (SLEDAI) scores [24], histological activity index (AI) and chronicity index (CI) scores [25] at renal biopsy are shown as Table 1. This study was approved by the ethical committee of Tsukuba University Hospital (no. 392). Prior written consent was given by the patients.

Immunohistological examinations

Five-μm-thick sections were obtained from the renal biopsy specimens of the SLE patients. Immunohistochemical

Table 1. Clinical characteristics of patient and positivity of dissected T cells.

No.	Age	Sex	Classification	Pre-s	UP (g/day)	Haematuria (RBC/HPF)	Urinary cast	Pyuria	BUN (mg/dl)	Cr (mg/dl)	Ccr (ml/min)	ADNA (U/ml)	CH50 (U/ml)	SLEDAI	TCR-cβ/ β-actin (%)	AI	CI
1	45	F	III (A)	No	5·0	10–19	+	–	10·1	0·54	92·1	> 300	4·8	17	18/28 (64·3%)	5	2
2	52	F	III (C)	Yes	0·46	1–4	–	–	20·7	1·01	38·9	< 2	40·2	0	15/34 (44·1%)	1	8
3	52	F	III (A) + V	No	0·3	1–4	–	–	10·0	0·57	84·0	64·7	18·2	9	18/26 (69·2%)	0	3
4	29	F	IV-G (A/C)	No	8·0	56	+	+	31·1	1·10	48·1	100·0	12·0	21	28/36 (77·8%)	16	3
5	25	F	IV-G (A/C)	Yes	5·0	5–9	+	–	15·0	0·60	93·0	> 300	2·1	19	18/25 (72·0%)	11	3
6	58	F	IV-S(A/C)+V	No	6·1	1–4	+	–	10·0	0·56	159·0	45	24·5	16	53/67 (79·1%)	12	4
7	59	F	IV-G(A/C)+V	No	2·3	1–4	–	+	26·1	0·95	51·5	64·7	18·2	9	19/28 (67·9%)	5	2
8	55	F	IV-G (A) + V	No	0·8	1–4	–	–	13·1	0·49	100·0	94·6	7·4	13	14/27 (51·9%)	10	0
9	18	F	IV-S (A) + V	Yes	0·46	1–4	–	–	12·3	0·48	108·5	58·1	9·5	18	22/31 (70·1%)	6	3
10	28	F	V	No	7·7	0–1	–	–	13·8	0·70	96·7	16·7	41·6	5	18/26 (69·2%)	1	2
11	39	F	V	Yes	5·3	0–1	–	–	12·4	0·51	138·1	5·4	50·7	4	22/32 (68·8%)	0	2
12	26	F	V	Yes	2·1	5–9	–	–	13·5	0·60	95·4	23·1	42·9	19	46/62 (74·2%)	0	0
13	22	M	IV-G(A/C)+V	Yes	4·15	30–50	+	–	16·0	0·90	110·8	< 5·0	20·5	10	7/20 (35·0%)	8	6
14	38	F	III (A/C)	No	0·85	1–4	+	–	18·7	0·62	141·7	> 300	23·2	13	18/31 (58·1%)	6	4
15	60	F	V	No	0·49	20–99	–	–	16·6	0·73	99·6	20·3	54·0	22	27/40 (67·5%)	0	3

S: segmental; G: global; A: active; C: chronic; Pre-s: pretreatment with steroid; UP: urine protein; RBC/HPF: red blood cell/high power field; BUN: blood urea nitrogen; Cr: serum creatinine; Ccr: creatinine clearance; Anti-ds: anti-double-stranded DNA; CH50: 50% haemolytic unit of complement serum; SLEDAI: systemic lupus erythematosus Disease Activity Index; AI: activity index score; CI: chronicity index score.

staining was performed by the avidin–biotin complex technique. Primary antibodies used included murine anti-human IFN-γ (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-IL-4, 10 (Research & Diagnostics Systems, Minneapolis, MN, USA); and polyclonal rabbit anti-human IL-17 and IL-13 (Santa Cruz Biotechnology). Staining was performed on the sections using normal murine IgG or rabbit immunoglobulin (Ig)G, a primary antibody, as a negative control. We also performed staining on sections of the renal biopsy samples of MGA and MCNS patients using anti-human IL-17 as the control.

Tissue sampling by laser microdissection

Frozen sections (10 μm thick) from the renal biopsy specimens of the SLE patients were stained with 0·05% toluidine blue solution (pH 7·0) (Wako Pure Chemical Industries, Osaka, Japan) and the individual single cells infiltrating into glomeruli and interstitiums were selected and dissected with laser-microdissection system (AS-LMD; Leica Microsystems Japan, Tokyo, Japan) (Fig. 2A).

RNA extraction and nested RT–PCR

Total RNA was extracted from the LMD samples by the Isogen method (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. First-strand cDNA was prepared from total RNA using the ThermoScript RT–PCR System (Invitrogen Life Technologies, Carlsbad, CA, USA) and amplified with primers specific to β-actin, T cell receptor β chain (TCR-Cβ), IL-2, IL-4, IL-10, IL-13, IL-17 and IFN-γ for nested RT–PCR (Table 2).

Statistical analysis

All data were expressed as mean ± standard error of the mean. Statistically significant differences between groups were determined using the Mann–Whitney *U*-test. A simple linear regression analysis was used to evaluate the correlation between the two parameters. The statistical significance was defined as $P < 0.05$.

