Multiplex determination of murine seminal fluid cytokine profiles

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Abstract

Seminal fluid is known to be responsible for orchestrating mating-induced immunomodulation. Central to this process are numerous cytokines that modulate uterine leukocyte recruitment and trafficking. Despite this, a comprehensive analysis of the cytokine profile of murine seminal fluid is lacking. This study addressed this issue by using multiplex immunoassays to characterise the profile of interleukin (IL)-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, eotaxin, granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), interferon (IFN)- γ , keratinocyte-derived chemokine (KC), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1 α , MIP-1 β , regulated upon activation normal T-cell expressed and secreted (RANTES), and tumour necrosis factor (TNF)- α in fluid drawn from the seminal vesicles of single mice ($n = 18$). Their levels and ratios were compared with those found in serum. IL-1 α , IL-1 β , IL-2, IL-5, IL-9, IL-12 (p40), IL-12 (p70), IL-13, IL-17, GM-CSF, IFN- γ , MCP-1 and TNF- α levels were significantly higher in serum; IL-4, G-CSF, eotaxin, KC and RANTES exhibited the opposite trend. Based on these findings, we propose a model of mating-induced immunomodulation that implicates seminal eotaxin, RANTES and MIP-1 α in the relocation and concentration of extravasated migrating endometrial eosinophils to the luminal epithelium. Furthermore, KC may participate in uterine neutrophil chemotaxis and activation. Eotaxin and MIP- α , together with IL-1 β and IL-9, may also enhance further cytokine synthesis for endometrial antigen-presenting cell recruitment for processing paternal ejaculate antigens. IL-4 and G-CSF could also minimise deleterious cell-mediated immunity and modulate IFN- γ production, thereby supporting the establishment of pregnancy.

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Introduction

The opinion that seminal plasma is not just a transport medium for the transfer of spermatozoa to the maternal tract is not recent, and its immunomodulatory properties in protecting spermatozoa, priming the uterus for pregnancy and modulating killer cell activities have long been recognized (Koch & Ellendorff 1985, Rees *et al.* 1986, Saxena *et al.* 1988, Cardoso *et al.* 1993, Liang *et al.* 1993). In the mouse, mating induces a marked, yet transient, inflammatory response associated with endometrial leukocyte infiltration, which commonly dissipates by the time of blastocyst hatching and implantation (Robertson *et al.* 1997). By this stage, remaining endometrial leukocytes exhibit an immunosuppressive phenotype (Hunt *et al.* 1984). Studies using vasectomised males have indicated that the activation and expansion of female lymphocyte populations which occurs after mating is triggered by seminal vesicle gland constituents, and that it is independent of

the presence of sperm. These lymphocytes relocate to embryo implantation sites and other mucosal tissues/lymph nodes as part of the development of maternal tolerance of the fetal allograft (Johansson *et al.* 2004).

This mating-induced phenomenon relies on an array of interrelated immunosuppressive mediators, including both prostaglandins, steroid binding proteins and cytokines (Tarter *et al.* 1986, Kelly 1995, Miao *et al.* 1996, Denison *et al.* 1999, Maccioni *et al.* 2001). With respect to cytokines, maternal hyporesponsiveness to paternal major histocompatibility complex (MHC) class I antigens is thought to be mediated by – at least – transforming growth factor (TGF)- β 1, regulated upon activation normal T-cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1 α , MIP-1 β and monocyte chemotactic protein (MCP)-1 (Robertson *et al.* 1997). Intriguingly, in man, activation of seminal plasma latent TGF- β 1 appears to be delayed until triggered by the acidic environment of

the vagina (Nocera & Chu 1995, Chu *et al.* 1996). Although alterations in the profile of some of these cytokines is not recorded in the peripheral circulation, it has been noted that murine coitus results in a fall in serum interferon (IFN)- γ and interleukin (IL)-12 (p70) concentrations, paralleled by a rise in keratinocyte-derived chemokine (KC) and granulocyte-colony stimulating factor (G-CSF) levels in serum (Orsi *et al.* 2006). The immunomodulatory properties of seminal plasma have been highlighted by studies indicating that exposure to seminal fluid can: induce IL-1 β , IL-6 and leukaemia inhibitory factor (LIF) expression in human cultured endometrial epithelial cells (Gutsche *et al.* 2003); alter IL-8 and IL-10 release from human cervical explants, peripheral blood and monocyte cell lines *in vitro* (Denison *et al.* 1999); and elicit a rise in granulocyte macrophage-colony stimulating factor (GM-CSF) in murine uterine luminal epithelial cells (Robertson *et al.* 1996a). The alterations in cytokine ratios (e.g. IL-10:IL-12) activated by semen deposition are believed to exercise a key inhibitory control over vital immune defences in the lower genital tract, with ablation of cell-mediated responses and immunosurveillance (Kelly *et al.* 1997). A comprehensive review of the role of seminal plasma and male factor signalling in the maternal tract has recently been conducted by Robertson (2005).

