Peripheral Blood Mononuclear Cells

Notch Signaling Pathways in Human

Inhibition of IL-5 through Type I IFN and

TLR7 Stimulation of APCs Results in

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TLR7 Stimulation of APCs Results in Inhibition of IL-5
through Type I IFN and Notch Signaling Pathways in Human Peripheral Blood Mononuclear Cells

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TLR7 agonists modulate Th2 immune responses through mechanisms that have not been fully elucidated. Suppression of IL-5 production from Ag- or phytohemagglutinin-stimulated human PBMCs by the TLR7 antedrug AZ12441970 was mediated via type I IFN–dependent and type I IFN–independent mechanisms through TLR7 activation of plasmacytoid dendritic cells, B cells, and monocytes. The type I IFN–dependent inhibition of T cell–derived IL-5 was mediated by IFN-α acting directly on activated T cells. IL-10 was shown not to be involved in the type I IFN–independent inhibition of IL-5 and the mechanism of inhibition required cell–cell interaction. Notch signaling was implicated in the inhibition of IL-5, because addition of a γ-secretase inhibitor blocked the type I IFN–independent suppression of IL-5. Accordingly, AZ12441970 induced high levels of the notch ligands Dll1 and Dll4 mRNA, whereas immobilized DLL4 resulted in the suppression of IL-5 production. Therefore, we have elucidated two mechanisms whereby TLR7 agonists can modulate IL-5 production in human T cells. The suppression of Th2 cytokines, including IL-5, would be of benefit in diseases such as atopic asthma, so we assessed TLR7 function in PBMC from asthmatics and showed equivalent activity compared with healthy volunteers. Demonstrating this function is intact in asthmatics and knowing it links to suppression of Th2 cytokines support the case for developing such compounds for the treatment of allergic disease. The Journal of Immunology, 2013, 190: 000–000.

A nergic disease is associated with an immune deviation leading to an enhanced Th2 cell–mediated immune response (1), which is characterized by the cytokines IL-4, IL-5, and IL-13 that drive the allergic response (2). The global burden of allergic diseases such as asthma has continued to rise over the past decade (3). Epidemiological studies have suggested a link between allergic disease and reduced microbial exposure of children, leading to the “hygiene hypothesis” (4–6), whereas other studies have linked respiratory viral infections to a greater risk of the development of asthma and induction of exacerbations (7). These effects are brought about by microbes containing pathogen-associated molecular patterns that interact with TLRs expressed on cells of the innate immune system, resulting in production of cytokines and expression of costimulatory molecules that modulate the adaptive immune response (8).

Ten distinct TLRs have been identified (9) including TLR7, a receptor that has been demonstrated to recognize ssRNA (10, 11). Stimulation of TLR7 modulates many of the end points associated with allergy in disease models including suppression of goblet cell hyperplasia, reduction in Th2 cytokines, lung eosinophilia, lung inflammation, and Ag-specific IgE (12–18). These data strongly support the concept of developing a TLR7 agonist to treat allergic disease, although current TLR7 agonists, used clinically in nonallergic indications, have side effects. These include influenza-like symptoms and lymphopenia and are linked to levels of compound in the plasma and the associated cytokine induction (19–21).

In a bid to overcome this issue, AstraZeneca, in collaboration with Dainippon Sumitomo, has developed a series of 8-oxoadenine TLR7 antedrugs designed to be rapidly metabolized to a less active form upon entry into the circulation (16, 22). Although these compounds have short-term lung exposure, it is sufficient to preferentially activate the IFN pathway leading to the production of IFN-α (22). TLR7 agonists have been shown to reduce Th2 cytokine production in vitro (23–25) and have shown efficacy in animal models of allergy in vivo (13–15, 17, 18, 22, 26).

A number of mediators have been implicated in Th2 cytokine suppression by TLR7 agonists including IFN-α, IFN-γ, IL-10, IL-12 (15, 18, 23, 25), and notch ligands (27, 28). We have used a selective TLR7 agonist, AZ12441970 (22), to further investigate the mechanisms resulting in suppression of IL-5, a representative Th2 cytokine, in vitro human cellular assays. In both Ag-activated and polyclonally stimulated PBMC, IFN-α–dependent and IFN-α–independent pathways were elucidated. Plasmacytoid dendritic cells (pDCs), monocytes, and B cells were all individually able to mediate suppression of IL-5 production by a TLR7 agonist. IFN-α mediated its inhibitory effect directly on T cells and the IFN-α–independent suppression pathway required cell–cell contact. AZ12441970 effectively induced the notch ligand DLL4, which suppressed the production of IL-5. The ability of TLR7 agonists to work through multiple APC leading to type I IFN and notch signaling pathway–dependent suppression of IL-5...
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production enhances the understanding of how these compounds may provide therapeutic benefit in allergic disorders such as asthma. We have further confirmed that TLR7 induction of IFN-α from PBMC of asthmatics is not significantly different to production from nonasthmatics, further supporting the potential utility of this class of compound in the treatment of such allergic diseases.

