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Interleukin 17 sustains rather than induces inflammation

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Abstract

Interleukin (IL)17 is a novel cytokine that has been suggested to play a key role in sustaining chronic inflammation in autoimmune diseases. Since its discovery, much attention has been given to mediators and factors responsible for the development of IL-17-producing cells while very few studies have investigated the inflammatory properties of this cytokine. Here we aimed to characterize the inflammatory properties of IL-17 and to establish if this cytokine *per se* can initiate an inflammatory reaction or if its main role is to contribute to the exacerbation of ongoing inflammation. To this aim we used two different mouse models of inflammation: the paw oedema and the airpouch. Interestingly, injection of IL-17 in the hind paw did not cause oedema while administration in the pre-inflamed tissues of 6-day-old air pouches induced a long lasting inflammatory reaction that was sustained between 4 and 24 h post-injection. Phenotypic analysis of cellular infiltrates demonstrated selective PMN recruitment in exudates and pouch lining tissues. This event was accompanied by induction of a distinct set of pro-inflammatory cytokines including IL-1 β , IL-6, TNF- α and chemokines KC and MCP-1. Co-administration of a neutralizing anti-KC antibody with IL-17 significantly reduced its inflammatory response suggesting a key role for this chemokine in mediating the inflammatory effects of IL-17. In conclusion these results demonstrate that IL-17 does not initiate an inflammatory reaction while, if injected in pre-inflamed tissues, is able to further amplify biochemical and cellular events characteristic of the early stages of the inflammatory reaction.

Keywords: Interleukin-17; inflammation; airpouch; paw oedema; Th17.

1. Introduction

CD4⁺ T cells play an important role in immune responses by coordinating the function and action of cells of the innate immune system such as macrophages and neutrophils. Upon antigenic stimulation naive CD4⁺ T cells expand and differentiate into different effector subsets termed T helper (Th)1, Th2 and Th17 [1, 2]. While Th1 and Th2 cells protect the host against intracellular and extracellular pathogens, respectively, Th17 cells have recently emerged as a specific subset of cells that significantly contribute to the development of autoimmune inflammatory diseases [3, 4].

Th17 cells were named after the cytokine they produce, IL-17, whose pleiotropic activities include the induction of proinflammatory cytokines TNF α , IL-1, and IL-6, as well as chemokines like IL-8 and monocyte chemoattractant protein 1(MCP1) in various cell types [5], [6]. Studies using IL-17 or IL-17 receptor (IL-17R) knock-out mice have highlighted the key role of this cytokine in several animal models of autoimmune diseases including collagen-induced arthritis [7], experimental autoimmune encephalomyelitis [8], experimental colitis [9, 10] and allergic asthma [11].

The clinical relevance of these findings has been confirmed by a number of studies showing for instance the presence of IL-17 in the synovial fluid of patients with rheumatoid arthritis (RA) [12, 13], the production of this cytokine by T cell clones established from RA patients [14, 15] and the expression of IL-17 mRNA in mononuclear cells present in the cerebrospinal fluid of multiple sclerosis patients [16]. These “by-the-day” growing number of evidences on the role of IL-17 in chronic inflammatory diseases have surely established the importance of this cytokine in autoimmunity. However, very little is still known on the biological effects promoted by this cytokine *in vivo*.

Therefore, in this study, we set out to investigate and characterize the effects of IL-17 in two classical models of inflammation reasoning that this would expand our knowledge on the pathological role of this cytokine and at the same time provide information on experimental models that could be helpful to develop novel anti-IL-17 strategies.

2. Materials and methods

2.1 Reagents

Recombinant mouse IL-17 and anti-mouse KC antibody (clone 48415) were purchased from R&D System (Abingdon, UK) and dissolved in carboxymethylcellulose (CMC 0.5% w/v in sterile PBS). λ -carragenan (1% w/v) was dissolved in sterile PBS. Unless otherwise specified, all the other reagents were from Sigma-Aldrich Co (Dorset, UK).

2.2 Mice

Male C57BL/6 mice (24 to 28 g; Harlan, UK) were used for all experiments. Animals were kept under standard conditions and maintained in a 12h/12h light/dark cycle at $22 \pm 1^\circ\text{C}$ in accordance with United Kingdom Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act 1986) and of the European Union directives.