Results

Detection of T cells in glomeruli and interstitium

Stained IL-4, IL-10 and IL-13 were observed in the glomerular and interstitial areas of the specimens from the SLE patients of the Class III-predominant, IV-predominant and Class V groups, especially in the latter area of the Class IV-predominant group (Fig. 1A) (the immunohistochemical data for the Class III-predominant and Class V groups are not shown). Many IL-4 cells were observed predominantly, mainly in the glomerular and interstitial cells, especially in intraglomerular infiltrating cells, in the Class IV-predominant group, while there were only a few IL-4-positive cells in the tubular epithelial cells (TEC) (Fig. 1Aa, b). IL-10- and IL-13-positive cells were observed prominently in the glomerular and interstitial infiltrating cells (Fig. 1Ac–f). Some stained IL-10-positive cells were observed in TEC (Fig. 1Ac, d). IL-17-positive cells were observed mainly in the glomerular and interstitial infiltrating cells and TECs, especially in intraglomerular cells of the Class IV-predominant group (Fig. 1Ag, h). Almost no IL-17-positive cells were observed in the glomeruli of the Class III-predominant (Fig. 1Ba) and Class V group (not shown) samples. However, IFN-γ cells were not observed in all the specimens (Fig. 1Bb) (the immunohistochemical data for the Class III-predominant groups are shown). Normal rabbit IgG was used as a negative control (Fig. 1Bc). IL-17-positive cells were not observed in all the specimens from the MGA and MCNS patients (Fig. 1C). This demonstrates that IL-17 may be produced preferentially in SLE patients.

Analysis of gene expression by laser microdissection and nested RT–PCR

Of 622 glomerular and interstitial infiltrating cells, 513 (82·5%) were β-actin-positive, among which 343 (66·7%) were TCR-Cβ-positive; these 343 cells were deemed to be T cells and used for cytokine analysis (Table 1). The number of positive samples for each cytokine/ TCR-Cβ$^+$ cells was expressed as a percentage.

The glomerular and interstitial infiltrating T cells produced IL-2, IL-4, IL-10, IL-13 and IL-17 cytokines in the Class III-predominant, Class IV-predominant and Class V groups. The positivity of cytokines is shown in Table 3 and Fig. 2B. The percentages of positive IL-4, IL-10 and IL-13 samples were more than 70%, 67% and 41%, respectively, in all the groups. The expression levels of IL-2 were low in each of the predominant groups. IFN-γ was detected only in the glomeruli of the Class III-predominant and Class V groups (32·3 ± 12·9% and 24·0 ± 10·0%, $P < 0.05$) (Table 3 and Fig. 2B). In the glomerular lesions, the percentage of positive IL-17 samples was 64·7 ± 10·1% and 70·7 ± 6·0% in the Class IV-predominant and V groups, while it was significantly greater than in the Class III-predominant group (44·7 ± 5·9%, $P < 0.05$) (Fig. 2Bb). In the interstitial lesions, the positivity of IL-17 (48·0 ± 4·2%) was also significantly lower in the Class III-predominant groups than that in the Class IV-predominant group (69·1 ± 8·9%, $P < 0.05$) (Fig. 2Bc).

Correlation between the expression levels of cytokines and clinical parameters in SLE patients

We analysed the correlation between the expression levels of Th1 (IL-2), Th2 (IL-4, IL-10, and IL-13) and Th17 (IL-17)

Table 2. Oligonucleotide primer sequences.

PCR products		Oligonucleotide sequence	Product size (bp)	RT–PCR cycles
β-actin				
First PCR	5′ sense	GGCATCCTCACCCTGAAGTA	496	25
	3′ anti-sense	CCATCTCTTGCTCGAAGTCC		
Nested PCR	5′ sense	AAATCTGGCACCACACCTTC	262	25
	3′ anti-sense	AGGGCATACCCCTCGTAGAT		
TCR-Cβ				
First PCR	5′ sense	ACATAAGGAAGGCTGCATGG	249	30
	3′ anti-sense	CGTTTTGATCATGGTGTGTGG		
Nested PCR	5′ sense	ATCAGGTGTGTGGGACTTTG	217	30
	3′ anti-sense	GACTCAGGACAGTGACATCA		
IFN-γ				
First PCR	5′ sense	TCTGCATCGTTTTGGGTTCTC	346	25
	3′ anti-sense	TCAGCTTTTCGAAGTCATCTC		
Nested PCR	5′ sense	TGTTACTGCCAGGACCCATAT	242	30
	3′ anti-sense	ACTCTTTTGGATGCTCTGGTC		
IL-2				
First PCR	5′ sense	ACTACCAGGATGCTCACATT	267	25
	3′ anti-sense	AAGGTAATCCATCTGTTCAGA		
Nested PCR	5′ sense	GCCACAGAACTGAAACATCTT	201	30
	3′ anti-sense	TTCTACAATGGTTGCTGTCTC		
IL-4				
First PCR	5′ sense	CTTCCCCCTCTGTTCTTCCT	318	25
	3′ anti-sense	TTCCTGTCGAGCCGTTTCAG		
Nested PCR	5′ sense	CTAGCATGTGCCGGCAACTTT	273	25
	3′ anti-sense	TCGGATCAGCTGCTTGTGCCT		
IL-10				
First PCR	5′ sense	ACAGCTCAGCACTGCTCTGT	327	30
	3′ anti-sense	AGTTCACATGCGCCTTGATG		
Nested PCR	5′ sense	CCCAGTCTGAGAACAGCTGCAA	210	30
	3′ anti-sense	CTGGGTCTTGGTTCTCAGCTT		
IL-13				
First PCR	5′ sense	CTATGCATCCGCTCCTCAAT	391	30
	3′ anti-sense	TTTACAAACTGGGCCACCTC		
Nested PCR	5′ sense	ATTGCTCTCACTTGCCTTGG	229	25
	3′ anti-sense	TCCTGTGGGTCTTCTCGATC		
IL-17				
First PCR	5′ sense	CTTCACCCTGTGGAACGAAT	262	30
	3′ anti-sense	CGGAATTGGTTCTGGAGTGT		
Nested PCR	5′ sense	GAGCACATGCACCACATACC	170	25
	3′ anti-sense	AGGAAACAGTCGCGGAGTGT		