Materials and Methods

Reagents

Methyl2-(3-((6-amino-2-butoxy-8-oxo-7H-purin-9(8H)-yl)propyl)(3-(dimethylamino)propyl)amino)phenyl)acetate (AZ12441970) was synthesized in the Medicinal Chemistry Facilities, AstraZeneca Research and Development Charnwood (Loughborough, Leicestershire, U.K.), and Soluprick SQ Der p 1 was purchased from ALK Abello (Hungerford, Berkshire, U.K.). Human TLR7 Ribogreen RNA assay kit, GATA3 Thorpe primers, and Dynabeads Human T Activator CD3/CD28 beads were purchased from Invitrogen Life Technologies (Paisley, U.K.). Miltenyi kits were purchased from Miltenyi Biotec (Bisley, Surrey, U.K.). Human IFN-α ELISA, IL-4, anti-IL-12 (IgG1), anti–IFN-α (IgG1), anti–IL-10 (IgG2b), DLL1, and DLL4 were purchased from R&D Systems (Abingdon, U.K.). Anti–IFN-α/βR (IgG1) was from AstraZeneca. Anti-CD28, anti-CD3, human TNF-α ELISA, and human IFN-γ ELISA were purchased from BD Biosciences (Oxford, U.K.). Costar Transwell plates were purchased from Fisher Scientific (Loughborough, Leicestershire, U.K.). RNeasy lysis buffer (RLT), RNeasy 96 kit, and QuantiTect Probe RT-PCR kit were purchased from Qiagen (Crawley, U.K.). Dibenazepine was purchased from Merck Chemicals (Nottingham, U.K.). Phytomenegglutinin (PHA) was purchased from Sigma-Aldrich (Dorset, U.K.). Aneuractin was made up of RPMI 1640 medium with 25 mM HEPES, 10% FCS (v/v), 2 mM l-glutamine, 10 U/ml penicillin, and 10 μg/ml streptomycin.

PBMC preparations

Blood was collected from healthy, consenting volunteers or from consenting atopic individuals who were skin prick positive for the house dust allergen Der p 1. In the study comparing mild asthma and healthy volunteers, blood was collected by Parexel in accordance with the ethical principles in the Declaration of Helsinki, Good Clinical Practice, and applicable regulatory requirements. Mild allergic asthma, but otherwise healthy, men or women aged 18–65 y were included if they had an FEV1 of at least 70% of predicted (prebronchodilator) and an FEV1/FVC ratio >70% and had not been using any asthma medication 4 wk prior to the visit. Subjects were confirmed as being atopic by a positive reaction to at least one allergen in a skin prick test at visit 1. Donor age, sex, and atopic status are shown in Supplemental Table I. Blood from healthy, consenting volunteers or from healthy, consenting individuals who were skin prick positive for the house dust allergen Der p 1 was obtained from Applied Biosystems (catalog number Hs00231122_m1), and others were designed using Applied Biosystems Primer Designer software, and the details are in Supplemental Table II. Normalization was carried out using the Quant-iT Ribogreen RNA assay kit, with readings measured using the Spectramax M5 plate reader. Relative normalized expression was determined.

Results

IFN-α dependence of TLR7-mediated suppression of IL-5

Incubations where cells were reconstituted used cells at the following concentrations: T cells at 150,000–200,000 cells/well; B cells at 80,000–100,000 cells/well; monocytes at 80,000–100,000 cells/well, and pDC at 10,000–20,000 cells/well.

Compound dilutions

For all assays, test compounds were made up in DMSO and serially diluted in DMSO to concentrations 1000-fold greater than the final incubation concentration. Stocks were diluted 100-fold into medium to generate 10X stock concentrations. DMSO vehicle controls were similarly prepared.

Stimulation with plate-bound CD3/CD28

Anti-CD3 (2 μg/ml) and anti-CD28 (0.5 μg/ml) were made up in PBS and 300 μl/well added to a 24-well tissue culture plate and incubated at 37°C for 2 h. The solution was then removed, and each well was washed with 400 μl PBS before addition of cells in medium to the wells.

Stimulation with immobilized notch ligands

DLL1 or DLL4 was diluted in PBS to 5 μg/ml. Seventy microliters was added per well of a 96-well plate and incubated at 37°C for 2 h. The solution was removed, the well was washed with 200 μl PBS, and cells and additions were then made to the well.

Transwell experiment

Costar 6.5 mm Transwell plates with 0.4-μm Pore Polycarbonate Membrane (catalog number 3413) had 600,000 T cells placed in the lower chamber. PBMC depleted of T cells (300,000 cells) were placed, where stipulated, in either the upper or lower chamber. T cells were stimulated by addition of 150,000 CD3/CD28 beads in the lower chamber. Compound or vehicle was added in a 10th of the final concentration to both upper and lower chambers. Neutralizing type I IFN Abs were included in all wells, and incubations were for a period of 2 d.

