EXERCISE IMMUNOLOGY REVIEW

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Exercise Immunology Review

Editorial Statement

Exercise Immunology Review, an official publication of the International Society of Exercise Immunology and of the German Society of Sports Medicine and Prevention, is committed to developing and. enriching knowledge in all aspects of immunology that relate to sport, exercise, and regular physical ativity. In recognition of the broad range of disciplines that contribute to the understanding of immune function, the journal has adopted an interdisciplinary focus. This allows dissemination of research findings from such disciplines as exercise science, medicine, immunology, physiology, behavioral science, endocrinology, pharmacology, and psychology.

Exercise Immunology Review publishes review articles that explore: (a) fundamental aspects of immune function and regulation during exercise; (b) interactions of exercise and immunology in the optimization of health and protection against acute infections: (c) deterioration of immune function resulting from competitive stress and overtraining; (d) modulation of the effect of aging on immune function by exercise; (e) exercise in the prevention and treatment of autoimmune disease, HIV infection, and cancer; and (f) the role of exercise in modulating immune function after tissue transplantation.

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From the editors

Exercise Immunology Review has entered a new realm in 2006. Since August, EIR has been available online. The first electronically available volume is EIR10, 2004. The number of hits on PubMed is growing from month to month (510-773). This year's volume of EIR again delivers a mixture of focussed reviews, papers presenting original data, and papers presenting novel ideas/hypotheses or defining new areas of potential interest for our discipline.

The first two reviews both pertain to the question of how chronic exercise may exert its beneficial anti-inflammatory effects. The first article by Fischer is an in-depth report on the role of interleukin (IL)-6 in exercise. IL-6 is now accepted as important metabolic player in the reaction of the body to exercise stress. This review addresses the mechanisms linking muscle activity to IL-6 synthesis, the effects of IL-6 on different organs, and effect of training on IL-6 release.
The next review by Gleeson et al. focuses on the effects of chronic training on Toll-like receptor (TLR) expression. This review evaluates the possible role of down-regulation of TLR in mediating the beneficial long term anti-inflammatory effects of exercise.
The third review by Nagatomi discusses the similarities and differences between alterations in peripheral cell numbers after exercise and during pathological conditions.
The following three articles present original data. The first by Peake et al. compares changes in markers of muscle damage and systemic inflammation after submaximal and maximal lengthening muscle contractions. Despite evidence of greater muscle damage following the maximal contractions, no differences in markers of systemic inflammation were seen.
The article by Smith et al. describes the physiological, health and economic impact of a comprehensive exercise-based rehabilitation program. It is encouraging to see that chronically ill elderly patients benefited from exercise training on all three levels.
The article by Lowder et al. illustrates that moderate exercise has a dramatic influence on inflammatory responses within the lungs of mice following influenza virus infection. Exercise caused a shift from a TH1 response to a TH2 response following infection, without any significant change in interferon-beta, leading to improved survival in the given situation.

This volume of EIR closes with an article by Simon et al. that is focused on immediate early genes (IEG) and their expression following exercise. IEG are limited in number, but they operate via novel post-transcriptional pathways that accelerate the rate of signal transduction. The pattern of IEG expression is an integral component of resultant cellular responses. The authors hypothesise that gene expression analysis of IEG may facilitate the discovery and definition of exercise-specific transcriptional fingerprints. If true, then this analytical technique may greatly improve our understanding of the mechanisms regulating responses to exercise.

Hinnak Northoff

Interleukin-6 in acute exercise and training: what is the biological relevance?