2.3 Paw oedema

Male C57BL/6 mice were divided into groups of 7, lightly anaesthetized with enflurane 4% mixed in $\text{O}_2/\text{N}_2\text{O}$ 1:1 atmosphere and injected intraplantarly with IL-17 (0.1, 0.5 or 1.0 μg in 50 μl of CMC 0.5% w/v) or the same volume of the vehicle or 50 μl of 1% λ -carrageenan. Oedema, measured with a hydropletismometer (Ugo Basile, Milan, Italy), was calculated by subtracting the initial paw volume to the paw volume measured at each time point.

2.4 Myeloperoxidase assay

Leukocyte myeloperoxidase (MPO) activity was assessed by measuring the H_2O_2 -dependent oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) as previously reported [17]. Paw tissues were homogenized for 35 sec in a solution composed of hexadecyltrimethylammonium bromide (HTAB; 0.5% w/v) in 50 mM sodium phosphate buffer pH 5.4 using Precellys 24 ceramic beads (Bertin technologies, France). After homogenization, samples were centrifuged (4000 g) for 20 min and the supernatants collected and used for the assay. Aliquots of 20 μl were incubated with 160 μl of TMB and 20 μl of H_2O_2 (in 80 mM phosphate buffer, pH 5.4) in 96-well plates. Plates were incubated for 5 min at room temperature and optical density was read at 620nm using GENios (Tecan, Reading, UK). Assay were performed in duplicates and normalized for protein content.

2.5 Airpouch

Dorsal airpouches were prepared by injection of 2.5 ml of air on day 0 and day 3. On day 6, mice received IL-17 (0.1, 0.5 or 1.0 µg) in 0.5 ml of 0.5% CMC, or CMC alone, directly into the pouch, modifying a protocol optimised for the response to IL-1 [18]. In some experiments IL-17 (1.0 µg) was co-injected with 10 µg of neutralizing anti-KC antibody [19]. Mice were sacrificed at different time-points, and airpouches washed thoroughly with 2 ml of PBS containing 50 U/ml heparin and EDTA (3 mM). Lavage fluid were centrifuged at 220g for 10 min at 4°C to separate the exudates from the inflammatory cells. Inflammatory exudates were collected and measured to evaluate the total volume and the protein concentration as index of plasma leakage. Protein concentration was determined using Biorad Protein Assay (Bio-Rad Laboratories, Hemel Hempstead, UK) according to the manufacturer's instructions. Total and differential cell counts were performed by light microscopy after staining in Turk's solution (crystal violet 0.01 % in 3% acetic acid).

2.6 Flow cytometry

Cells collected from the pouch cavities were washed with PBS and resuspended in FACS buffer (PBS containing 1% FCS and 0.02% NaN₂) containing CD16/CD32 FcγRII blocking antibody (clone 93; eBioscience, Wembley, UK) for 30 min at 4°C. Thereafter, cells were labeled for 30 min at 4°C with the following FITC or PE-conjugated antibodies (eBioscience, Wembley, UK): Gr1 (working dilution 1:500; clone AB6/8C5), F4/80 (1:100; clone BMT) and CD3 (1:100; clone 145 2C11), prior to analysis by FACS calibur using CellQuest software (Becton Dickinson, Franklin Lakes, NJ). At least 10⁴ cells were analyzed per sample, and determination of positive and negative populations was performed based on the staining attained with irrelevant IgG isotypes.

2.7 Histology

Histological analyses were conducted at the 6 h time-point: joints were trimmed, placed in decalcifying solution (EDTA 0.1mM in PBS) for 14 days and then embedded in paraffin. Sections (5 µm) were deparaffinized with xylene and stained with haematoxylin and eosin (H&E). Airpouch lining tissue samples were cut along the longitudinal axis at approximately the pouch midline, with particular care to preserve the original shape, fixed and embedded in paraffin blocks. Sections (5 µm) were permanently bonded to cover slips and stained with H&E. In all cases, a minimum ≥3 sections per animal were evaluated. Phase-contrast digital images were taken using the Image Pro image analysis software package.

