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Altered cytokine profile, pain sensitivity, and stress responsivity in mice with co-disruption of the developmental genes *Neuregulin-1* × *DISC1*

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Highlights

- Anxiety and stress responsivity phenotypes were examined in male and female mutant mice with simultaneous disruption of schizophrenia-associated genes NRG1 and DISC1
- Mice with partial knockout of NRG1 displayed reduced anxiety-related behaviour and stress-reactivity
- Disruption of DISC1, but not of NRG1, in the absence of epistasis, was associated with increased basal serum pro-inflammatory cytokine levels
- Partial NRG1 deletion in combination with DISC1 mutation diminished the DISC1-specific increases in the pro-inflammatory cytokines IL6 and TNF-α

Abstract

The complex genetic origins of many human disorders suggest that epistatic (gene × gene) interactions may contribute to a significant proportion of their heritability estimates and phenotypic heterogeneity. Simultaneous disruption of the developmental genes and schizophrenia risk factors Neuregulin-1 (NRG1) and Disrupted-in-schizophrenia 1 (DISC1) in mice has been shown to produce disease-relevant and domain-specific phenotypic profiles different from that observed following disruption of either gene alone. In the current study, anxiety and stress responsivity phenotypes in male and female mutant mice with simultaneous disruption of DISC1 and NRG1 were examined. NRG1 × DISC1 mutant mice were generated and adult mice from each genotype were assessed for pain sensitivity (hot plate and tail flick tests), anxiety (light-dark box), and stress-induced hypothermia. Serum samples were assayed to measure circulating levels of pro-inflammatory cytokines. Mice with the NRG1 mutation, irrespective of DISC1 mutation, spent significantly more time in the light chamber, displayed increased core body temperature following acute stress, and decreased pain sensitivity. Basal serum levels of cytokines IL8, IL1β and IL10 were decreased in NRG1 mutants. Mutation of DISC1, in the absence of epistatic interaction with NRG1, was associated with increased serum levels of IL1β. Epistatic effects were evident for IL6, IL12 and TNFα. NRG1 mutation alters stress and pain responsivity, anxiety, and is associated with changes in basal cytokine levels.

Epistasis resulting from synergistic NRG1 and DISC1 gene mutations altered pro-inflammatory cytokine levels relative to the effects of each of these genes individually, highlighting the importance of epistatic mechanisms in immune-related pathology.

Keywords: Neuregulin-1; Disrupted-in-Schizophrenia-1; Anxiety; Stress Responsivity; Cytokines

Introduction

Despite recent advances in our understanding of the critical pathophysiological mechanisms underlying genetic risk for schizophrenia and other neuropsychiatric disorders [22, 24], elucidating the contribution of factors including genetic heterogeneity, gene × environment (G × E), and gene × gene (G × G) interactions remains a challenge [11, 15]. Andreasen and colleagues investigated the significance of G × G interactions in relation to the contribution of candidate susceptibility genes to the development of schizophrenia [2]. These authors identified 11 interactions involving 5 genes and 17 SNPs (5 of which had been previously identified as schizophrenia vulnerability markers or implicate cognitive deficits in schizophrenia) that had a significant relationship with the emergence of schizophrenia-related anatomical endophenotypes in at least two brain regions. These interactions included interactions between susceptibility genes ErbB4 and disrupted-in-schizophrenia-1 (DISC1), ErbB4 and PDE4B, and neuregulin-1 (NRG1) and RELN [2].

Employing a preclinical genetic strategy, we have recently reported that simultaneous disruption of the developmental genes and schizophrenia risk factors NRG1 and DISC1 in mice produced a disease-relevant and domain-specific phenotypic profile different from that

observed following disruption of either gene alone. Specifically, co-disruption of NRG1 and DISC1, indicative of epistasis, produced impairment in sociability and increased social anxiety, with these behavioural changes accompanied by changes in hypothalamic oxytocin and vasopressin gene expression, two neuropeptides implicated in mammalian social behaviour [37].

Stressful events have been linked to the developmental course of schizophrenia and symptomatic exacerbation, although less is known about how stress reactivity interacts with genetic risk to promote disease trajectory [18, 27]. Higher levels of pro-inflammatory cytokines are associated with increased risk for schizophrenia [19], and patients with schizophrenia exhibit abnormal immune function, which can manifest as imbalance in basal cytokine levels [7, 34]. The active phase of schizophrenia is associated with chronic, low-grade inflammatory change that is largely attenuated by effective treatment [30]. Recent re-conceptualisations of 'stress-vulnerability' models of schizophrenia have emphasized how early life adversity, as well as acute and chronic biological insult, and exposure to psychosocial stressors, at critical developmental periods, appear to interact with genetic background to determine risk for psychotic illness [15, 43, 44].