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Running title: Interleukin-6 in acute exercise and training

Keywords: Cortisol; Cytokines; Inflammation; Glucose metabolism; Lipid metabolism; Skeletal muscle

ABSTRACT

It is now recognized that contracting skeletal muscle may synthesize and release interleukin-6 (IL-6) into the interstitium as well as into the systemic circulation in response to a bout of exercise. Although several sources of IL-6 have been demonstrated, contracting muscles contributes to most of the IL-6 present in the circulation in response to exercise. The magnitude of the exercise-induced IL-6 response is dependent on intensity and especially duration of the exercise, while the mode of exercise has little effect. Several mechanisms may link muscle contractions to IL-6 synthesis: Changes in calcium homeostasis, impaired glucose availability, and increased formation of reactive oxygen species (ROS) are all capable of activating transcription factors known to regulate IL-6 synthesis. Via its effects on liver, adipose tissue, hypothalamic-pituitary-adrenal (HPA) axis and leukocytes, IL-6 may modulate the immunological and metabolic response to exercise. However, prolonged exercise involving a significant muscle mass in the contractile activity is necessary in order to produce a marked systemic IL-6 response. Furthermore, exercise training may reduce basal IL-6 production as well as the magnitude of the acute exercise IL-6 response by counteracting several potential stimuli of IL-6. Accordingly, a decreased plasma IL-6 concentration at rest as well as in response to exercise appears to characterize normal training adaptation.
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INTRODUCTION

Since the first study in 1991 (115), several studies have consistently reported that the plasma interleukin-6 (IL-6) concentration increases in response to exercise

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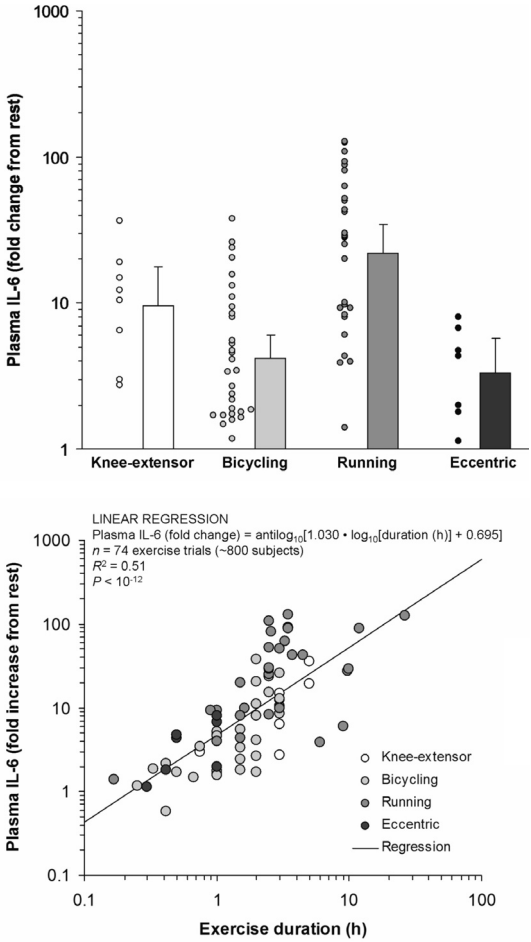

Fig. 1. Effect of mode and duration of exercise on post-exercise plasma IL-6.
Different modes of exercise (dynamic knee-extensor, bicycling, running, eccentric) and the corresponding increase in plasma IL-6 (fold change from pre-exercise level), based on the 67 exercise trials listed in Table 1 as well as 7 trials representing various eccentric exercise protocols (17, 53, 90, 144, 182, 194). Accordingly, the graphs represent approximately 800 subjects. Each dot represents one exercise trial, while the corresponding bars show geometric means with 95% confidence intervals (A). The overall $\log_{10}$-$\log_{10}$ linear relation (straight solid line) between exercise duration and increase in plasma IL-6 (fold change from pre-exercise level) indicates that 51% of the variation in fold plasma IL-6 increase can be explained by the duration of exercise (B).

(Table 1 & Fig. 1). Although the plasma concentration of several other cytokines may be affected by exercise, IL-6 increases more dramatically than any other cytokine investigated to date (120, 126). But what determines the magnitude and time course of the increase of IL-6 with exercise? What is the effect of exercise training on IL-6? And what is the possible biological relevance of IL-6 in acute and chronic physical activity? These are some of questions addressed in this review.