2.8 Cytokine ELISA

Aliquots (50 µl) of the air pouch inflammatory fluids obtained at different time after IL-17 or vehicle injection were diluted 1:1 with assay diluents and analyzed for the levels of IL-1, TNFα, IL-6, MCP-1 and KC levels by ELISA according to the manufacturer's instructions (eBioscience, UK).

2.9 RT-PCR

Paw and air pouch tissues were homogenized in Trizol reagent (Applied Biosystems, Warrington, UK) using Precellys 24 ceramic beads (Bertin technologies, France). Total RNA was extracted according to the manufacturer's protocol and reverse transcribed using 2µg oligo(dT)15 primer, 10 units AMV reverse transcriptase, 40units RNase inhibitor (all from Promega, Southampton, UK), and 1.25mM each dNTP (Bioline, London, UK) for 45min at 42°C. PCR was carried out using REDExtract-N-AmpTM PCR ReadyMixTM (Sigma) and previously validated [20] IL-17 receptor specific primers 3'-CCACTCTGTAGCACCCCAATG-5' sense and 3'-CCTGGAGATGTAGCCCTGGTC-5' anti-sense. Samples were normalised using ribosomal 18S QuantiTect Primer Assay oligonucleotides (QIAGEN).

2.10 Statistical Analysis

Prism software (GraphPad software) was used run analysis of variance (ANOVA), or Student's t-test for two selected groups. P-values lower than 0.05 were considered significant. Data are presented as mean ± S.E.M of *n* samples per group.

3. Results

3.1 IL-17 does not induce paw inflammation

To investigate the inflammatory properties of IL-17 *in vivo* we injected increasing doses of recombinant IL-17 (0.1, 0.5 or 1.0 µg) in the mouse hind paw and monitored changes in paw volume over 72 h, using the response to λ-carrageenan as positive control. IL-17 produced a very small and non-significant increase in paw volume at 2 h whereas λ-carrageenan produced typical oedema response, peaking at 6 and 48 h (Figure 1). We measured inflammatory leukocyte infiltration at 2 and 6 h measuring MPO activity in paw homogenates and observed similar results: a significant increase in λ-carrageenan but not IL-17 injected tissues compared to vehicle treated mice (Figure 2A). Consistent with these findings, H&E staining of λ-carrageenan-injected paws clearly showed the presence of inflammatory leukocytes (Figure 2B, right panel) whereas very little cell infiltrates was observed in tissues from mice administered with vehicle or IL-17 (Figure 2B, left or middle panel, respectively).

3.2 IL-17 amplifies on going inflammation

We next tested the effects of recombinant IL-17 in a mouse model of ongoing inflammation, the air pouch, where a lining tissue infiltrated by inflammatory cells forms over 6 days. As shown in Figure 3, injection of vehicle produced a modest infiltration of cells into the pouch. In contrast, administration of IL-17 (1 µg) produced an intense accumulation of inflammatory cells that peaked at 24 h, declining over 48 h (Figure 3A). This effect was accompanied by a similar pattern of accumulation of inflammatory fluid and increase in plasma leakage (Figure 3B and C, respectively); moreover, this response was dose dependent, with significant differences observed at 0.5 and 1.0 µg of IL-17 (Figure 3D).

3.3 Increased expression of IL-17 receptor in inflamed tissues

We hypothesized that IL-17 induced an inflammatory response in the air pouch but not in the paw because of different levels of expression of IL-17R in these tissues. Consistent with our expectations, we observed a marked and detectable expression of IL-17R mRNA in the naïve air pouch tissues but not in the paw (Figure 4A and B). In addition, comparison of IL-17R expression in vehicle and IL-17 treated air pouch tissues revealed a significant higher level in the latter (data not shown) suggesting a self-amplificatory inflammatory loop promoted by IL-17.