If the relative influence of a given gene on risk for an endophenotype of the overall schizophrenia syndrome depends upon exposure to one or more adverse environmental stressor(s), behavioural phenotyping strategies in risk factor mutants need to integrate assessment of relevant phenotypes following acute or chronic exposure to such stressor(s). To that end, in the current study, we examined anxiety and stress responsivity phenotypes in male and female mutant mice with simultaneous disruption of NRG1 and DISC1.

Methods

Mice

NRG1 × DISC1 mutant mice were generated by intercrossing the heterozygous NRG1 mutant line with the DISC1 (100P) mutant line and genotyping resultant offspring, as described previously [37]. Heterozygous NRG1 and DISC1 mutants were crossed and offspring heterozygous for *both* NRG1 and DISC1 were then used as breeding pairs to generate the following experimental groups involving wildtype (WT), heterozygous (HET) and homozygous (HOM) genotypes: $NRG1^{WT}$/ $DISC1^{WT}$, $NRG1^{WT}$/$DISC1^{HET}$, $NRG1^{WT}$/$DISC1^{HOM}$, $NRG1^{HET}$/$DISC1^{WT}$, $NRG1^{HET}$/$DISC1^{HET}$, $NRG1^{HET}$/$DISC1^{HOM}$; mice homozygous for the NRG1 mutation, with or without disruption of DISC1, are conceived but do not survive postnatally due to cardiac defects. Mice were housed in groups of three to five per cage and maintained on a standard 12⁄12 h light⁄dark cycle (08:00 h on; 20:00 h off) with *ad libitum* access to food and water. For all behavioural testing, the same testing cohort was used, and animals were tested sequentially as follows: hot plate test, tail flick test, light-dark test, stress-induced hypothermia. Serum cytokine analysis was completed in a separate cohort of experimental animals. Table 1 provides a summary of group sizes. These studies were approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland and were conducted under licence from the Department of Health and Children in accordance with Irish legislation and the European Communities Council Directive 86/609/EEC for the care and use of experimental animals, and from the Environmental Protection Agency in relation to the contained use of genetically modified organisms.

Hot plate / tail flick test

Thermal nociception measurements were conducted using the hot plate test (model 7280, Ugo Basile, Comerio, Italy) and the tail flick assay (model 7360, Ugo Basile, Comerio, Italy) as

described previously [45]. In the hot plate test, the temperature was 55° C with a cut-off time of 30 s; the mouse was placed on the hot plate and latency to lick a hindpaw or jump was measured. In the tail flick assay, all mice were habituated to a restraint tube (60 ml syringe, Becton Dickinson, Dublin, Ireland) for 20 min, 24 h prior to testing. On the day of testing, mice were placed in the same restraint for 10 min, after which the mouse, in the tube, was placed on the tail flick unit such that a point on the tail approximately 15 mm from the tip was located above the light source. This source, set to an intensity of 90 (range 10–99), was activated by the investigator and latency to flick the tail away from the light beam measured, with a cut-off time of 8 s. For both tests, latency measures were assumed to reflect thermal pain sensitivity. For each mouse, the hot plate test was performed 30 min prior to the tail flick assay. All assessments were carried out by an investigator who was blind to genotype.

Light-dark test

Mice were placed into a test chamber (43 × 43 × 33 cm) with a white Plexiglas floor and clear walls (ENV-515-16; Med Associates, St Albans, VT, USA); infrared detection beams on the x-, y- and *z*-axes track the mouse position and activity over the course of the experiment as described previously [16]. The chamber was equipped with a light-impermeable dark box insert, which covered half the area of the chamber. The light and dark compartments were connected via a small hole in the partition wall. Each compartment was differentially illuminated. A LED lamp (containing 9 × white Nichia LED bulbs, > 15 lux) was placed 30 cm above the light chamber compartment. The time spent in, entries into, and ambulatory time and counts in the light *vs* dark compartments were recorded for 10 min.