Two decades ago, IL-6 was first sequenced and described as a cytokine facilitating the differentiation of B-lymphocytes into immunoglobulin-secreting plasma cells (55, 56). Later, several other immunological properties was ascribed to this pleiotropic cytokine, which received its present name in 1987 (139). IL-6 belongs to a family of cytokines that also includes leukemia inhibitory factor, interleukin-11, ciliary neurotrophic factor, cardiotrophin-1, and oncostatin M. In addition to structural similarities, these cytokines share the gp130 receptor subunit (76).

Transcription and translation of the human gene encoding IL-6 – consisting of a ~5 kilobase long sequence containing 5 exons located on chromosome 7 (155) – leads to the synthesis of a propeptide containing 212 amino acids, which is cleaved in

order to obtain the mature IL-6 peptide containing 184 amino acids (56). Interestingly, a variant IL-6 peptide lacking the sequence encoded by exon II – thus unable to signal via the gp130 receptor – may be released from stimulated lymphocytes and monocytes in concert with the full-length IL-6 (74). Further post-translational modifications include varying degrees of glycosylation and phos-

Exercise mode											
Knee-extensor				Bicycling				Running			
n	Duration (h)	IL-6 (fold change)	Ref	*n*	Duration (h)	IL-6 (fold change)	Ref	*n*	Duration (h)	IL-6 (fold change)	Ref
7	3.0	3	(38)	9	0.4	1	(33)	12	0.2	1	(195)
7	0.8	3	(52)	9	0.3	1	(188)	19	6.0	4	(30)
7	3.0	6	(127)	16	0.7	1	(96)	7	1.0	4	(113)
6	3.0	11	(71)	7	1.0	2	(12)	8	1.5	4	(178)
7	3.0	12	(37)	17	1.0	2	(186)	6	9.1	6	(132)
6	3.0	15	(168)	6	2.0	2	(59)	8	1.5	8	(179)
6	5.0	19	(172)	9	0.5	2	(17)	30	2.5	8	(102)
7	5.0	36	(165)	8	1.0	2	(87)	7	1.0	9	(163)
				9	1.5	2	(86)	12	0.9	9	(114)
				7	0.3	2	(42)	10	1.6	10	(159)
				7	0.3	2	(42)	16	3.0	10	(107)
				8	0.4	2	(33)	10	1.5	20	(134)
				8	1.5	2	(177)	10	2.5	25	(119)
				6	2.0	3	(59)	13	9.8	28	(108)
				11	1.5	3	(181)	7	9.9	29	(110)
				6	0.8	3	(189)	7	2.5	29	(170)
				8	2.0	4	(11)	9	2.5	30	(169)
				8	1.0	5	(89)	50	4.5	42	(112)
				7	1.0	5	(163)	18	3.7	43	(21)
				9	1.0	5	(146)	6	3.0	50	(84)
				7	1.5	6	(164)	10	2.5	52	(109)
				6	2.0	8	(31)	16	3.3	63	(121)
				18	3.0	8	(128)	10	2.6	80	(175)
				8	1.0	9	(118)	18	3.5	88	(18)
				8	2.0	11	(60)	10	3.5	92	(183)
				8	3.0	13	(69)	16	2.5	109	(176)
				15	2.5	16	(106)	60	26.3	126	(111)
				6	2.0	20	(162)	10	3.5	128	(120)
				10	2.5	24	(109)				
				6	3.0	26	(117)				
				8	2.0	38	(47)				

Table 1. Effect of acute exercise on plasma IL-6 in humans.
Shown is the relation between exercise mode (dynamic knee-extensor, bicycling, and running), exercise duration, and plasma IL-6 increase (fold change from pre-exercise level). In studies investigating the effect of an intervention on the IL-6 response to exercise, e.g. carbohydrate supplementation, only the result from the control group (exercise without intervention) is presented. Hence, the n value may be lower than the n value presented in the original study.

phorylation, and several isoforms ranging from 21-30 kDa have been described (7, 46, 51, 95). Whether the biological effects *in vivo* of these isoforms differ is not established.