In man, there has been extensive interest in seminal plasma cytokine profile, which has been characterised for IL-1 α/β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-11, IL-12, tumour necrosis factor (TNF)- α/β , IFN- α/γ , TGF- β 1, RANTES, CSF-1, G-CSF, macrophage-CSF, stem cell factor, monocyte chemotactic and activating factor, macrophage migration inhibitory factor and vascular endothelial growth factor as well as an array of their soluble receptors (Naz & Stanley 1995, Shimoya *et al.* 1995, Frenette *et al.* 1998, Fujisawa *et al.* 1998a,b, Matalliotakis *et al.* 1998a,b, 2002, Omu *et al.* 1998, Huleihel *et al.* 1999, Naz & Leslie 2000, Maegawa *et al.* 2002, Gutsche *et al.* 2003, Paulis *et al.* 2003, Basu *et al.* 2004). However, these studies have been limited in the array of cytokines investigated in individual patients, and their focus has principally been on the association of these inflammatory mediators with a variety of disorders resulting in male factor infertility and/or characterised by inflammatory processes. By contrast, surprisingly little is known about both the identity and the concentration of cytokines in murine seminal fluid, principally due to the small volume of analysable sample.

The present study therefore aimed to: (a) establish the physiological profiles of cytokines in murine seminal fluid from single animals, and their relative ratios, and (b) compare these profiles with those measured in serum. Fluid-phase multiplex immunoassays were used to overcome the difficulties associated with multiple analyses on small sample volumes from individual animals (Orsi *et al.* 2006). Seminal vesicle fluid was used since it was straightforward to collect, and because it is understood to contain the immunomodulatory factors that act on the maternal tract. There is no evidence to implicate the accessory

glands in this process as seminal vesicle-deficient males are unable to elicit the typical response exhibited by their entire counterparts in terms of GM-CSF rise in the female tract (Robertson *et al.* 1996a).

Materials and Methods

CD1 virgin male mice (10–12 weeks old; $n = 18$) were killed by cervical dislocation in compliance with the Animals (Scientific Procedures) Act, 1986. Seminal vesicles were dissected while avoiding exposure to fluid from the coagulating gland, and collection was carried out by gentle massaging. Since difficulties were anticipated in analysing pure seminal fluid due to its density, samples were immediately diluted with 200 μ l sterile PBS with 0.5% BSA (as recommended by A Liversage, BioRad Laboratories) and thoroughly vortexed for 45 s. This dilution was taken into account for each sample when determining sample-specific cytokine profiles. In parallel, serum was isolated from blood obtained by post-mortem cardiac puncture. Blood and seminal fluid samples were all centrifuged at 9000 r.p.m. for 3 min on a microcentrifuge (Micro Centaur, MSE Scientific, Loughborough, UK). The supernatant was frozen at -80°C until analysed simultaneously for the following 23 cytokines: IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, eotaxin, G-CSF, GM-CSF, IFN- γ , KC, MCP-1, MIP-1 α , MIP-1 β , RANTES and TNF- α . This was achieved by 23-plex fluid-phase immunoassay using custom kits (BioRad Laboratories) run on a Luminex-100 cytometer (Luminex Corporation, Austin, TX, USA), equipped with StarStation software (Applied Cytometry Systems, Dinnington, UK) (Vignali 2000, Powell *et al.* 2004). Serum diluent was used in all cases to avoid false positive/negatives and dilution adjusted to 1:1 in order to maximise sensitivity to baseline levels (Orsi *et al.* 2006). All samples were analysed in duplicate. Cytokine levels were expressed in picogrammes per millilitre. Biofluid cytokine ratios were determined to highlight the interrelationships between cytokines. In rare cases where no analyte was detected for a specific cytokine, ratios could not be performed for that individual animal. All data were expressed as means $\pm$ S.E.M. Data distributions were assessed by Anderson–Darling tests and significant differences between groups were determined using Student's *t* tests or Mann–Whitney *U* tests, as appropriate.

Results

Serum and seminal fluid cytokine profiles

The profiles of all cytokines analysed were detected in both biofluids, with the exception of serum IL-3, which was below the sensitivity level of the assay (Fig. 1). The lowest detectable serum cytokine concentrations were noted for IL-4 and RANTES (both <1 pg/ml); low levels were recorded for IL-2, IL-5, IL-6, IL-10 and MIP-1 β (5–20 pg/ml range); intermediate levels for IL-1 α , IL-12

Figure 1 Serum and seminal fluid concentrations in male CD1 mice: (a) IL-2, IL-6, IL-10, MIP-1 β , G-CSF, GM-CSF and KC; (b) IL-3, IL-4 and IL-5; (c) IL-1 α , IL-1 β , IL-12 (p70), IL-13, IL-17 and MIP-1 α ; (d) IL-12 (p40), IL-9, IFN- γ , MCP-1 and TNF- α ; (e) eotaxin and RANTES.

(p70), G-CSF, GM-CSF and KC (25–80 pg/ml); and higher levels were identified for IL-1 β , IL-13, IL-17, MIP-1 α and TNF- α (100–250 pg/ml). The most prevalent cytokines in serum were IL-9, IL-12 (p40), eotaxin, MCP-1 and IFN- γ (250–400 pg/ml).

The lowest levels detected in seminal fluid were for IL-3, IL-4 and IL-5 (all <1 pg/ml). Relatively low concentrations of IL-1 α , IL-2, IL-6, IL-12 (p40), IL-12 (p70), IL-17, MIP-1 β and GM-CSF (<12 pg/ml) were detected; IL-1 β , IL-9, IL-10, IL-13, G-CSF, MCP-1, MIP-1 α , KC, IFN- γ and TNF- α were intermediate (20–150 pg/ml range), while by far the most prevalent cytokines in seminal fluid were RANTES and eotaxin (500–900 pg/ml range).