TagMan analysis

Cells were lysed in RLT buffer, and RNA was extracted using RNeasy 96 kit as described in the manufacturer’s protocol, including the optional on-column DNAse digestion. RNA was diluted 1/5 for TaqMan reactions, using the QuantiTect Probe RT-PCR kit, according to the manufacturer’s protocol. A total reaction volume of 25 μl was used and run on the Stratagene MX3000P instrument. Primer and probe sequences for GATA3 were obtained from Applied Biosystems (catalog number Hs00173021_m1), and others were designed using Applied Biosystems Primer Designer software, and the details are shown in Supplemental Table II. Normalization was carried out using the Quant-iT Ribogreen RNA assay kit, with readings measured using the Spectramax M5 plate reader. Relative normalized expression was determined.

At concentrations above 2 nM, the TLR7 agonist AZ12441970 induced IFN-α from human PBMC with a bell-shaped dose–response curve (Fig. 1A). The bell-shaped curve resulted from differences in the rates of production of IFN-α (results not shown). Other studies in our laboratory have shown that initially, as expected, the higher concentrations of AZ12441970 induced more IFN-α. However, this production was terminated after 4–6 h, whereas the production of IFN-α induced by lower concentrations of AZ12441970 was continued for longer. As a result, at the time point used, more IFN-α has accumulated at the lower concentrations of agonist, thereby generating a bell-shaped dose–response curve. We have no evidence to attribute the bell-shaped dose–response curve to cytotoxicity. AZ12441970 inhibited IL-5 production from PHA-stimulated PBMC with an IC50 of 2 nM (Fig. 1B). Induction of IL-4 was often below the limit of detection, so this cytokine could not be accurately quantified (results not shown).

Previously, we had shown a direct correlation in the inhibition of IL-5 and IL-13 by TLR7 agonists (22), so in these studies, we focused on IL-5. Because IFN-α mediated the suppression of IL-5 (Fig. 1C), the similarity between the concentrations of AZ12441970
required to induce IFN-α and suppress IL-5 production indicated a possible causal relationship. To test this, conditions were established that specifically blocked the effect of type I IFN signaling on IL-5 production using blocking/neutralizing Abs to IFN-α and IFN-α/βR (Fig. 1C). Whereas the suppression of IL-5 by IFN-α was completely blocked, there was only a small shift in the IC₅₀ for AZ12441970 from 2 nM, as stated above, to 5 nM when neutralizing Abs were included (Fig. 1B). Isotype controls had no effect on the inhibition of IL-5 by AZ12441970 (results not shown). These data highlighted that although IFN-α inhibited IL-5 production there were other mechanisms by which a TLR7 agonist suppressed the production of IL-5.

PHA stimulation of PBMC to induce IL-5 may represent a stimulus that is significantly stronger than a physiological one. Therefore, we also assessed the role of type I IFNs in the inhibition of IL-5 induced by Ag presentation to T cells in PBMC derived from atopic individuals. IFN-α dose-dependently inhibited the production of IL-5 from PBMC derived from house dust mite-sensitive donors, using the Ag Der p 1 (Fig. 2A). AZ12441970 inhibited Der p 1–induced IL-5 production (Fig. 2B) with an IC₅₀ of 4 nM, similar to the potency observed with PHA-stimulated PBMC. Inclusion of neutralizing Abs to type I IFNs again had little effect on suppression of IL-5 by AZ12441970, with the IC₅₀ shifting to 14 nM. These data showed that irrespective of the mode of stimulation both type I IFN–dependent and –independent pathways were operating.

**FIGURE 1.** IFN-α dependence of PHA-induced IL-5 production from PBMC. (A) Human PBMC were stimulated with AZ12441970 for 20 h, and IFN-α levels in the medium were determined. Data are from a single experiment performed in triplicate representative of determinations from five different donors. (B) Dose–response curve of effect of AZ12441970 on IL-5 production from PHA-stimulated PBMC in the absence (○) or presence (●) of 10 μg/ml anti–IFN-α + 1 μg/ml anti–IFN-α/βR Abs. Mean ± SEM from individual experiments performed on four different donors. (C) Suppression of IL-5 by 100 nM AZ12441970 or 5000 U/ml IFN-α in PHA-stimulated PBMC was assessed in the absence or presence of neutralizing anti–IFN-α + anti–IFN-α/βR Abs. Data from a single experiment performed in triplicate representative of determinations from three different donors. *p < 0.01; statistical analysis by Student paired t test compared with control or for paired results shown with the horizontal bar.

**FIGURE 2.** IFN-α dependence of Der p 1–induced IL-5 production from PBMC. (A) Human PBMC were stimulated with the Ag, Der p 1, in the presence of IFN-α, and the suppression of IL-5 was determined. Mean ± SEM from three individual donors. (B) Dose–response curve of effect of AZ12441970 on Der p 1–stimulated IL-5 production from PBMC in the absence (●) or presence (○) of neutralizing anti–IFN-α + anti–IFN-α/βR Abs. Mean ± range from two experiments performed in triplicate from PBMC derived from different donors.

**FIGURE 3.** Inhibition of IL-5 production from purified APC and T cells stimulated with Der p 1. Purified cellular subsets derived from PBMC were incubated with purified T cells and stimulated by addition of Der p 1. AZ12441970 was included at a concentration of 100 nM, and IFN-α was present at 5000 U/ml. IL-5 was assayed after 7-d incubation, and result is mean ± SEM from PBMC derived from a minimum of three different donors.