RT–PCR: reverse transcription polymerase chain reaction; TCR-Cβ: T cell receptor β chain; IL: interleukin; IFN-γ: interferon-gamma; bp: base pairs.

Table 3. Positivity of cytokines in glomeruli and interstitiums (%).

	Glomeruli			Interstitiums		
	Class III predominant	Class IV predominant	Class V	Class III predominant	Class IV predominant	Class V
IL-2	19·7 ± 10·3	23·7 ± 20·3	44·6 ± 12·8	25·6 ± 10·5	2·8 ± 21·7	27·9 ± 12·3
IFN-γ	32·3 ± 12·9	n.d.	24·0 ± 10·0	3·1 ± 3·8	n.d.	1·3 ± 1·6
IL-4	88·6 ± 9·1	80·4 ± 13·5	85·7 ± 7·1	90·6 ± 7·5	84·6 ± 13·5	70·9 ± 16·7
IL-10	67·2 ± 5·1	67·7 ± 14·3	70·0 ± 10·3	70·5 ± 6·4	84·3 ± 6·5	79·8 ± 8·1
IL-13	60·6 ± 15·7	47·6 ± 20·5	62·3 ± 9·3	52·0 ± 9·2	41·5 ± 13·4	62·3 ± 7·6
IL-17	44·7 ± 5·9	64·7 ± 10·1*	70·7 ± 6·0*	48·0 ± 4·2	69·1 ± 8·9*	62·6 ± 12·3

*$P < 0.05$ *versus* Class III-predominant groups. Results are expressed as mean ± standard error of the mean. Statistical significance was determined using the Mann–Whitney *U*-test. IL; interleukin; IFN-γ: interferon-gamma; n.d.: not determined.