Cytokine levels in serum and seminal fluid were markedly different: the majority were significantly higher in serum, as indicated for IL-1 α , IL-1 β , IL-2, IL-5, IL-9, IL-12 (p40), IL-12 (p70), IL-13, IL-17, GM-CSF, IFN- γ , MCP-1 and TNF- α . By contrast, only IL-4, G-CSF, KC, eotaxin and RANTES levels were significantly higher in seminal fluid. No significant differences in IL-6, IL-10 and MIP-1 α and MIP-1 β were detected between seminal plasma and serum.

Comparisons of serum and seminal fluid cytokine ratios

With very few exceptions, the ratios between the different cytokines were strikingly different between serum and seminal fluid (Tables 1 and 2). Because IL-3 serum levels were not quantified, serum ratios and seminal fluid comparisons are not available for this cytokine. Likewise, the single detectable serum RANTES sample prevented meaningful ratio comparisons between serum and seminal fluid.

Discussion

To the best of our knowledge, this study is the first study to characterise extensively the profile of 23 cytokines

(many of these novel) in the serum and seminal fluid of single mice using multiplex immunoassays. The serum cytokine concentrations reported here are comparable, in some respects, to those identified in C57/BL6 female mice during the oestrous cycle and in early pregnancy using multiplex immunoassays (Orsi *et al.* 2006). Noteworthy exceptions in CD1 males were: much higher MIP-1 α and IFN- γ levels; elevated IL-1 α , IL-17, TNF- α and GM-CSF concentrations; and lower IL-6, IL-10, G-CSF, RANTES and IL-12 (p40) profiles. Whether these effects are attributable to animal strain, gender, or both, remains unclear.

It is well documented that factors within seminal plasma act to enhance the receptivity of the maternal tract and immune system for the subsequent pregnancy. In particular, immunomodulatory moieties within murine seminal plasma elicit the relocation of antigen-presenting cells to the uterus where they participate in the presentation of paternal ejaculate antigens to activate lymphocytes (Robertson *et al.* 2003, O'Leary *et al.* 2004). Furthermore, they orchestrate leukocyte trafficking and activate uterine epithelial cytokine expression, such as GM-CSF and IL-6 (Robertson *et al.* 1992, Tremellen *et al.* 1998). Several of the cytokines found in murine seminal plasma have been credited with immunosuppressive activity, including IL-1, IL-6 and IL-8 (Kelly 1999, Robertson & Sharkey 2001).

Certain cytokine profiles were modest in both serum and semen – as noted for IL-2, IL-3, IL-4, IL-5 and IL-6. Although this may reflect their lesser importance in the early cytokine signalling induced by seminal plasma, these interleukins may nonetheless be involved in subsequent events associated with uterine priming and the establishment of pregnancy. In this respect, seminal plasma has been shown to elicit a 200-fold increase in IL-6 levels in oestrogen-primed uterine epithelial cells (Robertson *et al.* 1997), while the circulatory level of all these cytokines has been shown to increase significantly in the latter half of gestation (Orsi *et al.* 2006).

Table 1 Serum cytokine ratios (statistical comparisons with seminal fluid are indicated in Table 3. Ratios represent cytokines in columns divided by those in rows.

	IL-1 β	IL-2	IL-3	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 (p40)	IL-12 (p70)	IL-13	IL-17
IL-1 α	1.83 $\pm$ 0.06	0.15 $\pm$ 0.02	*	0.01 $\pm$ 0.01	0.08 $\pm$ 0.01	0.17 $\pm$ 0.04	4.54 $\pm$ 0.45	0.30 $\pm$ 0.06	4.96 $\pm$ 0.48	1.29 $\pm$ 0.16	2.91 $\pm$ 0.22	2.56 $\pm$ 0.42
IL-1 β	*	0.08 $\pm$ 0.01	*	0.01 $\pm$ 0.01	0.04 $\pm$ 0.01	0.09 $\pm$ 0.02	2.55 $\pm$ 0.31	0.16 $\pm$ 0.03	2.73 $\pm$ 0.25	0.71 $\pm$ 0.09	1.58 $\pm$ 0.12	1.39 $\pm$ 0.22
IL-2	*	*	*	0.07 $\pm$ 0.04	5.79 $\pm$ 3.62	1.81 $\pm$ 0.46	229.46 $\pm$ 135.12	10.71 $\pm$ 6.27	328.02 $\pm$ 176.45	56.91 $\pm$ 39.92	227.75 $\pm$ 186.40	121.79 $\pm$ 65.55
IL-3	*	*	*	*	*	*	*	*	*	*	*	*
IL-4	*	*	*	*	78.88 $\pm$ 13.16	126.46 $\pm$ 22.71	4906.65 $\pm$ 890.35	216.93 $\pm$ 22.09	5425.36 $\pm$ 880.93	1318.67 $\pm$ 202.10	3012.91 $\pm$ 486.63	2427.36 $\pm$ 337.04
IL-5	*	*	*	*	*	2.15 $\pm$ 0.57	69.38 $\pm$ 10.07	4.24 $\pm$ 0.97	80.01 $\pm$ 12.08	20.53 $\pm$ 4.29	39.90 $\pm$ 2.96	40.68 $\pm$ 8.20
IL-6	*	*	*	*	*	*	65.97 $\pm$ 19.99	2.35 $\pm$ 0.79	80.12 $\pm$ 24.34	11.42 $\pm$ 2.19	38.19 $\pm$ 13.46	25.49 $\pm$ 4.84
IL-9	*	*	*	*	*	*	*	0.07 $\pm$ 0.02	1.63 $\pm$ 0.41	0.30 $\pm$ 0.04	0.66 $\pm$ 0.07	0.72 $\pm$ 0.13
IL-10	*	*	*	*	*	*	*	*	25.33 $\pm$ 4.46	6.35 $\pm$ 0.94	15.17 $\pm$ 2.86	12.41 $\pm$ 1.52
IL-12 (p40)	*	*	*	*	*	*	*	*	*	0.29 $\pm$ 0.04	0.75 $\pm$ 0.17	0.55 $\pm$ 0.09
IL-12 (p70)	*	*	*	*	*	*	*	*	*	*	2.96 $\pm$ 0.52	2.05 $\pm$ 0.16
IL-13	*	*	*	*	*	*	*	*	*	*	*	1.06 $\pm$ 0.20