Stress-induced hyperthermia

All experimental mice were single housed during 24 h prior to test, with *ad libitum* access to food and water. The procedure was adapted from that described previously [14]. Rectal temperature was measured in each mouse twice (T1 = 0 min; T2 = +15 min), both before and after an acute restraint session in a restraint tube (60 ml syringe, Becton Dickinson, Dublin, Ireland). Stress-induced hyperthermia is measured as the difference in temperature between T1 and T2.

Cytokine analysis Serum samples were analysed for IFN-γ, IL1β, TNF-α, IL6, IL8 and IL10 using a multiplex cytokine immunoassay (MSD® 96-Well MULTI-SPOT kit, Meso Scale Discovery, Maryland, USA) [17]. 25 µl of each sample was assayed in duplicate according to manufacturers' instructions. Briefly, samples and standards were incubated in multi-spot wells under vigorous shaking (900 rpm) for 2 hr at room temperature. 25 µl of detection antibody was added and incubated for a further 2 hr. Plates were then washed with PBS (+0.05% Tween), 150 µl of Read Buffer was added and plates analysed by electrochemiluminescent detection using a SECTOR Imager. Concentrations were extrapolated from an 8-point standard curve ranging from 0-2500 pg/ml. Assay detection limits for each cytokine were as follows: IFN-γ, 0.5-2 pg/ml; IL1β, 0.5-2 pg/ml; IL6, 5-20 pg/ml; IL8, 5-20 pg/ml; IL10, 5-20 pg/ml; TNF-α, 0.5-2 pg/ml.

Data Analysis

Statistical analysis was carried using procedures similar to those described previously [37]. Group differences were assessed using between-subjects two-way ANOVA with main factors of genotype and sex. *Post-hoc* comparisons were carried out using independent t-tests, in which the p value was adjusted (p adj < 0.05) by Holm-Sidak's method. Where the data were not

normally distributed, analyses were conducted following square-root transformation. All results are presented as means ± SEM. A p value < 0.05 was considered significant. Statistical analyses were performed using the Statistical Package for the Social Sciences program 19.0 (SPSS, Chicago, IL).

Results

Hot Plate and Tail Flick Tests. $NRG1^{HET}$ demonstrated increased hot plate latency, indicative of decreased pain sensitivity [NRG1, $F(1, 81) = 6.25$, $p < 0.05$; Fig 1A]. No effects of DISC1 genotype, sex, or interactions, were evident for either the hot plate or tail flick tests [Fig 1B].

Light-Dark Test. In the light-dark test, where longer time in the brightly lit chamber signifies reduced anxiety-related behaviour, $NRG1^{HET}$ spent more ambulatory time in the light chamber [NRG1, $F(1, 82) = 5.21$, $p = 0.02$; NRG1 × sex, $F(1, 82) = 5.58$, $p = 0.02$; Fig 1C], more so in females than in males. No DISC1 genotypic effect, sex, or genotype × sex or interaction was evident.

Stress-induced Hyperthermia. Stress-induced hyperthermia is a hallmark of the stress response in mammals. There were no genotypic differences in change in basal body temperature at baseline [T1]. $NRG1^{HET}$ demonstrated attenuation in the increase in body temperature following 1 h of restraint stress [NRG1, $F(1,81) = 6.06$, $p = 0.02$; Fig 1D]. No DISC1 genotypic effect or genotype × sex interactions were evident.

Serum Cytokine Levels. Basal serum levels of cytokines IL8 and IL10 were decreased in female $NRG1^{HET}$ [IL8: NRG1 × sex, $F(1,52) = 12.66$, $p < 0.001$; IL10: NRG1 × sex, $F(1,52)$

= 8.45, $p < 0.005$]. IL1β levels were selectively decreased in male NRG1^{HET} [NRG1 × sex, $F(1,52) = 30.42$, $p < 0.001$], and decreased in DISC1 mutants of both sexes [DISC1, $F(2,52) = 11.16$, $p < 0.001$]. While levels of IL6 were reduced in female NRG1^{HET} but not in those with disruption of DISC1, the change in IL6 levels in female NRG1^{HET} varied with extent of co-disruption of DISC1 [NRG1 × DISC1 × sex, $F(2,52) = 14.70$, $p < 0.001$]. A decrease in levels of IL12 in male DISC1 mutants was reversed to an increase on co-disruption of NRG1 [NRG1 × DISC1 × sex, $F(2,49) = 6.02$, $p < 0.005$]. While TNF-α levels were increased in female NRG1^{HET} but decreased in male NRG1^{HET} [NRG1 × sex, $F(1,52) = 25.15$, $p < 0.001$], an increase in levels of TNF-α in male DISC1 mutants was reversed to a decrease on co-disruption of NRG1 [NRG1 × DISC1 × sex, $F(2,52) = 15.23$, $p < 0.001$; see Table 2].

