Original Article

Macrophage-derived interleukin-18 in experimental renal allograft rejection

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Abstract

Background. Interleukin 18 (IL-18) is primarily a macrophage-derived, pro-inflammatory cytokine. As macrophages can act as effector cells in acute rejection, we examined the role of IL-18 in a rat model of acute renal allograft rejection.

Methods. Life-sustaining orthotopic DA to Lewis allograft and Lewis-Lewis isograft kidney transplants were performed. In the same model, macrophage-depleted animals, achieved with liposomal-clodronate therapy, were also studied. Macrophage (ED1+) accumulation and IL-18 expression was assessed by immunohistochemistry. CD11b+ cells (macrophages) were isolated from kidney and spleen by micro beads. Real-time PCR was used to assess IL-18 and INF-γ mRNA expression in tissue and cell isolates.

Results. Allografts, but not isografts, developed severe tubulo-interstitial damage and increased serum creatinine by day 5 (P<0.001). Immunohistochemistry revealed a greater ED1+ cell accumulation in day 5 allografts compared with isografts (P<0.001). IL-18 mRNA expression was increased 3-fold in allografts compared to isografts (P<0.001). Accordingly, IL-18 protein was increased in allografts (P<0.001), and was predominantly expressed by ED1+ macrophages. CD11b+ macrophages isolated from allografts had a 6-fold upregulation of IL-18 mRNA expression compared to isograft macrophages (P<0.001). Macrophage depletion resulted in a marked attenuation of allograft rejection, ED1+ and IL-18+ cells were significantly reduced (P<0.05) as was IL-18 mRNA expression (29.28±2.85 vs 62.48±3.05, P<0.001). INF-γ mRNA expression (P<0.01) and iNOS (P<0.001) production were also significantly reduced in the macrophage-depleted animals.

Conclusion. This study demonstrates that IL-18 is significantly increased during acute rejection and is principally produced by intra-graft macrophages. We hypothesize that IL-18 upregulation may be an important macrophage effector mechanism during the acute rejection process.

Keywords: acute allograft rejection; interleukin-18; kidney transplantation; macrophages

Introduction

Macrophages are known to contribute to kidney damage during acute rejection; however, the mechanisms involved remain to be fully elucidated. This study examined the largely macrophage-derived cytokine, interleukin-18 (IL-18), as a potential effector molecule in the process of rejection.

Macrophages are versatile cells capable of participation in both innate and adaptive immune responses [1]. Macrophage accumulation within acutely rejecting allografts has been reported for many years [2], and is known to occur through both recruitment from circulation and through proliferation within the rejecting graft [3]. Until recently, the role of accumulated macrophages in the process of acute rejection has been ill-defined. We have shown that macrophage depletion provided protection from acute rejection in a rat kidney allograft model. In this experiment, protection was associated with a reduction in iNOS (inducible nitric oxide synthase) expression and nitric oxide (NO) generation, but no change in T-cell infiltration or activation, indicating that macrophages themselves may act as effectors of allograft damage [4]. Graft rejection by activated macrophages...
in the absence of T cells has been elegantly demonstrated in a xenograft islet model, and although different mechanisms may be operative during xenograft rejection, the ability of macrophages to reject a graft was confirmed [5]. What remains to be defined are the mechanisms by which macrophages mediate graft damage.

IL-18 is a potent pro-inflammatory cytokine, produced by several different cell types, but is primarily a product of macrophages [6]. Originally designated interferon gamma (INF-γ)-inducing factor, IL-18 is now known to be a pleiotrophic cytokine capable of interacting with the key cell types involved in the rejection process through both INF-γ dependent and independent mechanisms [7,8]. Relevant activities include T-cell chemotraction and polarization [9], and activation of NK cells [10] and macrophages [11].

IL-18 is a member of the IL-1 cytokine family and shares a signal transduction pathway with the IL-1 receptor, recruiting MyD88 (an adaptor protein) and IRAK (IL-1 receptor activating kinase) molecules, culminating in the activation of nuclear factor-κB (NF-κB) [12]. Thus, IL-18 stimulation of macrophages induces the transcription of a diverse set of target genes including IFN-γ, IL-1, iNOS and tumor necrosis factor-α (TNF-α), which are known to be involved in the pathogenesis of acute allograft rejection.