3.4 IL-17 specifically recruits PMNs to the site of inflammation

Phenotypic analysis of the air pouch infiltrated cells by FACS demonstrated a large influx of Gr1⁺ cells at 4 and 24h post-IL-17 compared to vehicle control (Figure 5A). This influx of PMNs appeared to be specific since it was not accompanied by a significant increase in the number of F4/80⁺ or CD3⁺ cells (markers of macrophage or T cell, respectively) at any time point (Figure 5B and 3C). This pattern was conserved over a number of experiments and cumulative results, expressed as total number of Gr1⁺, F4/80⁺ and CD3⁺ cells, are summarized in Figure 6. Histological analyses supported exudates determinations. H&E stained lining pouch tissues displayed large degree of infiltrated leucocytes in the pouch wall of IL-17-treated mice compared to those obtained from vehicle-injected (Figure 7).

3.5 IL-17 induces a selective pattern of inflammatory cytokines

Next, we investigated whether IL-17 induced a specific pattern of inflammatory cytokine and chemokines. Interestingly, whereas IL-17 induced a significant, yet modest increase in IL-1 β levels (around 50-60% at 4-24 h; Figure 8B), levels of TNF α and IL-6 were increased to a much greater extent (~160 and 950%, respectively), and significantly different only at 4 h when compared to vehicle-injected mice (Figure 8A and C, respectively). Similarly, IL-17 augmented KC levels much more than those of MCP-1 (3-fold vs. ~60%; Figure 8D and E, respectively). No significant differences were observed for levels of IL-2, IL-10, IFN γ and TGF β (data not shown).

3.6 The CXC chemokine KC mediates the inflammatory effect of IL-17

To establish if KC levels detected in association with the PMN recruitment induced by IL-17 was functional, a neutralizing anti-KC antibody was co-injected together with IL-17. Treatment of mice with anti-KC antibody significantly reduced IL-17-induced cell infiltration, exudate accumulation and plasma leakage (Figure 9 A, B and C, respectively). These effects were paralleled by a similar inhibition of TNF α , IL-1 and IL-6 contents detected in the inflammatory exudates (Figure 9 D, E and F, respectively).

4. Discussion

Many studies over the last decade have given IL-17 a unique role in the context of the inflammatory response [21, 22]. This cytokine is produced mainly by T cells rather than by macrophages or other cells of the innate immune system and thus it is believed to play an important role in the inflammatory events triggered by the adaptive or memory immune system [23, 24]. In line with this hypothesis, studies *in vivo* as well as *in vitro* have suggested that IL-17 cooperates either additively or synergistically with various cytokines or mediators in inducing inflammation [25, 26]. Surprisingly, however, very little is known about the specific effects induced by this cytokine alone. The aim of this study was to investigate if IL-17 could induce an inflammatory response *per se* or whether it acted predominantly as an amplifier of ongoing inflammation.

We began our study by testing the effects of IL-17 in the mouse paw oedema model, a well-known experimental system for the study of phlogogenic compounds or testing antiinflammatory drugs *in vivo* [27, 28]. Interestingly, injection of IL-17 into the paw did not induce any significant increases in paw volume or inflammatory leukocyte infiltration. These results were independent of mouse strain (data not shown) and quite surprising since obtained using three different doses that have been reported to cause a significant inflammatory response in other *in vitro* [29-31] or *in vivo* systems [32, 33].

Administration of IL-17 in the airpouch induced a markedly different response: a large number of PMNs accumulated into the pouch between 4 and 24 hours post-IL-17, while no significant changes in the number of T cells or macrophages were measured. This result begs the question on why does IL-17 cause inflammation in the airpouch model. The main difference between the two models is that, in the airpouch, phlogogenic compounds are injected in an “encapsulated” artificial cavity made of inflamed granulomatous tissue, while in the paw oedema test compounds are administered into a non-inflamed physiological tissue [34, 35]. In light of these considerations our results suggest that IL-17 is not a classical pro-inflammatory cytokine such as IL-1 β or TNF α but it plays a very specific and unique role in the context of on-going inflammatory diseases by exacerbating cellular and biochemical events activated during the acute phase of the inflammatory response.