**IFN-α mediates suppression of IL-5 by acting directly on T cells**

We investigated which cell types might be mediating suppression of IL-5 by incubating purified APCs with purified T cells and stimulating them with Der p 1 (Fig. 3). Inhibition of IL-5 by AZ12441970 was observed when pDC, B cells, or monocytes were used as the sole source of APC. In the above experiments, suppression of IL-5 production by IFN-α was observed irrespective of the type of purified APC used (Fig. 3). From these experiments, it was not possible to deduce whether IFN-α acted on the APC and/or the T cells to bring about this reduction. To identify which cell type was being modulated by IFN-α, the intention was to treat APC or T cells separately with IFN-α and then to wash the cells to remove the IFN-α before recombining the cells with their untreated counterparts to see which combination supported suppression of IL-5. To determine whether this was possible, PBMC were treated with IFN-α for 24 h, before washing the cells thoroughly, subsequent to stimulation with PHA (Supplemental Fig. 1). Under these conditions, prior exposure to IFN-α had no long-term suppressive effect on IL-5 production, although inclusion of IFN-α after the wash step, along with addition of PHA, resulted in suppression of IL-5.

Having identified the requirement for activation of T cells to observe an effect of IFN-α, purified T cells were activated by plate-bound CD3/CD28 Abs in the absence or presence of IFN-α. In addition, PBMC depleted of T cells (termed APC) were also incubated with or without IFN-α for 24 h. At the end of this period, cells
were washed extensively, and APCs and T cells were incubated in fresh wells together with PHA as the stimulus (Fig. 4). T cells preincubated with IFN-α suppressed IL-5 production, whereas pretreatment of APC with IFN-α did not. In all instances, reintroduction of IFN-α to the cells when they were recombined resulted in suppression of IL-5. These data identified that IFN-α acted on activated T cells resulting in suppression of the production of IL-5.

Type I IFN–independent inhibition is not mediated by IL-10 and requires cell–cell contact

To investigate the type I IFN–independent mechanism of IL-5 inhibition, we investigated the role of the anti-inflammatory cytokine IL-10. AZ12441970 induced the production of IL-10 from purified B cells and monocytes (Fig. 5A), making it a possible candidate in the suppression of IL-5. Exogenous IL-10 effectively suppressed the production of IL-5 when purified monocytes (Fig. 5B) or purified B cells (Fig. 5C) presented Der p 1 to purified T cells. The inhibitory effect of added IL-10 was fully reversed by inclusion of a neutralizing anti–IL-10 Ab, whereas the Ab had no effect on the suppression by AZ12441970. Inclusion of an isotype control had no effect on the production or inhibition of IL-5. Therefore, although AZ12441970 induced IL-10, these data pointed to the role of a different mediator in the suppression of IL-5.

Role of DLL4 in mediating suppression of IL-5 following stimulation by AZ12441970

Neutralizing Abs to a range of cell surface markers including CD40, CD40L, CD80, and CD86 did not result in any reversal of the IFN-α–independent inhibition of IL-5 by AZ12441970 (results not shown). The potential for a role of the notch signaling pathway in the cell–cell-mediated suppression of IL-5 was investigated by treating cells with the γ-secretase inhibitor dibenzazepine. γ-Secretase is involved in downstream signaling from the notch receptors. In PHA-stimulated PBMC, in the presence of neutralizing IFN-α/βR Abs, dibenzazepine reversed the inhibition by AZ12441970 (Fig. 7A). In the absence of neutralizing IFN-α/βR Abs, no effect of the γ-secretase inhibitor was observed, showing its effects were masked by the type I IFN generated (results not shown). These data suggested a role for the notch pathway in mediating IFN-α–independent IL-5 suppression. Whereas dibenzazepine had no effect on IL-5 production induced by PHA alone, 1 μM dibenzazepine inhibited Der p 1-induced IL-5 production by 64 ± 18% (n = 4; data not shown). This difference suggested a possible role for notch signaling in the production of IL-5 by Ag but made it difficult to look at effects of AZ12441970 on top of this. Therefore, PHA was used as the stimulus in subsequent studies.

The data with dibenzazepine did not conclusively prove there was an actual involvement of the notch pathway, given that selectivity data in a cellular context for this compound was not available, and it was possible that other pathways might be linked to γ-secretase–dependent processing. To establish whether AZ12441970 could modulate the notch pathway, its ability to induce mRNA for the notch ligands Jag1, Jag2, Dll1, and Dll4 was determined by TaqMan analysis. Jag2 was not detectable, so data are not included (also see Fig. 7C-E). AZ12441970 had minimal effects on induction of Jag1 and Dll1 in human PBMC though it gave a large induction of Dll4 (Fig. 7B). As inhibition of IL-5 by AZ12441970 was determined in the presence of PHA, the modulation of notch ligands with PHA was also assessed. Jag1, Dll1, and Dll4 were all induced, but when combined with AZ12441970, the only ligand showing a difference compared with PHA alone was Dll4, which showed an extensive increase in expression.