The pro-inflammatory characteristics of IL-18 and the significant macrophage accumulation evident during graft rejection suggest a role for IL-18 as a potential macrophage effector molecule. We therefore examined IL-18 expression in a model of acute renal rejection with the aim of better understanding the mechanisms of macrophage induced allograft injury.

**Methods**

**Experimental design**

Inbred 250–300 g male Lewis (RT1^b^) and Dark Agouti (DA) (RT1^av^) rats were obtained through Sydney University Animal Services. This study was performed in accordance with the requirements of the University of Sydney Animal Ethics Committee. Life-sustaining orthotopic kidney transplants were performed following bilateral nephrectomies on the animals as previously described [4]. Twenty-one DA to Lewis allografts and 12 Lewis to Lewis isografts were performed; there were two technical failures. No immunosuppressive therapy was administered.

Animals were killed on day 1 (five allografts, four isografts), day 3 (six allografts, five isografts) and day 5 (eight allografts, three isografts) post-transplantation; blood and tissue (kidney and spleen) were harvested at the time of sacrifice. Serum creatinine levels were measured by the Department of Biochemistry at Royal Prince Alfred Hospital. Blood (n = 3) and tissue samples (n = 4) were also taken from normal age-matched Lewis rats as controls.

In a second experiment, macrophage-depleted tissue from previous work [4] was studied.

Using the same rat model of AR as described above, liposomal-clodronate treated animals experienced a 60% reduction in blood and allograft macrophages resulting in protection from rejection, defined by allograft function and histology, as compared to control animals receiving either liposomal-PBS or saline.

**Antibodies**

ED1, a monoclonal anti-rat CD68 antibody (Serotec), labels tissue macrophages and monocytes. A polyclonal goat anti-rat IL-18 antibody (R&D Systems Inc., MN, USA) was used to detect the expression of IL-18. Mouse anti-rat CD11b (Clone Ox-42) (Serotec) antibody recognizes CD11b antigen, which is expressed on most macrophages, and also recognizes some dendritic cells and granulocytes. Anti-mouse IgG micro beads (Miltenyi Biotec) were used for positive selection of CD11b⁺ cells. Biotinylated secondary antibodies used were anti-mouse IgG and anti-goat IgG (Vector Labs., CA).

In the double labelling experiments, anti-T cell receptor (TCR clone 73) (BD Pharmingen CA, USA) antibody, antimouse Alexa Fluor 488 and Streptavidin Alexa Fluor (594) antibody (Molecular Probes Eugene, USA) were used. Isotype negative controls used were mouse and goat IgG1 (DAKO, Glostrup, Denmark).

**Histopathology**

Harvested kidney and spleen tissue was fixed in 4% paraformaldehyde, paraffin sections 5 μm thick were stained with Haematoxylin and Eosin. A blinded assessment of the degree of rejection was based on the Banff interstitial inflammation score [13], a graded scale of 0–3 according to the percentage of cortical tissue affected by mononuclear infiltrate; i0 = <10% of cortex affected, i1 = 10–25%, i2 = 25–50%, i3 = >50%.

**Immunohistochemistry**

A standard three step avidin-biotin immunoperoxidase technique was used. The 5 μm thick paraffin-embedded tissue sections were de-paraffinized with xylene, and treated with 3% H2O2 in methanol to suppress endogenous peroxidase activity. Antigen retrieval with 0.5 mg/ml of protease (Sigma-Aldrich, MO, USA) was performed on the sections before they were blocked with 10% normal horse serum. Primary antibody was incubated with the tissue for one hour, then washed, followed by incubation with the appropriate biotinylated secondary antibody and a further washing. Streptavidin-peroxidase complex was then applied, followed by DAB solution (DAKO liquid DAB substrate-chromogen solution). The slides were counter stained with Harris haematoxylin. For immunofluorescent double staining, primary antibodies were applied sequentially, Streptavidin Fluor 594 was used for IL-18, and anti-mouse fluro 488 was used as a secondary antibody for both ED1 and TCR. Specificity of IL-18 antibody was evaluated by pre-incubation and quenching of the antibody with recombinant IL-18 protein (R&D Systems Inc., MN, USA) prior to tissue application, resulting in negligible staining of the IL-18 antibody.
Quantification of immunohistochemistry