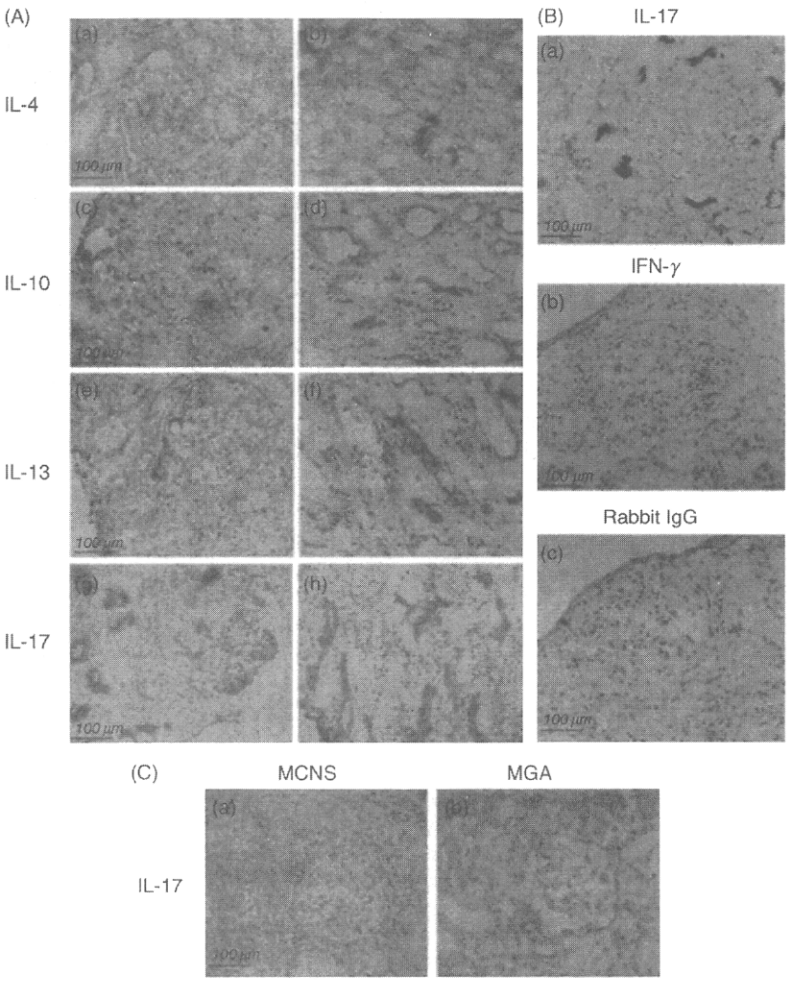

Fig. 1. Detection of T cells in glomeruli and interstitium. (A) Stained interleukin (IL)-4, IL-10, IL-13 and IL-17 were observed in glomeruli and interstitium areas of the Class IV-predominant groups. Many IL-4 cells are observed prominently, mainly in the cells infiltrating into the glomeruli and interstitium areas, especially in intraglomerular infiltrating cells. There are only a few IL-4 cells in the tubular epithelial cells (TEC) (a, b). IL-10 and IL-13 cells are observed prominently in some of the cells infiltrating into the glomeruli and interstitium areas (c–f). Some stained IL-10 cells were observed in TEC (d). Many IL-17 cells are observed prominently, mainly in the cells infiltrating into the glomeruli, interstitium areas and TEC (g, h), especially in intraglomerular cells and TEC (g) (original magnification × 100 in a–h). (B) In the Class III-predominant group, some stained IL-17 cells were observed in TEC but almost no IL-17-positive cells were observed in glomeruli (a). Interferon (IFN)-γ cells were not observed in all the specimens (b). Normal rabbit immunoglobulin (Ig)G was stained as negative control (c) (original magnification × 100). (C) IL-17-positive cells were not observed in all the specimens of minor glomerular abnormalities (MGA) and minimal change nephrotic syndrome (MCNS) (a, b) (original magnification × 100).

cytokines and clinical parameters in SLE patients, such as the urine protein (UP) level, haematuria, blood urea nitrogen (BUN) level, serum creatinine (Cr) level, creatinine clearance (Ccr), 50% haemolytic unit of complement serum (CH50), anti-double-strand DNA (anti-ds DNA) antibodies, SLEDAI scores, histological AI and CI (Table 4). Good and significant correlation data are shown in Fig. 3.

Correlation between Th1 cytokine and clinical parameters. In glomeruli, as known from the tendency of the point distribution on the charts, the parameters, BUN ($r = 0{\cdot}27$), Ccr ($r = 0{\cdot}31$), AI ($r = -0{\cdot}28$), CI ($r = 0{\cdot}39$) and SLEDAI ($r = -0{\cdot}21$) ($P < 0{\cdot}05$) showed a weak correlation with the expression level of IL-2 (Table 4). The expression level of IL-2 showed a good correlation with anti-ds DNA antibodies ($r = -0{\cdot}53$, Fig. 3Aa) and a significant correlation with CH50 ($r = 0{\cdot}80$, $P < 0{\cdot}001$, Fig. 3Ab). In the interstitium, haematuria ($r = -0{\cdot}36$), BUN ($r = -0{\cdot}24$), Cr ($r = -0{\cdot}35$) and CH50 ($r = 0{\cdot}37$) showed a weak correlation with the expression level of IL-2 (Table 4); Ccr ($r = 0{\cdot}63$) and CI ($r = 0{\cdot}404$) showed a good correlation with the expression level of IL-2 (Fig. 3Ac, d).

Correlation between Th2 and clinical parameters. In the glomeruli, haematuria ($r = 0{\cdot}44$), BUN ($r = -0{\cdot}44$), Cr ($r = -0{\cdot}41$) and CI ($r = -0{\cdot}59$) showed a good correlation with the expression level of IL-4 (Fig. 3Ba–d); SLEDAI ($r = -0{\cdot}36$) and AI ($r = -26$) showed a weak correlation with IL-4 (Table 4). The expression level of IL-10 showed a weak correlation with haematuria ($r = -0{\cdot}23$), BUN ($r = -0{\cdot}39$), Ccr ($r = 0{\cdot}27$), CI ($r = 0{\cdot}28$) and CH50 ($r = 0{\cdot}31$). However, there was almost no finding that showed any correlation with the expression level of IL-13 except for BUN ($r = -0{\cdot}21$), AI ($r = -0{\cdot}32$) and CH50 (Table 4).

In the interstitiums, there was a weak correlation in the expression level of IL-4 with haematuria ($r = 0{\cdot}24$), CH50 ($r = -0{\cdot}34$), AI ($r = 0{\cdot}22$), CI ($r = -0{\cdot}33$) and anti-ds DNA antibodies ($r = 0{\cdot}28$) (Table 4). IL-10 showed a good correlation with UP ($r = 0{\cdot}59$) (Fig. 3Be) and a weak correlation with SLEDAI ($r = -0{\cdot}26$) (Table 4). The percentage of IL-13