Table 2 Serum cytokine ratios (statistical comparisons with seminal fluid are indicated in Table 4).

	Eotaxin	G-CSF	GM-CSF	IFN- γ	KC	MCP-1	MIP-1 α	MIP-1 β	RANTES	TNF- α
IL-1 α	6.77 $\pm$ 0.46	0.48 $\pm$ 0.05	0.49 $\pm$ 0.04	6.23 $\pm$ 0.46	0.43 $\pm$ 0.02	4.91 $\pm$ 0.42	1.73 $\pm$ 0.18	0.26 $\pm$ 0.05	0.14	3.58 $\pm$ 0.42
IL-1 β	3.68 $\pm$ 0.24	0.27 $\pm$ 0.03	0.27 $\pm$ 0.02	3.41 $\pm$ 0.25	0.24 $\pm$ 0.01	2.70 $\pm$ 0.23	0.95 $\pm$ 0.09	0.14 $\pm$ 0.03	0.08	1.97 $\pm$ 0.23
IL-2	45.25 $\pm$ 6.29	47.62 $\pm$ 27.69	30.15 $\pm$ 18.07	574.95 $\pm$ 407.74	53.71 $\pm$ 33.64	449.23 $\pm$ 299.08	160.58 $\pm$ 96.03	4.63 $\pm$ 3.05	1.22	157.76 $\pm$ 89.14
IL-3	*	*	*	*	*	*	*	*	*	*
IL-4	7232.90 $\pm$ 1295.27	529.32 $\pm$ 98.73	510.04 $\pm$ 76.57	6792.30 $\pm$ 1169.26	520.20 $\pm$ 94.38	5485.62 $\pm$ 960.66	1740.93 $\pm$ 215.42	202.46 $\pm$ 30.23	17.76	3480.01 $\pm$ 447.94
IL-5	95.18 $\pm$ 6.77	8.20 $\pm$ 1.41	7.18 $\pm$ 0.59	91.65 $\pm$ 7.76	7.56 $\pm$ 1.32	76.16 $\pm$ 9.14	28.61 $\pm$ 4.36	3.48 $\pm$ 0.74	2.30	53.59 $\pm$ 7.62
IL-6	75.75 $\pm$ 20.58	9.18 $\pm$ 3.61	6.88 $\pm$ 1.88	87.33 $\pm$ 240.32	10.36 $\pm$ 5.04	72.61 $\pm$ 21.71	31.31 $\pm$ 13.45	2.01 $\pm$ 0.78	0.25	41.10 $\pm$ 10.21
IL-9	1.57 $\pm$ 0.16	0.17 $\pm$ 0.06	0.13 $\pm$ 0.01	1.65 $\pm$ 0.22	0.18 $\pm$ 0.08	1.37 $\pm$ 0.25	0.67 $\pm$ 0.23	0.06 $\pm$ 0.01	0.04	0.88 $\pm$ 0.11
IL-10	33.38 $\pm$ 5.01	2.80 $\pm$ 0.87	2.58 $\pm$ 0.43	34.71 $\pm$ 6.67	2.39 $\pm$ 0.58	28.97 $\pm$ 6.55	9.04 $\pm$ 1.24	0.96 $\pm$ 0.13	0.15	18.35 $\pm$ 2.67
IL-12 (p40)	1.59 $\pm$ 0.22	0.11 $\pm$ 0.01	0.12 $\pm$ 0.02	1.61 $\pm$ 0.34	0.11 $\pm$ 0.02	1.41 $\pm$ 0.43	0.40 $\pm$ 0.06	0.05 $\pm$ 0.01	0.03	0.85 $\pm$ 0.18
IL-12 (p70)	6.38 $\pm$ 0.74	0.46 $\pm$ 0.07	0.49 $\pm$ 0.07	6.34 $\pm$ 0.88	0.43 $\pm$ 0.07	5.07 $\pm$ 0.78	1.57 $\pm$ 0.19	0.18 $\pm$ 0.02	0.12	3.23 $\pm$ 0.29
IL-13	2.46 $\pm$ 0.16	0.19 $\pm$ 0.03	0.19 $\pm$ 0.01	2.30 $\pm$ 0.08	0.16 $\pm$ 0.02	1.82 $\pm$ 0.10	0.68 $\pm$ 0.10	0.09 $\pm$ 0.02	0.06	1.40 $\pm$ 0.15
IL-17	3.41 $\pm$ 0.45	0.28 $\pm$ 0.05	0.26 $\pm$ 0.03	3.38 $\pm$ 0.46	0.28 $\pm$ 0.06	2.75 $\pm$ 0.39	0.88 $\pm$ 0.14	0.09 $\pm$ 0.01	0.05	1.62 $\pm$ 0.17
Eotaxin	*	0.08 $\pm$ 0.01	0.08 $\pm$ 0.01	0.99 $\pm$ 0.06	0.07 $\pm$ 0.01	0.80 $\pm$ 0.08	0.27 $\pm$ 0.03	0.04 $\pm$ 0.01	0.02	0.57 $\pm$ 0.05
G-CSF	*	*	1.20 $\pm$ 0.16	15.53 $\pm$ 2.23	1.09 $\pm$ 0.14	12.93 $\pm$ 2.54	3.98 $\pm$ 0.47	0.55 $\pm$ 0.09	0.17	8.65 $\pm$ 1.26
GM-CSF	*	*	*	12.93 $\pm$ 0.66	1.11 $\pm$ 0.25	10.51 $\pm$ 0.76	4.13 $\pm$ 0.71	0.46 $\pm$ 0.08	0.27	7.08 $\pm$ 0.58
IFN- γ	*	*	*	*	0.08 $\pm$ 0.02	0.81 $\pm$ 0.04	0.33 $\pm$ 0.05	0.04 $\pm$ 0.01	0.03	0.57 $\pm$ 0.05
KC	*	*	*	*	*	11.85 $\pm$ 1.09	4.41 $\pm$ 0.59	0.65 $\pm$ 0.14	0.42	8.81 $\pm$ 1.16
MCP-1	*	*	*	*	*	*	0.40 $\pm$ 0.05	0.05 $\pm$ 0.01	0.04	0.74 $\pm$ 0.08
MIP-1 α	*	*	*	*	*	*	*	0.17 $\pm$ 0.05	0.07	2.60 $\pm$ 0.55
MIP-1 β	*	*	*	*	*	*	*	*	0.46	29.84 $\pm$ 7.71
RANTES	*	*	*	*	*	*	*	*	*	25.09