Analysis of the cellular infiltrate was performed blinded, by assessing 25 consecutive high-power fields (×400 magnification) of cortex for each section. Using an ocular grid, the number of cells staining positively (cells/mm²) for each antibody was counted.

Macrophage isolation

Harvested kidney and spleen of three day-5 isografts and two day-5 allografts was immediately placed in RPMI and passed through a 250 μm sieve (kidney) or a 70 μm sieve (spleen) before undergoing digestion with type I collagenase (Worthington, Freehold, NJ, USA) and deoxyribonuclease I, type IV (Sigma). Viable spleen cells were collected using a ficoll gradient, whereas enrichment of mononuclear cells from kidney was achieved using a separation gradient method as previously described [14]. The cells were incubated with mouse anti-rat CD11b:FITC (Serotec, Oxford, UK). MACS goat anti-mouse IgG micro beads (Miltenyi Biotec, CA, USA) were added to the single-cell suspensions which were then passed through a magnetic positive selection column as per the manufacturer’s instructions. An aliquot of the cells collected were analysed by flow cytometry to assess purity.

Extraction of RNA and cDNA synthesis

Total RNA was extracted from renal tissue or cell isolates using TRIzol (Invitrogen, CA, USA) according to the manufacturer’s instructions. First strand complementary DNA was synthesized using the SuperScript III Reverse Transcriptase kit (Invitrogen), using 1 μg of RNA and 2 μl of oligo (dT)₁₆.

Real-time PCR

A total of 5 μl of diluted cDNA (or PCR standards) was amplified by real-time PCR using 1x Universal Master Mix (PE Applied Biosystems, Foster City, CA, USA). Specific Taqman primers for IL-18 (sense 5'-GTGTTCCAG GACATGCTGAT; antisense, 5'-CCAGTCTCTTACTTT CACTATCTTTGTACA) and probe (FAMCGACCGAAC AGCCAAACATCCCTAMRA); and iNOS (sense 5'-TCC GCAAGGGAGTGTGTT; antisense, 5'-TCGTCGGCC AGCTTTTCT) and probe (CTGCCCGGAAAACTCCCA GGTCTACGT) were obtained from PE Applied Biosystems. Primers and probes used for GAPDH as a house keeping gene control and INF-γ have previously been described [15]. Triplicate real-time PCR experiments were performed.

Flow cytometry

Isolated cells labelled with mouse anti-rat CD11b:FITC antibody (Serotec) were analysed for purity by flow cytometry on a FACSscan (BD Biosciences, Mountain View, CA) gated to exclude non-viable cells. FACS analysis demonstrated that the CD11b⁺ cell populations isolated from kidney and spleen 5 days post-operatively, respectively, were 98 and 92% pure (data not shown).

Statistics

Data were recorded as mean±SEM. Statistical differences between the groups were analyzed by one-way analysis of variance. Repeated measures ANOVA (mean and standard error) was used in the macrophage isolation and depletion studies where PCR was performed three times on the samples. Pearson’s correlation was performed to correlate cell counts and creatinine values. All analyses were performed using SPSS 11.0 (SPSS Inc., Chicago, IL, USA). P-values <0.05 were considered significant.

Results

Assessment of acute rejection

Marked elevation of serum creatinine, indicating dysfunction, was seen in day 5 allografts. Isograft values did not differ significantly from normal (Figure 1a).

Allograft kidneys at day 5 post-transplant revealed an extensive mononuclear cell interstitial infiltrate consistent with severe acute rejection, whereas isografts showed minimal histological changes. The extent of severity of AR according to the Banff criteria for interstitial infiltration increased progressively in allografts, though not in isografts (Figure 1b).