The plasma IL-6 concentration is ~1 pg/ml or even lower in resting healthy subjects (17, 121). In contrast, the plasma IL-6 concentration may reach 10000 pg/ml in response to severe systemic infections (40). Less dramatic increases of plasma IL-6 are found in numerous inflammatory and infectious diseases. A pathogenic role for IL-6 in the development of the metabolic syndrome has been suggested, in part because the presence of a chronic low-level increase of plasma IL-6 (usually <10 pg/ml) is associated with obesity (6), low physical activity (36, 123), insulin-resistance (13), type 2 diabetes (67), cardiovascular disease (39) and may serve as a predictor of mortality (15).

Downstream signaling requires that IL-6 binds to the heterodimeric receptor complex consisting of the ubiquitously expressed gp130 receptor and the specific receptor IL-6Rα (50). This event triggers tyrosine-phosphorylation of gp130 by Janus-activated kinases (Jak) on the intracellular domain, whereby at least two distinct signalling pathways are activated: 1) the signal transducers and activators of transcription (STAT) 1 and 3, and 2) the mitogen-activated protein kinases (MAPK) (49). The two pathways are characterized by distinct effects; thus, the effect of IL-6 may vary in different tissues depending on the balance between the two pathways (54). A negative feedback mechanism of STAT activation involves transcription and translation of the suppressor of cytokine signaling 3 (SOCS3).

THE IL-6 RESPONSE TO ACUTE EXERCISE

Following exercise, the basal plasma IL-6 concentration may increase up to 100 fold, but less dramatic increases are more frequent (Table 1, Fig. 1A). Thus, the 8000-fold increase of plasma IL-6 following a 246 km "Spartathlon" race (92) represents an atypical and extreme response. Of note, the exercise-induced increase of plasma IL-6 is not linear over time; repeated measurements during exercise show an accelerating increase of the IL-6 in plasma in an almost exponential manner (37, 119, 172). Furthermore, the peak IL-6 level is reached at the end of the exercise or shortly thereafter (37, 119), followed by a rapid decrease towards pre-exercise levels.

Where does the exercise-induced IL-6 come from?

Importantly, the contracting skeletal muscle *per se* appears to be one of the main sources of the IL-6 in the circulation in response to exercise: In resting human skeletal muscle, the IL-6 mRNA content is very low, while small amounts of IL-6 protein predominantly in type I fibers may be detected using sensitive immunohistochemical methods (137). In response to exercise, an increase of the IL-6 mRNA content in the contracting skeletal muscle is detectable after 30 minutes of exercise, and up to 100-fold increases of the IL-6 mRNA content may be present at the end of the exercise bout (71, 168). Recently, further evidence that contracting muscle fibers themselves are a source of IL-6 mRNA and protein has been achieved by analysis of biopsies from the human *vastus lateralis using in situ* hybridization and immunohistochemistry (58, 128). In addition, assessment of the

interstitial IL-6 concentration using microdialysis indicates that the concentration of IL-6 within the contracting skeletal muscle may be 5-100 fold higher than the levels found in the circulation (84, 147). Accordingly, IL-6 appears to accumulate within the contracting muscle fibers as well in the interstitium during exercise. However, it has been the simultaneous measurement of arterio-venous IL-6 concentrations and blood flow across the leg that has demonstrated that large amounts of IL-6 can be released from the exercising leg (172). In the same study, the authors also estimated that the net release from the exercising leg could account for the systemic increase of plasma IL-6, assuming that IL-6 is distributed in the extracellular compartment and that IL-6 content in blood is the same in plasma and the cellular fraction. Since IL-6 appears to be transported solely in the non-cellular fraction of the blood (20), the net release of IL-6 from the exercising leg probably was overestimated. Yet, a simpler approach based on the close log-log linear relationship between recombinant human IL-6 (rhIL-6) dose and resulting steady state plasma IL-6 concentration (Fig. 2) supports the concept that IL-6 released from the exercising limb may account for systemic plasma IL-6 increase following exercise: At the end of the exercise, the average release of IL-6 from the contracting leg was 15 ng/min, while the systemic plasma IL-6 concentration was 14 pg/ml (172). Based on the dose-response relationship, the expected systemic plasma IL-6 concentration corresponding to an IL-6 dose of 15 ng/min is 16 pg/ml ($\text{antilog}_{10}[1.05 \cdot \log_{10}[15 \text{ ng/ml}] + 0.07]$), which corresponds well to the observed value.