By contrast, other cytokines were present at comparatively high concentrations in both serum and semen; namely eotaxin, MIP- α , IL-1 β and IL-9. However, while eotaxin and MIP-1 α consistently displayed proportionally higher ratios in semen than serum, only eotaxin levels were significantly higher in seminal plasma. In this respect, at oestrus, IL-5 recruits circulatory eosinophils into the uterine stroma, while the presence of ovarian steroids stimulates the synthesis of endometrial eotaxin, RANTES and MIP-1 α (Robertson *et al.* 2000) (Fig. 2). In turn, these elicit the extravasation and movement of eosinophils through the stroma to the epithelium (Robertson *et al.* 2000, Kayisli *et al.* 2002). In the presence of semen their abundance and proximity to the luminal surface peaks. Speculatively, high seminal eotaxin and RANTES levels may further operate directly on these eosinophils as they express – at least – an eotaxin-specific receptor which may regulate their selective recruitment (Mould *et al.* 1997). Unlike in man, murine coitus results in the deposition of semen directly into the uterus where, as allogenic material, it triggers an acute inflammatory response (Wood *et al.* 1997). Thus, seminal fluid constituents – potentially including eotaxin, MIP- α , IL-1 β and IL-9 – initiate a surge in the synthesis of other cytokines (e.g. IL-6 and GM-CSF) which subsequently elicits the recruitment and activation of antigen-presenting cells into

the endometrial stroma where they engulf and process paternal ejaculate antigens. Some of these may be eosinophils, which have recognised phagocytic and antigen-processing functions (Strath & Sanderson 1985, Xie *et al.* 2005). Intriguingly, the present data do not support a role for seminal IL-13 in eosinophil recruitment (Wills-Karp 1999). These then relocate to the para-aortic lymph nodes which drain the uterus (Robertson *et al.* 2003). In parallel, potentially in response to the array of cytokines described in this study, granulocytes and macrophages accumulate in the uterus to clear sperm and seminal debris (Wood *et al.* 1997). Thus, these cytokines, together with seminal TGF- β 1, may contribute to the localised inflammatory response which (a) acts to prime the uterus for subsequent embryo paracrine interactions, implantation and fetal growth, and (b) prepares the maternal immune system for exposure to paternal antigens, thus ensuring the successful establishment of pregnancy.