The dose dependence for induction of notch ligands in PBMC was assessed (Fig. 7C), and Dll4 was induced at concentrations of AZ12441970 above 1 nM, similar to the potency determined for inhibition of IL-5 (Fig. 1B). The dose dependency of the response in PBMC was the same from all donors tested, although the fold induction varied significantly from donor to donor. For Dll4, the maximum fold induction for individuals ranged from 20- to 6400-fold (results not shown). No induction of Jag1 or Jag2 was observed (Fig. 7C). Because pDC, monocytes, and B cells all mediate inhibition of IL-5 (Fig. 3), the induction of notch ligands in these cell types was assessed. In both monocytes (Fig. 7D) and pDC (Fig. 7E), AZ12441970 induced Dll4 mRNA levels over 1000-fold. Dll1 was poorly induced in monocytes and pDC, and there was a similar low level of induction of Jag1 but only at 1 μM AZ12441970. No induction of mRNA for any of the notch ligands was observed in B cells (results not shown). Thus, DLL4 looks to be the key candidate for modulation of IL-5. To confirm this, purified DLL1 and DLL4 were immobilized to the plastic surface of tissue culture plates, and PBMC were added with PHA as the stimulus (Fig. 7F). Under these conditions, DLL4 inhibited IL-5 production, which was completely reversed by the γ-secretase inhibitor dibenzazepine. Immobilized DLL1 had no effect on IL-5 production. To determine whether, apart from mediating a suppressive signal to inhibit production of the Th2 cytokine IL-5, it also had the ability to induce Th1 cytokines, we assessed the effect of immobilized DLL1 or DLL4 on induction of IFN-γ (Supplemental Fig. 2). DLL4 induced IFN-γ as did DLL1, although to a lesser extent, and the effects were inhibited by dibenzazepine.

In the literature, notch signaling has been linked to changes in Th1/Th2 phenotypes through upregulation of the transcription
and is representative of results from determinations in cells from three different donors. (C) Included to determine its effects on suppression by AZ12441970 or exogenously added IL-10. The result is from a single experiment performed in triplicate and is representative of results from determinations in cells from three different donors. (C) Identical conditions to (B), although using purified B cells in place of monocytes.

Factors Tbet and GATA3 (27, 29–31). We determined effects on the expression of the Th2 transcription factor Gata3 and the Th1 transcription factor Tbet in the presence of blocking type I IFN Abs (Fig. 8). PHA or AZ12441970 induced small but significant increases in Gata3 mRNA, and the PHA-induced increase was reduced by addition of AZ12441970. AZ12441970 had no effect on induction of Tbet, whereas PHA induced Tbet, and this induction was not affected by the inclusion of AZ12441970.

IFN-α induction by TLR7 agonists is intact in PBMC from asthmatic subjects

The characteristics of this class of compound give them potential for the treatment of allergic disease such as atopic asthma. Because IFN-α is important in the mechanism of suppression of the Th2 cytokine IL-5, we assessed TLR7 function in PBMC derived from asthmatic subjects and healthy volunteers (Fig. 9). Using the TLR7 agonists AZ12441970 and R848, we demonstrated that the ability of these compounds to induce IFN-α was not significantly different whether the PBMC were derived from asthmatics or healthy volunteers, thus providing confidence that they could have utility in the treatment of allergic asthma.

Discussion

Previously, we showed that AZ12441970, a selective TLR7 agonist, suppressed the production of IL-5 in human and murine assays (22). In that initial study, IFN-α also inhibited the production of IL-5, and given that TLR7 agonists are known inducers of IFN-α, we have now investigated the role IFN-α plays in the suppression of IL-5 production by AZ12441970. Although AZ12441970-induced IFN-α from PBMC, and the dose response of IFN-α induction correlated with the potency of IL-5 inhibition, the inclusion of Abs to block type I IFN signaling revealed that mechanisms apart from type I IFN signaling were involved. This was true in both PHA- and Ag-stimulated PBMC, showing the effect was independent of the mode of T cell activation. The use of different methodologies to stimulate the T cells to produce IL-5 was necessitated by the cellular needs in the systems being tested. T cells require APC when being stimulated by Ag or PHA, whereas isolated T cells were stimulated with anti-CD3/CD28 to compensate for the lack of APCs.

The ability of type I IFNs to suppress IL-5 production has been reported using IFN-α with polyclonally stimulated human CD4+ T cells (32–34) or IFN-β on Ag-stimulated PBMC (35). Others have investigated the role of IFN-α in the suppression of IL-5 by TLR7 agonists in murine studies and have shown that neutralizing IFN-α/β Abs reduced the inhibition of IL-5 by R848 (23). In human cells, R848 or SA2 redirected the differentiation of Ag-specific Th2 cell lines toward a Th1/Th0 phenotype, and a mixture of neutralizing Abs to IL-12, IFN-α, and IFN-γ cells was required to block this effect of SA2 (25). These data implied that IFN-α may be one of a number of TLR7-induced soluble mediators that regulate the production of Th2 cytokines, and our data also points to the role of mediators apart from type I IFNs.