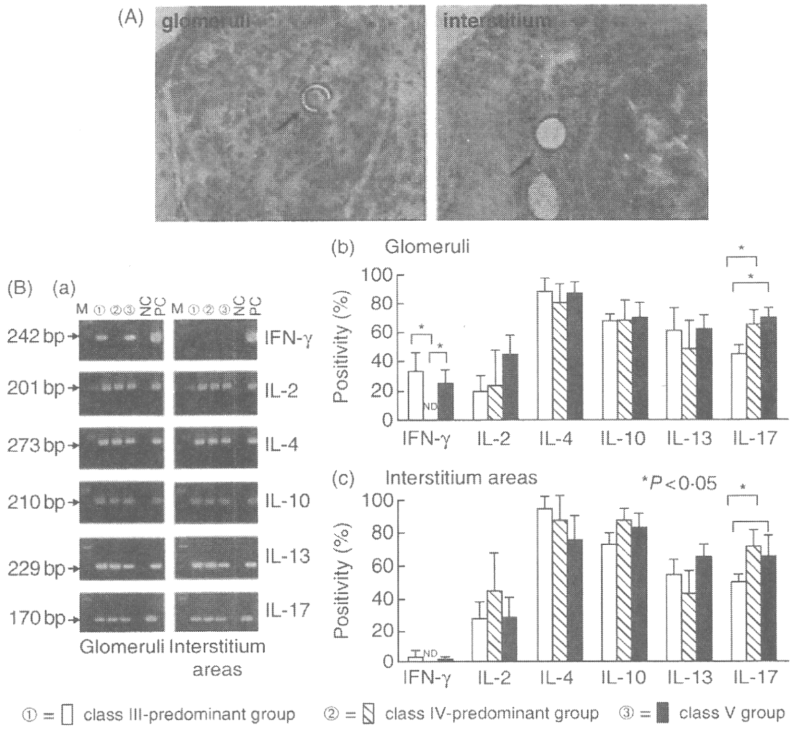

Fig. 2. (A) Targeted infiltrating cells selected and cut by laser microdissection (LMD). The glomeruli and interstitium areas of a single infiltrating cell (black arrows) were selected and dissected with a laser microbeam one by one. (B) Analysis of cytokine gene expression in lesions. (a) Detection of cytokines in the lesions of the renal biopsy specimens from the patients by nested polymerase chain reaction (PCR). Specific expression of interferon (IFN)-γ, interleukin (IL)-2, IL-4, IL-10, IL-13 and IL-17 were identified in the lesions of the glomeruli and interstitium areas from the renal biopsy specimens of the patients in the Class III-predominant groups ($n = 4$), Class IV-predominant groups ($n = 7$) and Class V groups ($n = 4$). M: molecular size marker; NC: negative control; PC: positive control cDNA clone. (b) Expression of IFN-γ, IL-2, IL-4, IL-10, IL-13 and IL-17 mRNAs in the glomeruli areas of the Class III-predominant (white bars), Class IV-predominant (hatched bars) and Class V (black bars) groups was analysed by nested reverse transcription–polymerase chain reaction (RT–PCR). (c) Expression of IFN-γ, IL-2, IL-4, IL-10, IL-13 and IL-17 mRNAs in the interstitium areas of the Class III-predominant (white bars), Class IV-predominant (hatched bars) and Class V (black bars) groups was analysed by nested RT–PCR (n.d. = not determined). The number of positive samples is shown as a percentage. Error bars represent ± standard error. $P < 0{\cdot}05$, by Mann–Whitney *U*-test.

samples showed a weak correlation with UP ($r = -0{\cdot}35$), haematuria ($r = -0{\cdot}31$) and Ccr ($r = 0{\cdot}37$) (Table 4), and a good correlation with BUN ($r = -0{\cdot}68$), Cr ($r = -0{\cdot}49$), CH50 ($r = 0{\cdot}48$), AI ($r = -0{\cdot}54$) and anti-ds DNA antibodies ($r = -0{\cdot}43$) (Fig. 3C).

Correlation between Th17 and clinical parameters. In the glomeruli, UP ($r = 0{\cdot}33$), AI ($r = 0{\cdot}26$), CI ($r = -0{\cdot}34$) and BUN ($r = 0{\cdot}26$) showed a weak correlation with the expression level of IL-17 (Table 4). Haematuria ($r = 0{\cdot}54$) and SLEDAI ($r = 0{\cdot}54$) showed a significantly positive correlation with the expression level of IL-17 (Fig. 3Da, b). In the interstitiums, the positive IL-17 samples showed a weak correlation with BUN ($r = 0{\cdot}37$), Cr ($r = 0{\cdot}38$), AI ($r = 0{\cdot}29$), CI ($r = -0{\cdot}27$) and Ccr ($r = -0{\cdot}36$) (Table 4), and a good correlation with haematuria ($r = 0{\cdot}47$) and SLEDAI ($r = 0{\cdot}54$) (Fig. 3Da, b). In particular, focusing upon patients whose SLEDAI scores are more than 10, there is a highly significant correlation between SLEDAI scores and the expression levels of IL-17 both in the glomeruli ($r = 0{\cdot}81$, $P < 0{\cdot}05$) and the interstitiums ($r = 0{\cdot}87$, $P < 0{\cdot}001$) (Fig. 3Dc).

Discussion

A cytokine balance of T helper cells in the kidneys of LN patients has drawn a great deal of attention [5,6]. We analysed the single-cell cytokine profile of the samples from the LN patients, including IL-13 and IL-17, by LMD. We observed the predominance of the Th2 cytokine both in the glomeruli and the interstitiums; this corresponds to the results of the study using whole kidneys by Murata *et al.* [6]. However, IFN-γ was observed only in the glomeruli of the ISN/RPS Class III-predominant and Class V groups. Chan *et al.* [19] reported that up-regulation of IFN-γ, IL-2 and T-bet (the Th1 transcription factor) was observed and no difference was observed in glomerular expression level of any

Table 4. Correlation between the levels of cytokines and clinical parameters.