However, although IL-6 released from the contracting muscles may account for most of the IL-6 found in the circulation, other studies have demonstrated that skeletal muscle is not the sole source of exercise-induced IL-6. Using oral supplementation with vitamins C and E for 4 weeks, the IL-6 net release from the exercising legs was almost blocked completely, yet the systemic increase of plasma IL-6 was only reduced by 50% (37). Very high concentrations of IL-6 along the Achilles' tendon has been detected using microdialysis in response to prolonged running (84), but since the muscle mass involved in exercise is much higher than the mass comprised by tendons, the mutual contribution of peritendinous versus muscle-derived IL-6 to the systemic IL-6 is unclear. In addition, a small net release of IL-6 from the internal jugular vein has been reported, suggesting that the central nervous system may contribute to the IL-6 found in the circulation (118). In contrast, a contribution from peripheral blood mononuclear cells to the IL-6 found in the circulation of healthy subjects is detected consistently neither at rest nor in response to exercise (121, 162, 186, 189). The adipose tissue may contribute markedly to IL-6 in the circulation at rest (98, 160), but measurement of arterio-venous plasma IL-6 differences across the abdominal subcutaneous adipose tissue bed shows that this compartment does not contribute to the exercise-induced IL-6 in the circulation until the recovery phase (88). However, since almost any cell type may synthesize IL-6 upon adequate stimulation (3), further studies may discover other sites contributing to the IL-6 in the circulation in response to exercise.

How is the exercise-induced IL-6 response regulated?

Overall, the combination of mode, intensity and duration of the exercise determines the magnitude of the exercise-induced increase of plasma IL-6. However,

although it was suggested that the IL-6 response was related to muscle damage (17), it now has become clear that eccentric exercise is not associated with more marked increases of plasma IL-6 than compared to exercise involving concentric muscle contractions (Fig 1A). Thus, muscle damage is not required in order to increase plasma IL-6 during exercise. Rather, eccentric exercise may result in a delayed peak and a slower decrease of plasma IL-6 during recovery (53, 90, 194).

In contrast, the IL-6 response is sensitive to the exercise intensity (122), which again indirectly represents the muscle mass involved in the contractile activity. Since contracting skeletal muscle *per se* is an important source of IL-6 found in the plasma (37, 172), it is therefore not surprising that exercise involving a limited muscle mass, e.g. the muscles of the upper extremities, may be insufficient in order to increase plasma IL-6 above pre-exercise level (8, 57, 116). In contrast, running – which involves several large muscle groups – is the mode of exercise where the most dramatic plasma IL-6 increases have been observed (Table 1, Fig. 1A).

Regardless, exercise duration is the single most important factor determining the post-exercise plasma IL-6 amplitude (Table 1, Fig. 1B); more than 50% of the variation in plasma IL-6 following exercise can be explained by exercise duration alone ($P < 10^{-12}$). Since exercise at high intensity often is associated with shorter duration of the exercise and *vice versa*, the relationship between the plasma IL-6 increase and the duration may be even more pronounced if adjusted for the exercise intensity. In accordance, 6 minutes of maximal rowing ergometer exercise may increase plasma IL-6 two-fold (105), but more than 10-fold increases of plasma IL-6 has not been observed in response to exercise lasting less than 1 h (Fig. 1B). Based on the log-log linear relationship between time and fold increase of plasma IL-6 (Fig. 1B), a 10-fold increase of plasma IL-6 requires exercise for 1.9 h (95% confidence interval, CI, 1.6 - 2.9 h, $P < 0.0001$) of exercise, while a 100-fold increase of plasma IL-6 requires exercise lasting 6.0 h (CI 4.5 - 8.1 h, $P < 0.0001$). This relationship is remarkably insensitive to the mode of exercise, although the highest increases of plasma IL-6 generally are found in response to running.