Many factors might account for inability of IL-17 to initiate an inflammatory reaction. We hypothesized that one could be the lack of IL-17R expression in non-inflamed tissue. Analysis of IL-17R mRNA expression confirmed our hypothesis and showed a marked expression in the air pouch but not in the paw tissue samples. These results suggest that the pre-inflamed microenvironment of the airpouch is associated with expression of IL-17R, plausibly in the pouch lining tissue which display a low degree of cell infiltration. Consistent with this hypothesis, studies have shown that up-regulation of IL-17R by inflammatory cytokines is a limiting factor for the development of downstream IL-17-induced effects, including IL-8-dependent recruitment of PMNs [36, 37].

Characterization of the phenotype of IL-17 induced cellular infiltrate revealed a selective accumulation of PMNs but not monocyte/macrophages or T cells. This was mainly due to a large release of KC from the airpouch since co-administration of a neutralizing anti-KC antibody together with IL-17 significantly reduced the inflammatory response and almost abolished the accumulation of TNF α , IL-1 and IL-6. These results provide further insights on the mechanism by which IL-17 sustain inflammation and highlight the importance of PMNs as the main cell type that mediate the inflammatory effects of this cytokine.

In a typical inflammatory reaction PMNs accumulate first at the site on injury or infection followed by macrophages and T cells. These latecomers have the specific function of clearing up pathogen-engulfed neutrophils (macrophages) and taking records of these events for further attacks (memory T cells). If the host is not able to clear the pathogens and start the resolution of inflammation, a different pattern of biochemical events takes place and another “wave” of pathogen-fighters, eg. PMNs, is required: we propose that, in this scenario, IL-17 takes center stage by further expanding and amplifying biochemical and cellular events that have occurred in the early stages of the inflammatory reaction.

Only few studies have shown that IL-17 induces PMN infiltration in other cavities such as the peritoneum or the lung [32, 38]. In our experiments, the inflammatory response induced by IL-17 in the airpouch was much greater in terms of both cellular infiltrate and cytokine production. Once again this might be explained by the specific reliance of IL-17 on a pre-inflamed tissue and a ‘primed’ cellular microenvironment to exert its full potential. It should be in fact considered that both peritoneum and lung are sites under immuno surveillance and,

as such, under continuous control of the adaptive and innate immune systems. Conversely, the airpouch is a newly formed structure that originates as result of an unrecognized (and by day 6 inflamed) tissue reorganization.

In agreement with this view, original studies on the airpouch have suggested that the inflammatory response that characterize this model partially resembles the inflammatory settings found in the synovium of RA patients [35, 39]. Synovial fluids contain large numbers of PMNs and T cells; it is believed their presence drives destruction and erosion of the joint. The IL-17 produced locally probably by activated memory T cells as well as by neutrophils might be the key factor for exacerbation of local inflammatory circuits [40, 41]. In conclusion, our results further confirm the role of IL-17 in the exacerbation of the inflammatory response and provide a useful and easier alternative way to test the effect of IL-17-targeted drugs *in vivo*. Furthermore, we suggest that novel anti-IL-17 compounds might be useful to limit disease progression in autoimmune diseases while ineffective in preventing the acute phase of the inflammatory response.

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Competing interest statement.

The authors have no conflicting financial interests.

Figure legends

Figure 1. IL-17 is inactive in the mouse paw oedema test. IL-17 (0.1, 0.5 or 1.0 µg), CMC vehicle (0.5% w/v) or λ-carrageenan (1% w/v) were administered in the dorsal surface of the mouse right hind paw and the swelling measured at 2, 4, 6, 24, 48 and 72 hours. The oedema is expressed as the difference between the initial basal paw volume and the paw volume at each time-point. Values are means ± S.E.M of three separate experiments with 7 mice * $P < 0.05$ and ** $P < 0.01$ vs. vehicle.

Figure 2. IL-17 does not induce leukocyte infiltration in the mouse paw oedema test.

(A) Myeloperoxidase activity of paw tissue homogenates obtained 2 and 6 h after injection of IL-17 (1.0 µg), CMC vehicle (0.5% w/v) or λ-carrageenan (1% w/v). Values are expressed as optical density at 600 nm per mg of protein. The results show are means ± S.E.M of three separate experiments with 7 mice ** $P < 0.01$ vs. vehicle. **(B)** Haematoxylin-eosin staining of paw tissues obtained from mice treated with IL-17 (1.0 µg), CMC vehicle (0.5% w/v) or λ-carrageenan (1% w/v) treated mice at the peak of the swelling (2 hours for IL-17 or CMC and 6 hours for λ-carrageenan). Pictures are representative of three separate experiments with similar results.