![Graph](https://example.com/graph1.png)

**Fig. 1.** (a) Serum creatinine was significantly elevated in allografts 5 days post-transplant. (**P < 0.001**); (b) Banff interstitial inflammation score grouped according to days post-transplant shows a significant increase in allografts at day 5 (**P < 0.001**).
Macrophage accumulation during acute rejection

Macrophages (ED1\(^+\) cells) were present in allograft tissue within the first 24 h post transplantation. Allografts showed a greater macrophage accumulation compared with isografts, becoming significant by day 5 (88.5 ± 20.6 vs 3.5 ± 2.2 cells/HPF, \(P < 0.001\)) by immunohistochemistry (Figure 2a). Accumulating cells at day 1 and 3 were predominantly peri-vascular, evolving to peri-tubular and peri-glomerular; by day 5, there was also evidence of tubulitis and tubular destruction in allografts.

Elevated IL-18 in acute rejection is chiefly a product of macrophages

IL-18\(^+\) cells were increased in number in renal allografts compared with isografts, reaching significance at day 5 (47.2 ± 12 vs 1.4 ± 1.3 cells/HPF, \(P < 0.001\)) (Figure 2b). Interstitial infiltrating cells were the primary cell type expressing IL-18, there were occasional glomerular epithelial cells staining positive for IL-18, but little if any tubular IL-18 expression. The serum creatinine, as an indicator of graft injury, strongly correlated to both ED1\(^+\) \((r = 0.77)\) and IL-18\(^+\) cells \((r = 0.70)\), \((P < 0.001)\). Furthermore, IL-18\(^+\) cell numbers strongly correlated with ED1\(^+\) cell accumulation \((r = 0.97, P < 0.001)\), and serial sections of allografts at day 3 and 5 stained for IL-18 and ED1 demonstrate that most cells producing IL-18 were ED1\(^+\) macrophages (Figure 3). Double staining for IL-18 and ED1 confirmed that macrophages are the predominant source of IL-18 (Figure 4a), supporting this is the lack of double staining of TCR\(^+\) and IL-18\(^+\) cells (Figure 4b).

IL-18 mRNA expression is increased in acute rejection

IL-18 was constitutively expressed at low levels by normal kidney, minimal increases were found in isograft tissue, while IL-18 gene expression was progressively upregulated in allograft tissue. IL-18 gene expression in whole kidney tissue from day 5 allografts was 3-fold higher compared with day 5 isografts (106.7 ± 27 vs 38.6 ± 12.65, \(P < 0.001\)) (Figure 5a).

\(\text{INF-}\gamma\) and iNOS mRNA expression is increased in this model of acute rejection

\(\text{INF-}\gamma\) mRNA expression was progressively increased in allografts. There was no significant difference between day 3 allograft and isograft \(\text{INF-}\gamma\); however, by day 5, allograft \(\text{INF-}\gamma\) levels were markedly elevated and isograft levels fell to normal (0.37 ± 0.21 vs 0.004 ± 0.004, \(P = 0.001\)) (Figure 5b). iNOS mRNA was significantly increased in day 5 compared to day 3 allografts (4.8 ± 2.0 vs 0.2 ± 0.3, \(P < 0.001\)) and all other groups (Figure 5c).

Specific upregulation of IL-18 mRNA expression by kidney macrophages

In macrophages (CD11b\(^+\) cells) isolated from kidneys 5 days post-transplant, IL-18 expression was upregulated 6-fold in allografts compared with isografts (55.6 ± 2.9 vs 7.12 ± 2.7, \(P < 0.001\)) (Figure 6). There was no significant increase in IL-18 mRNA expression from splenic macrophages isolated from allografts compared with isografts (\(P = 0.38\)).