↑, increase; ↓, decrease; ↔ no effect of the intervention.

Intervention	Effect on exercise-induced IL-6	References
Reduction of pre-exercise glycogen content	Muscle IL-6 mRNA ↑ Plasma IL-6 ↑	(24, 71, 171)
Supplementation with carbohydrates	Muscle IL-6 mRNA ↔ Plasma IL-6 ↓	(37, 179, 189)
Hyperglycemia in Type 1 diabetes	Plasma IL-6 ↑	(42)
Nicotinic acid (inhibits lipolysis)	Muscle IL-6 mRNA ↔ Adipose tissue IL-6 mRNA ↑ Plasma IL-6 ↑	(62)
Hot environment	Plasma IL-6 ↑	(164)
Indomethacin (NSAID)	Plasma IL-6 ↓	(143)
O_2 supplementation to COPD patients	Plasma IL-6 ↓	(188)
Supplementation with antioxidants	Muscle IL-6 mRNA ↔ Plasma IL-6 ↓	(37, 179, 189)

Table 2. Some interventions influencing the exercise-induced IL-6 response.

What mechanisms may explain why contractile activity leads to increased synthesis of IL-6? Since IL-6 is synthesized and released only from the contracting muscles and not from the resting muscles exposed to the same hormonal changes (66, 172), circulating systemic factors alone does not explain why contracting muscles synthesize and release IL-6. Instead, local factors seem necessary, although systemic factors may modulate the response.

The promoter region of the IL-6 gene contains binding sites for the nuclear factor kappa B (NF-κB) and nuclear factor interleukin-6 (NFIL6) (93). Additional transcription factors such as the nuclear factor of activated T cells (NFAT) (1) and heat shock factors 1 and 2 (HSF1 and HSF2) (141) may contribute to the activation of IL-6 gene transcription. *In vitro*, calcium activates both NFAT and NF-κB (29, 83), and incubation of muscle cell cultures with a calcium ionophore (ionomycin) increases IL-6 secretion in a p38 MAPK dependent manner (24). Human studies have shown increased total and nuclear content of phosphorylated p38 MAPK, but unaltered nuclear content of NFAT in muscle biopsies after 1 h of bicycling (97), while mRNA content of calcineurin A – which is involved in calcium signalling – is increased in muscle biopsies 6 h post 3 h of knee-extensor exercise (136). Activation of NF-κB has been demonstrated in rat skeletal muscle after exercise (65), but not consistently in humans (97). Noteworthy, NF-κB is a redox-sensitive transcription factor (154) that may be activated by reactive oxygen species (ROS). Increased ROS formation in exercising skeletal muscle following exercise has been demonstrated directly in animals (27, 63) and indirectly in humans (4). *In vitro*, murine skeletal myotubes release IL-6 when exposed to oxidative stress in a NF-κB-dependent way (81). In addition, supplementation with different antioxidants attenuates the systemic increase of IL-6 in response to exercise (179, 189). Using arterio-venous differences of IL-6 across the leg, we observed that the reduced systemic increase of IL-6 during exercise was due to an almost complete inhibition of the net leg release of IL-6 in the group pre-treated with vitamin C and E for 4 weeks (37). The observation that indomethacin – a member of the non-steroid anti-inflammatory drugs (NSAID), which are known to inhibit NF-κB activity – reduces the exercise-induced increase of IL-6 further supports that NF-κB is likely to serve as a link between contractile activity and IL-6 synthesis (80, 143). On the other hand, increased oxidative stress, as well as low glucose availability, low glycogen content, catecholamines, increased intracellular calcium levels, hyperthermia, ischemia-reperfusion are all features of exercise capable of inducing heat shock proteins (HSPs) (9, 22, 34, 125, 190, 193), which may in turn activate IL-6 synthesis via HSF1 and HSF2 (141). Accordingly, several regulators of IL-6 transcription are likely to be activated by an altered intramuscular milieu in response to exercise (Fig. 4). This point of view is supported by the various interventions that have demonstrated an effect on the exercise-induced IL-6 response (Table 2). For instance, reduction of intramuscular glycogen content prior to exercise results increased accumulation of IL-6 mRNA within the contracting muscle as well as increased release of IL-6 from the contracting muscle (24, 71, 171). This effect of glycogen reduction on the exercise-induced IL-6 response may be mediated through activation of p38 MAPK (24) and AMPK (89). In contrast, supplementation with carbohydrates during exercise inhibits the exercise-induced increase of IL-6 in plasma, whereas IL-6 mRNA expression within the contracting muscle is unaffected (32, 102, 109,