In addition, seminal fluid was also characterised by higher levels of IL-4 and G-CSF compared with serum, suggesting that these too may have a role, as yet undefined, in mating-induced immunomodulation. In man, IL-4 is thought to be necessary for the establishment and maintenance of pregnancy, by avoiding the harmful effects of cell-mediated immunity in the vicinity of putative embryo implantation sites and at the fetomaternal

Figure 2 Seminal plasma-induced immunomodulation. Seminal cytokines attract eosinophils (E) to the uterine luminal surface following their chemotactic relocation from the circulation in response to endometrial cytokines. Seminal cytokines may also contribute to neutrophil (N) chemoattraction while seminal antigens are processed by dendritic cells. These relocate to the para-aortic lymph nodes and induce the activation, proliferation and differentiation of T-cell (T) subsets. These subsequently relocate into the uterine endometrium by the time of embryo implantation, by which time macrophages (M) have cleared residual seminal debris.

interface (Lin *et al.* 1993, Piccinini 2005). Similarly, G-CSF, which participates in subsequent placental granulocytosis, can modulate the production of deleterious levels of IFN- γ (which we report to be comparatively low in seminal fluid), which is known to impair human trophoblast cell growth and function *in vitro* and to cause abortion in mice (Sugita *et al.* 2003; reviewed by Daher *et al.* 2004).

Seminal fluid and serum cytokines were expressed as ratios in order to further highlight their differences (Tables 3 and 4). Expressing cytokine profiles in this way is useful as these glycoproteins operate in a network system that involves numerous inhibitory and synergistic interactions. The evident ratio differences found in serum and seminal fluid probably reflect their differing specific functions. In particular, the most pronounced differences in serum and seminal ratios were noted for KC, RANTES (proportionally higher in semen), IL-17 and IL-12 (p40) (which showed the inverse relationship). The relatively high proportion (and indeed overall levels) of KC to other cytokines in seminal fluid compared with serum may belie its function in the chemotaxis and activation of neutrophils associated with murine coitus (Knudsen *et al.* 2002). Indeed, it has been reported that the most noteworthy effect of mating seminal vesicle-deficient stud mice was a complete absence of neutrophils in the uterine luminal cavity (Robertson *et al.* 1996b). We propose that this effect may be attributable, at least in part, to an absence of RANTES, eotaxin, G-CSF and KC in these animals, which would normally orchestrate neutrophil relocation to the site of seminal deposition. With respect to RANTES, in addition to its role in endometrial eosinophil recruitment, high levels may participate in immunomodulation of antigenicity of sperm cells in the male genital tract prior to ejaculation, and perhaps also in the female tract following mating (Naz & Leslie 2000, Kayisli *et al.* 2002). By contrast, the proportionally lower seminal fluid IL-17 levels (reflected in the ratios) may be accounted for by its adverse pro-inflammatory properties which – while participating in the induction of IL-1 β , IL-6, IL-8, G-CSF and MCP-1 – are actually associated with transplant rejection and other disorders of inflammatory origin (Fossiez *et al.* 1998, Zhang *et al.* 2005). Thus, the low levels of IL-17 we have identified in semen may be conducive to developing the immunopermissive environment required for tolerance of the conceptus upon its entry into the uterus. The exact meaning of the strikingly lower IL-12 (p40) ratios (and levels) in semen is likely to be very complex. As well as being a subunit of other cytokines (e.g. IL-12 (p70) and IL-23), it acts as an antagonist of the p70 heterodimer. However, high levels of IL-12 (p40) have been associated with an acceleration of allograft rejection *in vivo* (Sun *et al.* 2004), thereby suggesting that low levels of IL-12 (p40) may favour successful implantation as outlined for IL-17.

All cytokine profiles were determined on a homogeneous population of virgin CD1 male mice. Although these values are no doubt representative of real differences

Table 3 Seminal fluid cytokine ratios.

	IL-1 β	IL-2	IL-3	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 (p40)	IL-12 (p70)	IL-13	IL-17
IL-1 α	17.90 $\pm$ 4.74	0.47 $\pm$ 0.10	0.07 $\pm$ 0.02	0.02 $\pm$ 0.01	0.11 $\pm$ 0.03	0.71 $\pm$ 0.21	32.57 $\pm$ 11.50	3.61 $\pm$ 1.01	1.15 $\pm$ 0.38	2.16 $\pm$ 0.52	4.24 $\pm$ 1.05	0.96 $\pm$ 0.30
IL-1 β	*	0.03 $\pm$ 0.01	0.00 $\pm$ 0.01	0.00 $\pm$ 0.01	0.01 $\pm$ 0.01	0.04 $\pm$ 0.01	1.91 $\pm$ 0.58	0.21 $\pm$ 0.02	0.06 $\pm$ 0.01	0.13 $\pm$ 0.01	0.24 $\pm$ 0.01	0.05 $\pm$ 0.01
IL-2	*	*	0.16 $\pm$ 0.02	0.05 $\pm$ 0.01	0.24 $\pm$ 0.02	1.52 $\pm$ 0.12	80.21 $\pm$ 29.17	7.54 $\pm$ 0.60	2.47 $\pm$ 0.31	4.99 $\pm$ 0.53	9.31 $\pm$ 0.93	1.96 $\pm$ 0.21
IL-3	*	*	*	0.32 $\pm$ 0.04	1.56 $\pm$ 0.08	10.05 $\pm$ 0.45	450.00 $\pm$ 125.12	51.57 $\pm$ 3.88	15.16 $\pm$ 0.88	31.27 $\pm$ 0.54	59.31 $\pm$ 2.56	13.28 $\pm$ 1.27
IL-4	*	*	*	*	5.32 $\pm$ 0.52	34.50 $\pm$ 2.82	1.510.37 $\pm$ 429.10	182.35 $\pm$ 24.60	52.07 $\pm$ 4.92	105.98 $\pm$ 7.38	197.21 $\pm$ 11.46	46.98 $\pm$ 7.19
IL-5	*	*	*	*	*	6.64 $\pm$ 0.29	348.77 $\pm$ 33.30	33.30 $\pm$ 2.34	10.20 $\pm$ 0.84	21.15 $\pm$ 1.19	39.94 $\pm$ 2.09	8.36 $\pm$ 0.81
IL-6	*	*	*	*	*	*	51.13 $\pm$ 18.44	5.12 $\pm$ 0.37	1.56 $\pm$ 0.12	3.23 $\pm$ 0.19	6.14 $\pm$ 0.39	1.30 $\pm$ 0.13
IL-9	*	*	*	*	*	*	*	0.20 $\pm$ 0.02	0.05 $\pm$ 0.01	0.11 $\pm$ 0.01	0.21 $\pm$ 0.02	0.05 $\pm$ 0.01
IL-10	*	*	*	*	*	*	*	*	0.34 $\pm$ 0.05	0.70 $\pm$ 0.08	1.32 $\pm$ 0.13	0.25 $\pm$ 0.01
IL-12 (p40)	*	*	*	*	*	*	*	*	*	2.20 $\pm$ 0.14	4.22 $\pm$ 0.32	0.93 $\pm$ 0.12
IL-12 (p70)	*	*	*	*	*	*	*	*	*	*	1.91 $\pm$ 0.06	0.42 $\pm$ 0.05
IL-13	*	*	*	*	*	*	*	*	*	*	*	0.22 $\pm$ 0.03