				Glomeruli						
	SLEDAI	CH50	ADNA	haematuria	Cr	BUN	AI	CI	UP	Ccr
IL-2	−0.214*	0.795***	−0.53**	0.363*	0.114	−0.27*	−0.279*	0.387*	0.045	0.31*
	0.049	0.002	0.018	0.046	0.242	0.047	0.047	0.045	0.435	0.047
IL-4	−0.361*	−0.065	0.016	−0.437**	−0.405*	−0.441**	−0.262*	−0.591**	−0.115	−0.095
	0.046	0.592	0.47	0.042	0.041	0.044	−0.048	0.01	0.34	0.367
IL-10	−0.156	0.308*	0.194	−0.231*	0.019	−0.391*	−0.091	0.282*	0.187	0.265*
	0.268	0.047	0.143	0.048	0.472	0.045	0.626	0.046	0.049	0.047
IL-13	0.001	0.342*	−0.192	0.162	0.023	−0.213*	−0.319*	0.022	0.038	0.127
	0.499	0.047	0.045	0.146	0.467	0.049	0.047	0.531	0.445	0.325
IL-17	0.541**	0.123	−0.157	0.543**	0.029	0.264*	0.227*	−0.341*	0.333*	0.007
	0.017	0.33	0.278	0.018	0.458	0.049	0.049	0.046	0.047	0.488
				Interstitium						
	SLEDAI	CH50	ADNA	haematuria	Cr	BUN	AI	CI	UP	Ccr
IL-2	−0.125	0.37*	−0.175	−0.362*	−0.348*	−0.24*	0.173	0.404**	0.154	0.63**
	0.327	0.045	0.265	0.046	0.046	0.048	0.268	0.037	0.29	0.016
IL-4	0.178	−0.34*	0.279*	−0.24*	−0.168	0.07	0.221*	−0.333*	−0.062	−0.084
	0.262	0.046	0.047	0.044	0.273	0.401	0.048	0.046	0.413	0.381
IL-10	−0.26*	−0.116	−0.02	−0.094	0.207	0.037	0.195	0.061	0.586**	−0.058
	0.047	0.339	0.471	0.369	0.059	0.447	0.091	0.414	0.012	0.418
IL-13	0.058	0.483**	−0.436**	0.31*	−0.486**	−0.675**	−0.541**	−0.117	−0.35*	0.371*
	0.418	0.033	0.025	0.047	0.039	0.002	0.018	0.338	0.047	0.046
IL-17	0.544**	−0.134	0.476*	0.471*	0.379*	0.374*	0.294*	−0.273*	−0.028	−0.364*
	0.018	0.316	0.036	0.038	0.045	0.042	0.047	0.048	0.459	0.047

Correlation between clinical parameters and cytokines was assessed by using the Pearson correlation coefficient test (r-value showed in up, *r = 0·2–0·4, weak correlation; **r = 0·4–0·7, good correlation; ***r = 0·7–0·9, significant correlation. P-value showed in down). UP: urine protein; RBC/HPF: red blood cell/high power field; BUN: blood urea nitrogen; Cr: serum creatinine; Ccr: creatinine clearance; ADNA: anti-double-stranded DNA; CH50: 50% haemolytic unit of complement serum; SLEDAI: systemic lupus erythematosus Disease Activity Index; AI: activity index score; CI: chronicity index; IL: interleukin.

target genes between the WHO Classes. However, as they reported, they did not analyse at a single-cell level; therefore, they could not identify the cellular origin of the detected mRNA, which is likely to be the reason for the discrepancy between their results and our results. Morimoto *et al.* [8] reported that Th2 predominance in the peripheral blood might induce renal lesions, and the co-existence of Th1 and Th2 might cause haemolytic anaemia or pulmonary lesions in SLE patients. Our result demonstrates that Th1 has a role in protecting the kidneys of LN patients; this corresponds to the results of the experiments on the peripheral blood of the SLE patients reported by Morimoto *et al.* Although, conventionally, it was believed that enhanced Th1 cell activation and IFN-γ production might contribute to the development of autoimmune diseases [26,27], certain findings have exploded this general hypothesis. For example, experimental autoimmune nephritis and collagen-induced arthritis (CIA) was exacerbated in mice treated with anti-IFN-γ-neutralizing antibodies and in IFN-γ-deficient or IFN-γ receptor-deficient mice [28]. Haas *et al.* [29] reported that IFN-γ might play a key role in suppressing the development of nephritis in MRL/lpr mice (SLE models).

In addition to the helper T cells classified into Th1 and Th2 types, another helper T cell subset, Th17, has been discovered recently [9]. It has been observed that IL-17 has a proinflammatory role in many inflammatory conditions [9], contributing to the pathogenesis of autoimmune and inflammatory diseases, including SLE [30].

Elevated concentrations of proinflammatory cytokines (IL-18, IL-17, IL-12) and Th2 cytokine (IL-4) in the SLE patients were reported [31]. Dong *et al.* [32] reported that the cultured peripheral blood mononuclear cells (PBMC) of LN patients stimulated by IL-17 produced significantly high levels of IL-6, IgG and anti-ds DNA antibodies. However, IL-17 did not increase them in cultured PBMC of normal controls [32]. Crispin *et al.* [33] have demonstrated that $CD3^+$ $CD4^-CD8^-$ double-negative (DN) T cells from SLE patients produce significant amounts of IL-17 and IFN-γ. Furthermore, $IL\text{-}17^+$ and DN T cells are found in renal biopsy specimens from LN patients. In our study, we have confirmed successfully the production of IL-17 in infiltrating T cells in the kidneys (glomeruli and interstitiums) of the LN patients at a single-cell level. This suggests that IL-17 may play an important role in the LN patients. It was reported