163). While glucose availability may interfere with IL-6 gene expression through AMPK (2), other mechanisms regulating IL-6 at a posttranslational level appear to exist.

To make it even more complex, IL-6 appears to be capable of enhancing its own transcription (72), which may partly explain the almost exponential increase of IL-6 towards the end of exercise (Fig. 3). However, it should be noted that the IL-6 released into the circulation is cleared very quickly, thus the 'area under the curve' for plasma IL-6 in response is limited in particular in response to short bouts of exercise (Fig. 3). In mice, the halflife of ^{125}I-labelled IL-6 in the circulation is 2 minutes (99), which is accordance with the rapid decline of plasma IL-6 following rhIL-6 infusion from human studies (187). Most of the IL-6 is cleared by the kidneys and the liver (31, 99).

What are the effects of IL-6 in acute exercise?

Exercise is known to cause major physiological, hormonal, metabolic, and immunological effects. The question is whether exercise-induced IL-6 mediates some of these effects. Of note, IL-6 may act locally within the contracting muscle during exercise or within the adipose tissue during recovery, while most other cells and target organs are exposed only to IL-6 released into the systemic circulation. Regarding the systemic effects of IL-6, the dose-response relationship and timing has to be considered. First, it should be noted that marked increases of plasma IL-6 only occur if the exercise involves a considerable muscle mass working for a considerable amount of time at a considerable intensity. Otherwise, a systemic IL-6 increase may be small or absent. Regardless, the exercise-induced peak plasma IL-6 concentration will usually not exceed 100 pg/ml. Second, the peak plasma IL-6 concentration occurs at the cessation of the exercise (or shortly after), thus the systemic effects induced by IL-6 are for the most part expected to occur during recovery from exercise.

Metabolic and hormonal effects of exercise-induced IL-6. Whole body oxygen consumption and carbondioxide production increases in response to rhIL-6 infusion in the postabsorptive state as well as during a euglycemic hyperinsulinemic clamp (19, 184). This increase in energy turnover may occur without significant changes in body temperature, though a moderate increase in body temperature – which occurs when the plasma IL-6 concentration is 300 pg/ml or higher (174, 184, 185) – may *per se* be associated with an augmented energy turnover. However, since a relatively high plasma IL-6 concentration apparently is required in order to increase body temperature, it seems unlikely that the systemic increase of IL-6 in response to exercise modulates metabolism through changes in body temperature.

In rats, IL-6 injection may deplete hepatic glycogen content (173). *In vitro* and *in vivo* in animals, several studies have indicated that IL-6 interferes with insulin-signalling in hepatocytes and liver tissue (68, 77, 78, 156, 157), whereby hepatic glucose output may increase. However, even marked elevations of plasma IL-6 has little effect on glucose metabolism in resting humans: In subjects both with and without type 2 diabetes, an acute elevation of plasma IL-6 has no effect glucose rate of appearance (R_a), glucose disappearance (R_d) or plasma glucose in the postabsorptive state (133, 167). When combined with a euglycemic hyperinsulinemic clamp, an acute increase of plasma IL-6 to ~50 pg/ml has no effect on