We eliminated IL-10 as one of these mediators as, although AZ12441970 induced IL-10 from purified B cells and monocytes, IL-10 was an efficacious suppressor of Ag induced IL-5, when IL-10 was blocked with neutralizing Abs it had no effect on suppression of IL-5 production by AZ12441970. IL-10 has been shown to inhibit Ag presentation from monocytes; it has inhibitory effects on APCs and inhibits IL-5 production from Th2 cells (reviewed in Ref. 36). We cannot fully rule out an effect of IL-10 as a compensatory mechanism may have masked this. IL-10 is produced over a number of days by a TLR7 agonist, in vitro, and may not have been produced at high enough levels, soon enough, to have had any effect on the Ag challenge. It remains possible that TLR7-induced IL-10 acting on APC could inhibit subsequent Ag-induced IL-5 production.

The extent of the role that soluble mediators played in the suppression of IL-5 was investigated by separating the activated T cells from the APC in Transwell chambers. AZ12441970 inhibited the production of IL-5 and this inhibition was fully reversed by neutralizing the type I IFN signaling. We concluded that under these conditions IFN-α was the only soluble mediator.
having a suppressive effect on IL-5 production and other suppressive mechanisms were cell contact dependent.

TLR7 antedrugs were rapidly metabolized in vivo (16, 22) and short-term exposure of a TLR7 agonist was sufficient to induce IFN-\(\alpha\) (22). Therefore, if the efficacy of an antedrug is linked to its ability to induce IFN-\(\alpha\), which itself has a short biological \(t_{1/2}\), then IFN-\(\alpha\) has to induce effects that outlive its pharmacokinetic exposure. We were unaware of any investigation in the literature that addresses this, so initially we incubated PBMC with IFN-\(\alpha\) for a period of 24 h and, after washing away the IFN-\(\alpha\), found no evidence of a suppressive effect on IL-5 production. Given that readdition of IFN-\(\alpha\) resulted in suppression of IL-5, it was apparent that the cells had not become desensitized to IFN-\(\alpha\). Further investigation identified that activation of T cells was necessary for IFN-\(\alpha\) to have a suppressive effect on IL-5 production. These data therefore suggest that if a TLR7 agonist could be dosed to increase IFN-\(\alpha\) transiently, then suppression of Th2 cytokine production from activated T cells by the IFN-\(\alpha\) would be possible. Verification of the benefits of such a mechanism will come from testing such compounds in clinical trials. Demonstrating that TLR7 stimulation of PBMC derived from asthmatics induced IFN-\(\alpha\) showed that, at least peripherally, TLR7 was functional in such patients, giving further confidence to test such compounds in man.

We investigated which cell types mediated TLR7-dependent inhibition of IL-5 production. TLR7 is functional on pDC, monocytes, and B cells (37, 38), and each of these individually mediated inhibition in Ag-stimulated assays with purified T cells. Whereas pDC have been identified as the cellular source of TLR7 induced

![FIGURE 7. Role of notch signaling in IFN-\(\alpha\)-independent suppression of IL-5. In all these experiments, neutralizing type I IFN Abs were present. (A) PBMCs, stimulated with PHA, were incubated with or without 30 nM AZ12441970. The level of inhibition compared with vehicle (0.2% DMSO) control was determined, and the effect of dibenzazepine on the inhibition induced by AZ12441970 was determined. Data are mean ± SEM from determinations using three different donors. *p < 0.05; statistical analysis by Student paired t test compared with relevant control. (B) PBMCs were stimulated with PHA in the presence or absence of AZ12441970. Cells were quenched in RLT, RNA was extracted, and levels of notch ligand mRNA were quantified by TaqMan. Levels were calculated as fold change over the level in unstimulated cells. Data are the mean ± SEM from determinations using four different donors. (C) PBMC were incubated with a range of concentrations of AZ12441970, and induction of notch ligand mRNA was determined after 24 h. Data are mean ± SEM from determinations using a minimum of five different donors. (D) Induction of notch ligand mRNA by purified monocytes stimulated by AZ12441970 for 24 h. (E) Induction of notch ligand mRNA by purified pDC stimulated by AZ12441970 for 24 h. (F) Purified DLL1 and DLL4 were adsorbed to tissue culture wells to determine their effect on IL-5 production from PHA-stimulated PBMC. The result is mean ± SEM from determinations using a minimum of four different donors.](http://www.jimmunol.org/)

![FIGURE 8. Effect of AZ12441970 on GATA3 and Tbet induction. PBMC were stimulated with AZ12441970 or PHA in the presence of blocking IFN Abs, and the level of Gata3 or Tbet was determined by TaqMan after 24 h incubation. Data are mean ± SEM from determinations from four different donors. *p < 0.05; statistical analysis by Student paired t test compared with medium control or between indicated pairs of data.](http://www.jimmunol.org/)