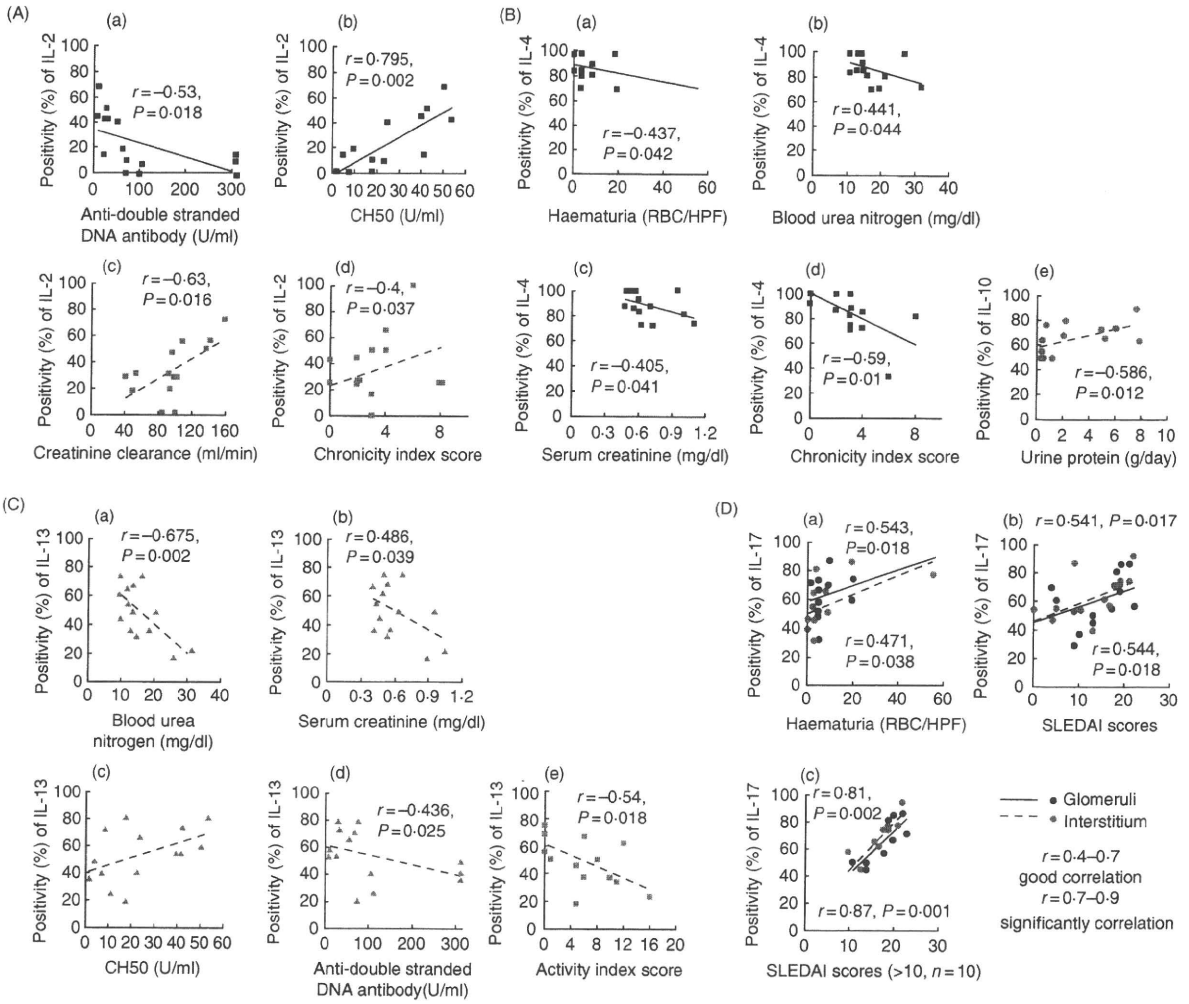

Fig. 3. Correlation between T helper type 1 (Th1), Th2 and Th17 cytokines and clinical and laboratory parameters in systemic lupus erythematosus (SLE). (A) Correlation between the levels of Th1 cytokine interleukin (IL)-2 and anti-double-strand (ds) DNA antibodies (a), 50% haemolytic unit of complement serum (CH50) (b), creatinine clearance (c) and chronicity index score (d) in glomeruli (black full line and points) and interstitium (black dashed line and grey points). (B) Correlation between the levels of Th2 cytokines–IL-4 and haematuria (a), blood urea nitrogen (b), serum creatinine (c) and chronicity index score (d) in glomeruli (black full line and points). Correlation between the levels of IL-10 and urine protein in interstitium (black dashed line and grey points). (C) Correlation between the levels of IL-13 and blood urea nitrogen (a), serum creatinine (b), 50% haemolytic unit of complement serum (CH50) (c), anti-ds DNA antibodies (d) and Activity Index scores (e) in interstitium. (D) Correlation between the levels of Th17 cytokine (IL-17), haematuria (a) and SLE Disease Activity Index (SLEDAI) scores (b) in glomeruli (black full line and points) and interstitium (black dashed line and grey points). Correlation between the level of IL-17 and SLEDAI scores (> 10) in glomeruli and interstitium (c). A simple linear regression analysis was used to evaluate the correlation between the two parameters, $P < 0{\cdot}05$.

that cyclosporine A might inhibit the production of IL-17 in the healthy control and RA patient groups [34]. Cyclosporine A also inhibits IL-15-induced IL-17 production in the $CD4^+$ T cells through down-regulation of PI3K/Akt and nuclear factor-kappa B (NF-κB) [35]. Inhibition of IL-15-induced IL-17 production by tacrolimus was also observed in $CD4^+$ T cells [35]. It may be considered that the inhibition of IL-17 is an important mechanism of the efficacy of these two kinds of calcineurin inhibitors in the steroid-resistant LN patients.