Shaded boxes highlight significantly different ratios from those reported in serum: light grey, $P < 0.05$; mid-grey, $P < 0.01$; dark grey, $P < 0.001$.

Table 4 Seminal fluid cytokine ratios.

	Eotaxin	G-CSF	GM-CSF	IFN- γ	KC	MCP-1	MIP-1 α	MIP-1 β	RANTES	TNF- α
IL-1 α	184.56 $\pm$ 52.84	9.35 $\pm$ 1.98	0.87 $\pm$ 0.24	9.76 $\pm$ 2.61	8.10 $\pm$ 2.42	6.06 $\pm$ 1.42	24.57 $\pm$ 6.36	1.33 $\pm$ 0.42	163.37 $\pm$ 50.39	20.73 $\pm$ 5.22
IL-1 β	10.43 $\pm$ 0.65	0.56 $\pm$ 0.03	0.05 $\pm$ 0.01	0.56 $\pm$ 0.03	0.44 $\pm$ 0.04	0.35 $\pm$ 0.01	1.39 $\pm$ 0.06	0.07 $\pm$ 0.01	8.19 $\pm$ 1.11	1.21 $\pm$ 0.03
IL-2	404.90 $\pm$ 49.24	22.58 $\pm$ 3.36	1.94 $\pm$ 0.22	21.71 $\pm$ 2.50	17.63 $\pm$ 2.23	13.90 $\pm$ 1.58	56.26 $\pm$ 7.20	2.63 $\pm$ 0.37	356.10 $\pm$ 70.95	47.93 $\pm$ 5.50
IL-3	2508.71 $\pm$ 140.93	134.82 $\pm$ 7.99	12.06 $\pm$ 0.31	136.70 $\pm$ 6.65	111.04 $\pm$ 11.04	87.23 $\pm$ 3.75	342.49 $\pm$ 16.71	17.42 $\pm$ 2.13	2065.90 $\pm$ 316.28	297.46 $\pm$ 7.99
IL-4	8420.08 $\pm$ 549.71	449.53 $\pm$ 31.09	40.76 $\pm$ 2.93	451.42 $\pm$ 27.96	356.82 $\pm$ 29.40	291.80 $\pm$ 18.52	1133.61 $\pm$ 73.40	61.87 $\pm$ 9.76	6690.93 $\pm$ 975.76	1001.66 $\pm$ 67.80
IL-5	1741.26 $\pm$ 142.13	93.15 $\pm$ 7.41	8.04 $\pm$ 0.41	92.76 $\pm$ 6.25	72.96 $\pm$ 6.44	58.69 $\pm$ 2.95	232.62 $\pm$ 17.12	10.95 $\pm$ 1.42	1389.11 $\pm$ 217.73	202.42 $\pm$ 12.94
IL-6	264.75 $\pm$ 20.20	14.27 $\pm$ 1.20	1.23 $\pm$ 0.07	14.09 $\pm$ 0.95	11.33 $\pm$ 1.09	9.08 $\pm$ 0.63	35.84 $\pm$ 2.95	1.67 $\pm$ 0.22	212.71 $\pm$ 31.83	30.91 $\pm$ 2.03
IL-9	8.59 $\pm$ 0.85	0.49 $\pm$ 0.06	0.04 $\pm$ 0.01	0.49 $\pm$ 0.05	0.39 $\pm$ 0.04	0.32 $\pm$ 0.03	1.21 $\pm$ 0.14	0.06 $\pm$ 0.01	6.53 $\pm$ 1.25	1.08 $\pm$ 0.12
IL-10	59.16 $\pm$ 8.21	3.14 $\pm$ 0.39	0.27 $\pm$ 0.03	3.07 $\pm$ 0.35	2.49 $\pm$ 0.34	1.93 $\pm$ 0.17	7.90 $\pm$ 1.02	0.37 $\pm$ 0.07	50.86 $\pm$ 9.87	6.70 $\pm$ 0.75
IL-12 (p40)	178.46 $\pm$ 13.74	9.52 $\pm$ 0.68	0.83 $\pm$ 0.04	9.62 $\pm$ 0.71	7.38 $\pm$ 0.57	6.16 $\pm$ 0.42	23.62 $\pm$ 1.41	1.14 $\pm$ 0.16	133.43 $\pm$ 17.68	20.79 $\pm$ 1.28
IL-12 (p70)	81.64 $\pm$ 4.07	4.37 $\pm$ 0.22	0.38 $\pm$ 0.01	4.36 $\pm$ 0.16	3.50 $\pm$ 0.29	2.81 $\pm$ 0.09	10.90 $\pm$ 0.37	0.54 $\pm$ 0.07	65.39 $\pm$ 8.91	9.50 $\pm$ 0.15
IL-13	43.33 $\pm$ 2.29	2.32 $\pm$ 0.14	0.20 $\pm$ 0.01	2.30 $\pm$ 0.08	1.85 $\pm$ 0.15	1.48 $\pm$ 0.04	5.79 $\pm$ 0.26	0.30 $\pm$ 0.04	34.54 $\pm$ 4.88	5.07 $\pm$ 0.18
IL-17	271.82 $\pm$ 49.98	14.12 $\pm$ 2.26	1.18 $\pm$ 0.17	13.79 $\pm$ 2.17	11.32 $\pm$ 2.08	8.61 $\pm$ 1.16	35.64 $\pm$ 6.16	1.62 $\pm$ 0.35	238.28 $\pm$ 54.48	29.94 $\pm$ 4.64
Eotaxin	*	0.06 $\pm$ 0.01	0.01 $\pm$ 0.01	0.06 $\pm$ 0.01	0.04 $\pm$ 0.01	0.04 $\pm$ 0.01	0.14 $\pm$ 0.01	0.01 $\pm$ 0.01	0.79 $\pm$ 0.11	0.12 $\pm$ 0.01