Graft injury and IL-18 mRNA expression is reduced in macrophage depletion studies

In order to better demonstrate the links between macrophages and IL-18 expression in acute renal allograft rejection, macrophage-depleted samples (achieved by treatment with liposomal-clodronate) were evaluated. The macrophage-depleted group showed marked attenuation of the histological parameters of acute rejection as compared to the two control groups (those treated with saline only or PBS-liposomes), and maintained normal renal function [4]. Graft macrophages were significantly reduced in the
liposome-clodronate treated group (73.7±24.1 ED1⁺ cells/HPF) compared with the two control groups (137.5±24.8, \( P = 0.01 \) and 147.2±27.1, ED1⁺ cells/HPF, \( P = 0.005 \)), but remained significantly higher than was observed in non-transplanted kidneys (\( P = 0.002 \)) (Figure 7). There was no reduction in other leukocytes (CD3⁺, CD4⁺, CD8⁺, neutrophils and NK cells) [4].

IL-18 positive cells were significantly lower in the macrophage-depleted group (45.5±16.6 cell/HPF) (Figure 7) compared with the two control groups (83.8±25.6, \( P = 0.02 \) and 100.1±27.8 cells/HPF, \( P = 0.002 \)). IL-18 expression in untransplanted controls was still significantly lower than in the liposomal-clodronate treated group (\( P = 0.003 \)).

Real-time PCR showed a significant decrease in IL-18 mRNA expression in renal allografts from macrophage-depleted animals compared to the control group (29.28±2.85 vs 62.477±3.048, \( P < 0.001 \)) (Figure 8a). IL-18 gene expression in the clodronate

<table>
<thead>
<tr>
<th>Day</th>
<th>ED1⁺ Macrophages</th>
<th>IL-18⁺ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td><img src="a" alt="Day 1 Allograft" /></td>
<td><img src="a" alt="Day 1 IL-18" /></td>
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<tr>
<td>Day 3</td>
<td><img src="b" alt="Day 3 Allograft" /></td>
<td><img src="b" alt="Day 3 IL-18" /></td>
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<td>Day 5</td>
<td><img src="c" alt="Day 5 Allograft" /></td>
<td><img src="c" alt="Day 5 IL-18" /></td>
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Fig. 3. ED1⁺ macrophages are progressively increased in renal allografts (a-e). There is an initial increase in isograft macrophage accumulation observed at day 3 which subsides by day 5 (d-f). IL-18⁺ cells progressively increase in allografts during rejection (g-i), whereas the initial increase in IL-18⁺ cells in isografts at day 3 resolves by day 5 (j-l). Serial sections of day 3 and 5 allograft tissue demonstrate co-localisation of ED1⁺ and IL-18⁺ cells (b, h and c, i).

Fig. 4. (a) In day 5 allografts, IL-18⁺ cells fluoresce red, and ED1⁺ cells fluoresce green—the majority of cells exhibit double staining for both IL-18 and ED1 and fluoresce yellow; (b) IL-18⁺ cells fluoresce red, and TCR⁺ cells fluoresce green—illustrating minimal double staining.

![Fig. 4](a) ![Fig. 4](b)
treated group remained higher than the untransplanted (normal) animals \((P = 0.001)\). There was no significant difference between the two control groups \((P = 0.8)\).

INF-\(\gamma\) and iNOS mRNA expression was significantly reduced \((P < 0.05\) and \(P < 0.001\), respectively) in the macrophage-depleted group compared to rejecting controls but remained elevated compared to untransplanted tissue (Figure 8b and c).

**Discussion**

This is the first study, to our knowledge, examining IL-18 expression during acute renal allograft rejection. IL-18 was up-regulated at both the gene and protein level, and macrophages were demonstrated to be the predominant source. This was verified by a marked increase in IL-18 mRNA expression in macrophages isolated from day 5 allografts, and conversely, by significant abrogation of IL-18 upregulation in macrophage-depleted allografts. This data will be discussed in the context of the known actions of IL-18 on cell types involved in the pathogenesis of acute rejection.

The contribution of macrophages to the pathogenesis of acute rejection is being increasingly recognized, not only as supporters of T-cell driven processes, but also as direct effectors of tissue injury \([4,5]\). Activated macrophages are known to produce IL-18 and our study demonstrates this in the setting of allograft rejection. These findings, in addition to the strong correlation between IL-18 expression and graft injury lead us to suggest that IL-18 may be a key factor in macrophage-mediated graft damage in acute rejection. The mechanisms thought to promote macrophage accumulation occurs both through recruitment from the circulation and also local proliferation. The process is directed by chemokines such as MCP-1, MIP-1, RANTES and M-CSF.