Figure 3. IL-17 induces inflammation in the mouse airpouch test. Time-course of cell infiltration **(A)**, exudate formation **(B)** and protein accumulation **(C)** into the inflammatory fluids of mice that received a local injection of CMC vehicle or 1.0 µg of IL-17. Values are means ± S.E.M of three separate experiments with n=5-6 mice * $P < 0.05$ and ** $P < 0.01$ vs. vehicle. **(D)** Dose-dependent increase in cell infiltration into the air pouch of mice sacrificed 24 hours after receiving the indicated doses of IL-17. The dotted line represents the average number of cells obtained from control vehicle-treated mice. Values are means ± S.E.M of three separate experiments with n=5-6 mice * $P < 0.05$ and ** $P < 0.01$ vs. vehicle.

Figure 4. Upregulation of IL-17R in inflamed tissues. **(A)** Comparative PCR analysis of IL-17R and ribosomal 18S expression in hind paw and 6-days air pouch tissue homogenates of 3 mice (upper panel). **(B)** Cumulative densitometric analysis of IL-17R expression in hind paw and 6-days air pouch tissue homogenates. Values were normalized for ribosomal 18S expression and are means ± S.E.M of three separate experiments with n=5-6 mice. **

$P < 0.01$ vs. paw tissues.

Figure 5. Phenotypic analysis of IL-17-induced inflammatory cellular infiltrates. Cells obtained from vehicle- or IL-17-injected air pouches were washed, stained with (A) GR1, (B) F4/80 or (C) CD3 and analyzed by FACS. The numbers in the histograms indicated the percentage of positively stained cells. The dotted lines show the profiles of cells stained with an irrelevant FITC or PE-conjugated IgG. Values are representative of three separate experiments with $n=5-6$ mice.

Figure 6. Selective recruitment of PMNs into the air pouch of IL-17 treated mice. Cumulative analysis of the number of (A) GR1-, (B) F4/80- or (C) CD3-positive cells obtained from vehicle- or IL-17-injected air pouches. Values are means $\pm$ S.E.M of three separate experiments with $n=5-6$ mice. ** $P < 0.01$ vs. vehicle.

Figure 7. Histological changes in the pouch tissues of IL-17-treated mice. Haematoxylin-eosin staining of air pouch lining tissues removed from the mice challenged for 4 hours with (A) vehicle or (B) IL-17 (1.0 μ g). Left and right panels are 100X and 40X magnifications, respectively. Pictures are representative of three separate experiments with similar results.

Figure 8. IL-17 induces a distinct profile of inflammatory cytokine and chemokine production. Levels of (A) TNF α , (B) IL-1 β , (C) IL-6, (D) KC and (E) MCP1 in the inflammatory fluids obtained from air pouches of mice challenged with vehicle or IL-17 (1.0 μ g). Values are means $\pm$ S.E.M of three separate experiments with $n=5-6$ mice * $P < 0.05$ and ** $P < 0.01$ vs. vehicle.

Figure 9. KC mediates the inflammatory effects of IL-17. Time-course of cell infiltration (A), exudate formation (B) and protein accumulation (C) into mouse airpouch of mice that have received CMC vehicle, IL-17 (1.0 μ g) or IL-17 plus anti-KC neutralizing antibody (10 μ g). Values are means $\pm$ S.E.M of three separate experiments with $n=5-6$ mice ** $P < 0.01$ vs. IL-17. Levels of (D) TNF α , (E) IL-1 β , (F) IL-6 in the inflammatory fluids obtained from air pouches of mice challenged with vehicle, IL-17 (1.0 μ g) or IL-17 plus anti-KC neutralizing antibody (10 μ g). Values are means $\pm$ S.E.M of three separate experiments with $n=5-6$ mice ** $P < 0.01$ vs. IL-17.

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A

B

Figure 3

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Figure 5

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