![FIGURE 9. IFN-\(\alpha\) induction by AZ12441970 in PBMC derived from asthmatics. PBMC were prepared from the blood of healthy volunteers (\(n = 16\) individuals) and asthmatic (\(n = 16\) individuals) patients and stimulated with vehicle (control), 100 nM AZ12441970, or 1000 nM R848 for 20 h. IFN-\(\alpha\) was determined by ELISA. A Wilcoxon test analysis of the induced IFN-\(\alpha\) production (basal level subtracted) for asthmatics compared to nonasthmatic subjects was conducted (no significant difference for either compound treatment was observed).](http://www.jimmunol.org/)
IFN-α in PBMC (37), TLR7 stimulation of purified monocytes or B cells did not induce IFN-α (results not shown). Because these cells supported TLR7-mediated inhibition of IL-5, this provides further evidence for a type I IFN–independent mechanism of IL-5 inhibition.

Addition of IFN-α resulted in inhibition of IL-5 production when monocytes and B cells were used as APC, and it was likely this was mediated by a direct effect of IFN-α on T cells (32–34). This was confirmed by showing that IFN-α pretreatment of activated T cells suppressed IL-5 production, whereas IFN-α–pretreated APC did not.

Reduction of the level of IL-5 inhibition with a γ-secretase inhibitor provided evidence for a possible role of the notch signaling pathway in the inhibition of IL-5 by a TLR7 agonist. The notch pathway is involved in developmental choices and differentiation of the hematolymphoid system (39). In mammalian cells, there are four notch receptors (notch 1–4) and five notch ligands (jagged1, jagged2, delta1, delta3, and delta4). Upon notch–ligand/receptor interaction, the intracellular domain of notch is cleaved by γ-secretase, it then translocates to the nucleus where it acts as a transcription factor. In mice, jagged ligands are associated with induction of Th2 responses, whereas DLL ligands are linked to Th1 responses (29). Stimulation of PBMC with PHA induced Jag1 and Dll4 with a very low induction of Dll1. It was possible the increase in Jag1 was driving the increased production of IL-5. However, AZ12441970 suppressed IL-5 production without any effect on PHA induced Jag1 or Dll1. Of note was the large increase in Dll4 expression on top of that induced by PHA, implicating this particular notch ligand as having a role in the suppression of IL-5. AZ12441970 dose-dependently induced Dll1 and Dll4 in PBMC, and the dose–response curve mirrored the inhibition of IL-5. Dll4 was strongly induced in pDC and monocytes, and Dll1 was also induced but with much lower levels of induction. Interestingly, we found no evidence of notch ligand induction in B cells. Immobilized DLL4 modulated IL-5 production, and the reversal of this effect with the γ-secretase inhibitor confirmed the role of this pathway. The role of DLL4 in modulating Th2 cytokine production is well described. In murine lymph node cells stimulated with immobilized DLL4 ligand, Th2 cytokine production was suppressed (40), whereas immobilized DLL4 reduced IL-4 and IL-13 production from T cells restimulated with respiratory syncytial virus (41). Blocking of DLL4 in animal models resulted in increased Th2 cytokine production and lead to exacerbated allergic lung disease in an in vivo model (40) and increased airway hyperresponsiveness (41).

TLR ligands have been shown to modulate DLL4 expression. Activation of TLR2 and TLR9 increased DLL4 expression in mice (31) and this was associated with reduced IL-4 production. Human immature monocyte–derived dendritic cells (DC) showed little induction of Dll4 with R848 and synergistic activation with TLR3 or TLR4 was required to increase Dll4 mRNA expression 100-fold (28). Maturation of DC by LPS or R848 resulted in a shift of polarizing activity from Th2 to Th1 and was associated with an increase of DLL4 and a decrease in jagged1 (27). Silencing of DLL4 in R848-matured DC by means of a specific small interfering RNA significantly reduced the ability of the DC to polarize T cells to a Th1 phenotype. The effects of R848 could have been attributed to its TLR7 or TLR8 activity (42). Using AZ12441970, which is devoid of TLR8 activity (22), we have been able to demonstrate that TLR7 is linked to increases in DLL4 that are associated with downregulation of the Th2 phenotype, as shown by reduction of IL-5, and upregulation of Th1 responses, as shown by induction of IFN-γ.

Previous studies have shown that DLL4 on pDCs in mice activated T cells to produce IL-10 (43). In initial studies, we did not observe induction of IL-10 by immobilized DLL4 in our human cellular assays (results not shown), but it is clearly an area of interest because it could potentially link to the generation of regulatory T cells that are induced by R848 in the mouse (44). The mechanism by which B cells inhibited IL-5 is currently unknown, because TLR7 stimulation of B cells did not produce IFN-α or upregulate Dll4, and the IL-10 produced was not implicated in the inhibition of IL-5 in our assays. We therefore conclude a further, and as yet undefined, mechanism is induced by AZ12441970 in B cells, which results in suppression of IL-5.