Discussion

Mice containing the NRG1 mutation displayed reduced anxiety-related behaviour and stress-reactivity across several measures; these data are consistent with reports that NRG1 variation modulates behavioural and neuroendocrine responses to acute or chronic exposure to both physical and psychological stressors [17, 28, 41, 42]. The reduced heat pain sensitivity demonstrated by mice with disruption of NRG1, as indicated by increased latency in the hot plate test, is also in accordance with a previous report [45] and confirms that NRG1 has pro-nociceptive functions.

There is accumulating evidence that NRG1 also plays an important regulatory and neuroprotective role in the peripheral and central immune system [33, 46]. It has been shown that a schizophrenia-associated missense mutation [valine to leucine] within the transmembrane region of NRG1 is associated with increased IL6, TNF-α, and IL8 protein secretion levels in mutation carriers relative to controls [33]. Similarly, investigations into

interactions between DISC1 point mutations and gestational exposure to immune system insults suggest an important role for pro-inflammatory cytokines, particularly IL6, in the pathophysiology that leads to exacerbation of schizophrenia-related behaviours in these mutants [31]. In the present study, basal IL6, IL8 and TNF-α, as well as IL1β, IL2, and IL10, protein levels were altered in NRG1 mutants. Exposure to aversive situations and negative social interactions has been linked with higher pro-inflammatory cytokine levels, notably increased IL6 and the type II soluble receptor for TNF-α [10]. In the present study decreased stress reactivity, pain sensitivity and an anxiety-like profile in NRG1 mutants was accompanied by decreased basal pro-inflammatory cytokine levels. Evidence from human and animal studies suggests that inflammatory mechanisms and stress vulnerability may interactively determine the course of psychopathology [3, 9, 20, 25], including impacting on specific dimensions of pathology such as pain sensitivity [29]. In humans, higher levels of negative affect were associated with lower tolerance to pain during vaccine-induced low-grade inflammation [29], suggesting that changes in basal pro-inflammatory cytokine levels may also contribute to the reduced pain sensitivity and blunted stress responsivity observed in NRG1 mutant mice.

Disruption of DISC1, but not of NRG1, in the absence of epistasis, was associated with increased basal serum pro-inflammatory cytokine levels in this model; that IL1, IL6, and TNF-α levels were altered in DISC1 mutants may reflect the observed reduction in interaction between DISC1 and GSK3ß, as the latter plays an important role in the innate and adaptive immune response [5]. In contrast to NRG1-mediated decreases in basal cytokine levels, DISC1 mutants displayed elevated basal IL6 levels which are consistent with the reported increase in the vulnerability to the behavioural and physiological effects of various stressors and immune insults in DISC1 mutant mice [1, 23, 31]. Of the pro-inflammatory cytokines shown to be increased in schizophrenia, IL-6 appears to play a central role in the inflammatory process and

has been associated with both the duration of the disorder and the resistance of patients to antipsychotic treatment [47].

Gene × gene interactions between NRG1 and DISC1 were detected on basal cytokine concentrations, where NRG1 mutation in combination with DISC1 deletion diminished the DISC1-specific increases in the pro-inflammatory cytokines IL6 and TNF-α observed in the absence of NRG1 mutation, but normalised DISC1-specific decreases in IL12. Although epistatic effects were not observed on pain sensitivity or stress-responsivity in the present study, the opposing effects of NRG1 × DISC1 epistasis on different cytokines may provide some insight into the divergent effects of these gene mutations on other physiological and behavioural pathological phenotypes. The balance between activation of Th1 (promoting cell-mediated immunity and including IL12, IFN-γ, TNF-α) and Th2 (promoting humoral immunity and including IL6) immune cells determines the type of immune response and has been implicated to varying extents in immune-related pathology in schizophrenia. However, inconsistent reports relating to Th2 cytokine increases in schizophrenia patients has meant that the importance of Th1/Th2 balance in the pathophysiology of schizophrenia is still a matter of debate [30, 35, 39]. It is possible that the contradictory reports of Th1 and Th2 imbalances in schizophrenia may be partially linked to the varying contributions of schizophrenia risk genes in sampled human populations and the respective effects of these genes on the different populations of T helper cells and associated cytokines. Interestingly, first episode psychosis patients with depression have been shown to exhibit greater Th2 cytokine activation relative to those without depression [36] and may result from mutations of the DISC1 gene which is well-established to impart phenotypes relevant to affective disorders [6].