G-CSF	*	*	0.09 $\pm$ 0.01	1.03 $\pm$ 0.05	0.82 $\pm$ 0.07	0.66 $\pm$ 0.03	2.56 $\pm$ 0.11	0.14 $\pm$ 0.02	14.78 $\pm$ 1.98	2.26 $\pm$ 0.11
GM-CSF	*	*	*	11.47 $\pm$ 0.48	9.13 $\pm$ 0.74	7.36 $\pm$ 0.26	28.51 $\pm$ 0.96	1.41 $\pm$ 0.18	167.44 $\pm$ 22.00	24.94 $\pm$ 0.58
IFN- γ	*	*	*	*	0.81 $\pm$ 0.08	0.66 $\pm$ 0.06	2.55 $\pm$ 0.11	0.13 $\pm$ 0.06	15.31 $\pm$ 0.42	2.23 $\pm$ 0.14
KC	*	*	*	*	*	0.87 $\pm$ 0.06	3.36 $\pm$ 0.21	0.18 $\pm$ 0.03	18.75 $\pm$ 2.49	3.00 $\pm$ 0.22
MCP-1	*	*	*	*	*	*	3.92 $\pm$ 0.16	0.20 $\pm$ 0.03	23.04 $\pm$ 3.07	3.44 $\pm$ 0.11
MIP-1 α	*	*	*	*	*	*	*	0.05 $\pm$ 0.01	5.74 $\pm$ 0.70	0.89 $\pm$ 0.03
MIP-1 β	*	*	*	*	*	*	*	*	204.24 $\pm$ 62.07	25.29 $\pm$ 5.36
RANTES	*	*	*	*	*	*	*	*	*	0.24 $\pm$ 0.04

Shaded boxes highlight significantly different ratios from those reported in serum: light grey, $P < 0.05$; mid-grey, $P < 0.01$; dark grey, $P < 0.001$. No Statistical analyses were performed for IL-3 and RANTES due to their absent/poor detection levels in serum.

in cytokine profile between serum and seminal fluid, and highlight which ones are likely candidates in mating-induced immunomodulation, the reported values may differ from those of older stud mice. In this respect it has been reported that male potency to elicit an immunomodulatory response in the female tract is linked to stud sexual status and maturity (Robertson *et al.* 1996b); older, more experienced males may thus exhibit slightly different cytokine profiles to those reported herein.

Although the array of cytokines investigated in the present study does not include all the potential participants in mating-induced immunomodulation (e.g. TGF- β_1), these findings nevertheless greatly extend our understanding of the immunoregulatory mediators of early pregnancy. The difficulty associated with investigating these factors *in vivo* relates to their dynamic interactions and often short-lived increases in concentration. However, our limited understanding of organ culture and immune/other somatic cell functions in culture also limits how representative *in vitro* models of cytokine-leukocyte networks are likely to be. Due to the inhibitory/synergistic *modus operandi* of many elements of the cytokine network, multiplex immunoassays will probably prove to be a useful tool in clarifying the mechanisms underlying many immunological phenomena including the establishment of pregnancy, pseudopregnancy (in rodents), miscarriage, pre-eclampsia and susceptibility to sexually transmitted diseases. In this respect, species-specific differences in the production site for seminal plasma cytokines will have to be taken into consideration.

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