To confirm cytokine production in the kidney by RT–PCR, we conducted immunohistochemical experiments. The production of IL-13 and IL-17 were also observed by immunohistochemistry. Stained IL-17-positive cells were observed not only in the glomeruli or interstitiums, but also in the tubular epitheliums of LN patients (Fig. 1). Crispin *et al.* [33] reported that IL-17-positive cells were found by immunofluorescence mainly in the tubule-interstitial zone, the area where cellular infiltration is mainly found. We made stains for IL-17-positive cells with anti-human IL-17 in

the specimens from MGA and MCNS patients; no IL-17-positive cells were observed (Fig. 1Ca, b). This has demonstrated that IL-17 may be produced preferentially in SLE patients. Matsumura *et al.* also found stained IL-17 in the tubular epitheliums of LN patients by immunohistochemistry (personal communication). Thus, production of IL-17 in the tubules was confirmed by the RT–PCR and LMD methods. We believe that the RT–PCR technique is more sensitive than immunohistochemistry and can be used for quantification of the production of each cytokine.

We analysed the correlation between the expression levels of Th1, Th2 and Th17 cytokines and clinical parameters. We found that the levels of IL-2, IL-4, IL-10, IL-13 and IL-17 have a correlation with some clinical and laboratory parameters (Fig. 3). A negative correlation was found between the level of IL-2 and haematuria, BUN, Cr, anti-ds DNA antibody and SLEDAI, except for Ccr, CH50 and CI. However, the IL-17 level was correlated positively with UP, haematuria, BUN, Cr, AI and SLEDAI, while correlating negatively with CI and Ccr (Fig. 3). These findings indicate that IL-2 and IL-17 play opposite roles in SLE development. It is suggested that IL-2 may play a role in protecting against SLE development, while IL-17 might have a reverse effect. Wong *et al.* [36] showed significant and positive correlations of plasma IL-17 concentrations with SLEDAI scores in the patients without renal disease. Yang *et al.* [37] showed that patients with active SLE (SLEDAI > 6) exhibit an increased proportion of Th17 cells in $CD3^{-}CD8^{-}$ T cells from PBMC compared with healthy individuals by flow cytometric analysis, and a significant positive correlation between the percentage of Th17 cells and the SLEDAI score. Doreau *et al.* [38] also found that the serum of patients with SLE had higher concentrations of IL-17 than did the serum of healthy people, and that IL-17 abundance correlated with the disease severity of SLE. In our study, the level of IL-17 correlated positively and significantly with SLEDAI scores both in the glomeruli and the interstitiums. A highly significant correlation was observed between SLEDAI scores and the level of IL-17 in both the glomeruli and the interstitiums of active SLE patients (SLEDAI > 10) (Fig. 3D). We also found that the level of IL-17 has positive correlations with AI and negative correlations with CI in both glomerulus and interstitium, although correlations were weak (Table 4). This suggests that IL-17 may play an important role in the inflammatory process of a renal disease during the acute phase of SLE patients. With few IFN-γ-positive samples, we did not analyse the correlation between IFN-γ and the clinical and laboratory parameters. IFN-γ was observed only in the glomeruli of ISN/RPS Class III-predominant and Class V groups; accordingly, IFN-γ might play a role in protecting against the inflammatory process in LN patients, as with IL-2. The IL-2 level correlatates good positively with CI, suggesting that IL-2 might act during the chronic stage of glomerulonephritis (Fig. 3A and Table 4). Nakae *et al.* [39] found that IL-17 can suppress Th1 cell differentiation in the presence of exogenous IL-12 *in vitro*, and IFN-γ can down-regulate Th17 cell differentiation. Not only IFN-γ but also IL-4 can suppress IL-17 production *in vitro* [40,41]. Chu *et al.* [42] demonstrated further that IFN-γ might regulate susceptibility to CIA through suppression of IL-17, and IFN-γ and IL-4 together had a synergistic effect on suppression of type II collagen (CII)-specific IL-17 production during CII restimulation *in vitro*. This might be the reason why the expression levels of IFN-γ and IL-4 were higher in the ISN/RPS Class III-predominant group than those of other classes, whereas that of IL-17 was lower. Th2 cytokine showed inconsistent results, but it seems likely that IL-13 plays a protective role in lupus nephritis (Fig. 3C, Table 4).

In conclusion, we have shown that the glomerular infiltrating T cells might act as Th1, Th2 and Th17 cells, while the interstitial infiltrating T cells, as Th2 and Th17 cells in the Class III-predominant and Class V groups. In contrast, both the glomerular and interstitial infiltrating T cells might act as Th2 and Th17 cells in the Class IV-predominant group. The cytokine balances may be dependent on the classification of renal pathology and IL-17 might play a critical role in SLE development.

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Disclosure

None of the authors have any conflict of interest with the subject matter or materials discussed in the manuscript.

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