The genes associated with risk for schizophrenia display notable overlap with other psychiatric disorders, including candidate genes for anxiety and bipolar disorder [4, 12, 13, 21]. This provides a molecular genetic basis for clinical co-morbidity and symptom overlap, and may suggest cross-utility of pharmacological agents. It has been suggested that the overlap between schizophrenia and anxiety [8, 38] may have to do with reactivity and stress reactivity [4]. Immune system imbalances have also been implicated in the pathophysiology of these overlapping disorders where altered expression of neuro-immune genes and increased levels of cytokines are observed, especially in schizophrenia patients with comorbid depression [36]. Specifically, patients with first episode psychosis and comorbid depression exhibit increased circulating levels of IL-6 which are associated with lowered AKT1 expression, an interacting genetic partner of NRG1 [26], suggesting a direct correlation between neuro-immune genes, emotionality and cytokine profiles in these subsets of schizophrenia patients [36].

In summary, these data suggest that the schizophrenia risk gene NRG1 impacts on stress-responsivity, pain sensitivity and anxiety phenotypes in a manner that is independent of DISC1 gene deletion, and correlates with effects on basal cytokine levels in these mice. Importantly, epistasis resulting from synergistic NRG1 and DISC1 gene mutations alters pro-inflammatory cytokines levels relative to the effects of each of these genes on inflammatory markers in isolation, highlighting the importance of epistatic mechanisms in immune-related pathology. Further investigation into NRG1 × DISC1 interactions on the immune system following more prolonged stress/immune challenge and the identification of common signalling pathways implicated in immune system dysregulation by these schizophrenia risk genes are warranted.

Declaration of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Figure and Table Legends

Figure 1: **(A)** Increased hot plate latency in NRG1^{HET} (* effect of NRG1 genotype, $P < 0.05$). (**B**) Latency to tail flick (TF). Data are expressed as means ± SEM. **(C)** Percentage (%) total time spent in the light chamber during the light-dark test in NRG1^{WT}/ DISC^{WT}, NRG1^{WT}/DISC1^{HET}, NRG1^{WT}/ DISC1^{HOM}, NRG1^{HET}/DISC1^{WT}, NRG1^{HET}/DISC1^{HET}, NRG1^{HET}/ DISC1^{HOM}. Increased % total time in the light chamber in NRG1^{HET}, more pronounced in females (* effect of NRG1 genotype, $P < 0.05$). Data are expressed as means ± SEM. **(D)** Rectal temperature measure on two occasions: T1 (baseline), T2 (10-min post-restraint stress). Attenuation in restraint stress-induced increase in body temperature in NRG1^{HET} (* effect of NRG1 genotype, $P < 0.05$). Data are expressed as means ± SEM. Group sizes are detailed in Table 1.

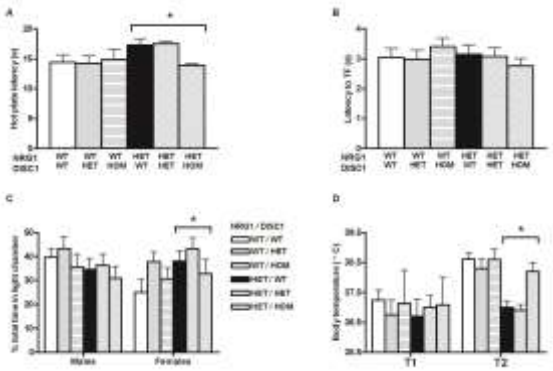

Table 1: Group sizes and sequence of testing. In order to minimise potentially confounding litter effects, no more than 2 experimental mice of a single sex and genotype were drawn from the same litter.