AZ12441970 induction of Dll4, inhibition of IL-5, and production of IFN-α all occurred with similar dose dependencies. Blockade of the type I IFN signaling resulted in only a 2- to 3-fold reduction in the IC50 for IL-5 inhibition, which suggested that at the lower concentrations of AZ12441970, the suppression of IL-5 was mediated predominantly by IFN-α, whereas at higher concentrations suppression by both IFN-α and DLL4 was possible. It remains a possibility that IFN-α could mediate its effects through induction of Dll4, but because exposure of PBMC to IFN-α for 24 h prior to stimulation with PHA did not result in any reduction in IL-5 production, then this suggests that IFN-α treatment does not result in sustained upregulation of Dll4.

We thought it was possible that regulation of IL-5 production might be mediated by the transcription factors Tbet and GATA3, which are linked to Th1 and Th2 responses, respectively. Suppression of Th2 responses by IFN-α was associated with enhanced Tbet expression with no effect on the level of Gata3 (33). In other studies, IFN-α induced the loss of Gata3 expression with no effect on Tbet levels (34). R848 reduced the expression of Gata3 (25) or led to a reduction in the Gata3/Tbet ratio (27). Overall, an increase in the Tbet/Gata3 ratio appears to be linked to a suppression of a Th2 response. Interestingly, PHA induced a large fold increase in Tbet with a very modest increase in Gata3, yet despite the increase in a Th1 transcription factor, IL-5 was still induced. AZ12441970 had no effect on the PHA-induced increase in Tbet, although it reduced the increase in PHA-induced Gata3. Given that AZ12441970 alone induced Gata3, the data suggest that, when type I IFN was blocked, modulation of IL-5 did not correlate to overall changes in Tbet and Gata3. Further investigation of the role of Gata3/Tbet in the cytokine producing cells is needed.

Using a selective TLR7 agonist, we have demonstrated that TLR7 can modulate IL-5 production through a number of mechanisms and via a number of cell types. pDC activation resulted in production of IFN-α, which acted on activated T cells to suppress IL-5 production. Induction of Dll4 in monocytes and pDC and subsequent activation of the notch signaling pathway lead to a further mechanism to reduce IL-5 production. Production of IL-10 from B cells and monocytes had the potential to reduce Ag presentation by APCs, whereas B cells also mediated inhibition by an as yet undefined mechanism. Given these properties and data showing that TLR7 function is retained in asthmatic PBMC, TLR7 agonists are good candidates to reverse the Th2 predisposition in allergic disease. However, although the hygiene hypothesis supports a role for microbes, via their activation of innate immune cells, in the prevention of asthma, it is also noted that viral respiratory infections increase the risk of asthma or exacerbations (7). The differences may be down to the route of exposure or the types of microorganisms involved. Although a virus may activate TLR7, any beneficial effect of activating TLR7 could be lost through stimulation of other TLRs (45). Indeed, an inhaled TLR3 ligand has been shown to induce allergic airway disease, whereas a TLR7–selective agonist in the mouse, R848, had no such effect (46). In addition, viruses stimulate a broader range of cells, including epithelial cells, cause damage by viral replication and provide a longer...
duration of stimulus than a TLR7 agonist. Asthmatics may be more prone to respiratory viral infection because they have a reduced innate immune to viruses through reduced IFN production (45). A TLR7 agonist by inducing type I and type II IFNs may actually prove beneficial in this process. The clarification of whether TLR7 antagonists provide clinically significant benefits to patients will ultimately be revealed via clinical trials.

Acknowledgments

We thank the work of Mike Dyvdon in providing the statistical analysis of patient IFN-α production.

Disclosures


References

**Supplementary Table 1: Donor characteristics**

The table displays the characteristics of the donors used for the evaluation of TLR7 induction of IFNα. Details of age, gender, asthmatic status and skin prick test reactivity are given.

**Supplementary Table 2: Primer and probe sequences used for Taqman**

Primer and probe sequences were designed using Applied Biosystems Primer Designer software and the sequence details are detailed below.

**Supplementary Figure 1: Pre-incubation of PBMC with IFNα has no effect on subsequent PHA stimulated IL-5 production**

PBMCs were incubated with or without IFNα for 1 d before washing the cells and splitting each incubation and stimulating with either PHA alone or with PHA + IFNα before incubating for a further 2 d. Data from 4 experiments with cells from different individual donors showing mean ± SEM.

**Supplementary Figure 2: IFNγ induction by immobilized notch ligands**

Purified DLL1 and DLL4 were adsorbed to tissue culture wells to determine their effect on IFNγ production from PBMC in the presence of antibodies to neutralize IFNα/βR signaling. Result is mean ± SEM from a minimum of 3 determinations.
Supplemental Figure 1

PBMC ± IFNα → Wash 3X → PHA ± IFNα → 20 hr → 2 days → harvest

IL-5 (% inhibition)

20 hr pre-incubation conditions
- control
- 1 μg/ml IFNα

no further addition after wash

IFNα added after wash
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**Range:** 19 - 60  
**Median:** 26.5  
**Mean:** 32.2

**Asthmatic subjects**

**Range:** 25 - 46  
**Median:** 32  
**Mean:** 32.5

**HDM = house dust mite**
**Supplementary Table 2:**

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