The means by which macrophage-derived IL-18 elicits tissue damage may be INF-\(\gamma\) dependent or
IL-18 in acute rejection

(a) IL-18 mRNA expression was upregulated following transplantation (control groups treated with saline or liposomal-PBS), but significantly attenuated by macrophage depletion (clodronate treated) (**P<0.01); (b) INF-γ mRNA expression was significantly reduced in macrophage-depleted animals compared to controls (**P<0.01); (c) iNOS mRNA expression was significantly reduced in macrophage-depleted animals compared to PBS-liposome treated controls (**P<0.01).

Fig. 8. (a) IL-18 mRNA expression was upregulated following transplantation (control groups treated with saline or liposomal-PBS), but significantly attenuated by macrophage depletion (clodronate treated) (**P<0.01); (b) INF-γ mRNA expression was significantly reduced in macrophage-depleted animals compared to controls (**P<0.01); (c) iNOS mRNA expression was significantly reduced in macrophage-depleted animals compared to PBS-liposome treated controls (**P<0.01).

Independent. As a potent inducer of INF-γ, IL-18 promotes cell-mediated immunity enhancing T-cell proliferation and chemoattraction [9] and natural killer cell cytotoxicity [10]. INF-γ exhibits diverse and complex roles in the process of allograft rejection [16]. In this model, a progressive increase in INF-γ mRNA expression was demonstrated. As a proven INF-γ inducer, IL-18 may potentially drive the process of allograft rejection via INF-γ upregulation. Interestingly the reduction in INF-γ mRNA expression in macrophage-depleted animals was observed despite preservation of the T-cell response, suggesting that either T-cell production of INF-γ was reduced due to the absence of macrophage support, or that macrophages themselves may have significantly contributed to the production of INF-γ during rejection.

INF-γ independent actions of IL-18 include autocrine activation of macrophages, leading to further cytokine release such as TNF-α and NO [17], thereby delivering direct cell damage and providing a supportive milieu for other key effector cells. In this study, we show that iNOS mRNA is increased in allografts compared to isografts, and reduced in the macrophage-depleted animals. In our earlier studies, iNOS and its product NO were similarly found to be elevated in allografts and to be significantly reduced in the clodronate-treated animals [4]. iNOS is predominantly found in macrophages and is responsible for the cytotoxic bursts of high NO concentrations observed in acute rejection [18]. As IL-18 is known to stimulate the production of NO from macrophages [17], the attenuation of IL-18 in the macrophage-depleted group probably contributed to the marked fall in iNOS and NO observed. This raises the possibility that INF-γ-independent pathways may also be contributing to graft damage (i.e. macrophage release of NO, TNF-α, IL-1), illustrating another possible mechanistic-link between IL-18 and the rejection process. Other downstream cytokine and chemokine responses to IL-18 need to be studied to clarify this.

The specific upregulation of IL-18 by isolated intragraft macrophages, not seen in spleen macrophages, suggests direct graft related influences on the maturation and function of these intragraft macrophages. Further investigation of intragraft macrophage phenotype and function during acute rejection may provide further insights into the pathogenesis of rejection.

Macrophage accumulation and increased IL-18 expression has been described in other inflammatory/immune-mediated processes, including contact
hypersensitivity [19], and lupus-like autoimmune disease [20]. The pathogenic role of IL-18 in these models is supported by the finding that blocking IL-18 resulted in significant protection from the pathological processes. Given these findings and our results, an interventional study blocking IL-18 in acute rejection is currently underway.

This study indicates that IL-18 may have a significant role in the pathogenesis of acute renal allograft rejection, potentially acting via both INF-γ dependent and independent mechanisms. We also present further evidence of active participation by macrophages in the acute rejection process and identify IL-18 as a potential mechanism of macrophage effector function. IL-18 neutralizing experiments will be required to confirm this link.

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Conflict of interest statement. None declared.

References

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