Table 1

Cohort	Procedure and Sequence	NRG1 / DISC1 Genotype					
		WT / WT	**WT / HET**	**WT / HOM**	**HET / WT**	**HET / HET**	**HET / HOM**
1	Hot Plate	9 m / 5 f	8 m / 7 f	7 m / 8 f	8 m / 8 f	9 m / 10 f	9 m / 7 f
	Tail flick	9 m / 4 f	6 m / 6 f	7 m / 8 f	6 m / 5 f	10 m / 9 f	8 m / 4 f
	Light dark test	10 m / 8 f	7 m / 10 f	6 m / 8 f	7 m / 9 f	8 m / 8 f	7 m / 5 f
	Stress-induced hypothermia	9 m / 5 f	8 m / 7 f	7 m / 8 f	8 m / 8 f	9 m / 10 f	9 m / 7 f
2	Plasma Cytokine analysis	4 m / 6 f	4 m / 6 f	5 m / 6 f	5 m / 6 f	6 m / 6 f	4 m / 6 f

Table 2: Concentrations (pg/ml) of serum cytokine levels in male and female $NRG1^{WT}/DISC^{WT}$, $NRG1^{WT}/DISC1^{HET}$, $NRG1^{WT}/DISC1^{HOM}$, $NRG1^{HET}/DISC1^{WT}$, $NRG1^{HET}/DISC1^{HET}$, $NRG1^{HET}/DISC1^{HOM}$. Data are expressed as means ± SEM. $^{\#\#}$ $P < 0.01$, effect of DISC1 genotype; ** $P < 0.01$, effect of NRG1 genotype; ‡ $P < 0.01$, NRG1 × DISC1 interaction.

Table 2

NRG1 / DISC1	WT / WT	WT / HET	WT / HOM	HET / WT	HET / HET	HET / HOM
Females	**(n=4)**	**(n=4)**	**(n=5)**	**(n=5)**	**(n=6)**	**(n=4)**
IL10	153.4 ± 34.6	103.6 ± 36.15	47.0 ± 19.1	22.1 ± 2.3 **	21.0 ± 2.7 **	26.3 ± 3.1 **
IL1β	7.4 ± 2.3	2.0 ± 0.3 ##	2.5 ± 0.2 ##	5.9 ± 0.3	5.2 ± 0.3	4.2 ± 1.5
IL6	41.0 ± 34.5	73.8 ± 25.5	38.8 ± 14.0	17.2 ± 7.1 **	6.9 ± 1.7 ** ‡	30.0 ± 15.8 ** ‡
TNF-α	2.1 ± 0.6	1.1 ± 0.2	1.3 ± 0.2	2.1 ± 0.3	2.3 ± 0.1	2.0 ± 0.3
IFN-γ	1.7 ± 0.4	6.5 ± 2.2	3.2 ± 1.1	2.8 ± 0.3	2.5 ± 0.4	2.7 ± 0.5
IL8	41.9 ± 3.3	59.9 ± 1.7	68.3 ± 7.2	44.8 ± 6.7 **	39.0 ± 1.4 **	55.9 ± 11.1 **
IL12	251.5 ± 21.5	220.7 ± 86.3	78.7 ± 44.9	19.6 ± 4.1 **	15.2 ± 3.7 **	20.8 ± 5.2 **
Males	**(n=6)**	**(n=6)**	**(n=6)**	**(n=6)**	**(n=6)**	**(n=6)**
IL10	32.9 ± 6.9	25.6 ± 3.6	21.9 ± 1.2	26.9 ± 8.0	29.4 ± 5.0	31.7 ± 1.3
IL1β	4.9 ± 2.0	6.5 ± 0.7	6.9 ± 0.6	5.4 ± 0.1 **	2.8 ± 0.5 **	2.9 ± 0.4 **
IL6	17.5 ± 4.3	25.8 ± 17.1	22.7 ± 10.0	11.5 ± 5.3	23.2 ± 3.0	22.4 ± 6.0
TNF-α	1.9 ± 0.4	2.8 ± 0.2	2.8 ± 0.3	2.3 ± 0.1 **	1.3 ± 0.2 ** ‡	1.7 ± 0.2 ** ‡
IFN-γ	7.3 ± 4.1	2.9 ± 0.2	2.6 ± 0.2	3.5 ± 0.6	2.5 ± 0.2	2.7 ± 1.2
IL8	56.2 ± 6.2	38.2 ± 4.8	37.8 ± 5.0	62.1 ± 15.7	55.3 ± 2.8	69.3 ± 11.9
IL12	45.1 ± 15.6	23.9 ± 5.2	18.4 ± 3.3	30.6 ± 19.1	32.6 ± 13.8 ‡	46.3 ± 18.4 ‡