plasma glucose, glucose R_a or R_d (82), while an acute increase of plasma IL-6 to ~200 pg/ml increases glucose R_d and glucose oxidation (19). However, a much lower increase of plasma IL-6 increases both glucose R_a and R_d during exercise (35). The mechanism behind the apparent discrepancy between the effect of IL-6 at rest and during exercise is unknown, but the presence of additional "exercise cofactors" capable of modulating the effect of IL-6 has been suggested (35). Alternatively, the effect of IL-6 on glucose metabolism is only detectable when glucose fluxes are high as in response to exercise or insulin stimulation. Accordingly, a systemic increase IL-6 in response to exercise may augment hepatic glucose output, while other tissues increase the uptake of glucose, whereby the plasma glucose concentration is unaffected. Thus, it is possible that the enhanced hepatic output is balanced by increased glucose uptake in the contracting skeletal muscle during exercise. However, conflicting results regarding the effect of IL-6 on glucose uptake in skeletal muscle exist: In mice, IL-6 decreases insulin-mediated glucose uptake in skeletal muscle (75), while L6 myotubes exposed to IL-6 *in vitro* demonstrate increased insulin-sensitivity (19).

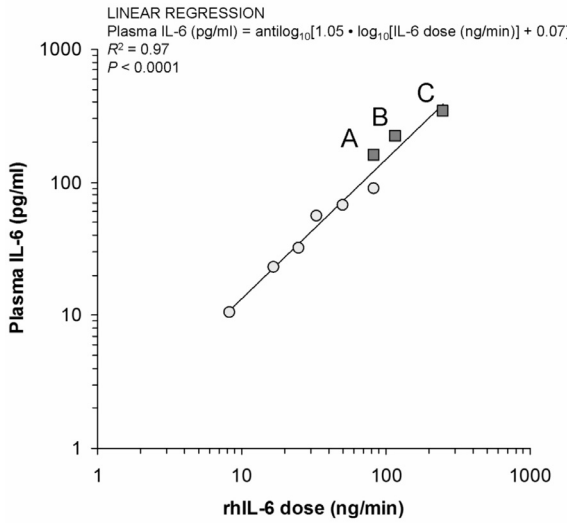

Fig. 2. Dose-response curve for rhIL-6.
Shown is the plasma IL-6 concentration in response to different infusion rates of rhIL-6 diluted in saline containing human albumin. The equation describes the log10-log10 linear regression (straight solid line). The light grey circles represent data from a pilot study, while the dark grey squares represent published data: A, (72); B, (133); C, (187). Although the shown dose-response relationship has been established in resting subjects, it has been proven useful also in exercise trials (35).

Infusion of rhIL-6 increases lipolysis and fat oxidation after 2 h in healthy subjects (187) and in subjects with type 2 diabetes (133). The lipolytic effect of IL-6 is also observed in cultured adipocytes, suggesting a direct effect of IL-6 on adipose tissue (133). Increased IL-6 mRNA content in the adipose tissue is observed in response to exercise (69), and this increase appears to be mediated by catecholamines (73). If the IL-6 mRNA is translated into protein, an additive effect together with the IL-6 derived from the circulation is possible. Accordingly, IL-6 and adrenaline may enhance the lipolytic capacity of each other in response to exercise. As for the liver, the effect of IL-6 in adipocytes may partly be due to a decrease in insulin-signalling (148, 158). Although adipose tissue mRNA expression of the hormone-sensitive lipase (HSL) is increased by rhIL-6 infusion, the corresponding HSL protein is not affected (192).

Does IL-6 affect other hormones, which in part may explain the apparent metabolic effects of IL-6? Table 3 summarizes some of the effects of an acute increase of plasma IL-6 on some major hormones in humans. IL-6 injection increases adrenocorticotropic hormone (ACTH) in a corticotropin-releasing hormone (CRH) dependent manner in rats (101), while injection of an anti-IL-6 antibody abrogate the endotoxin-induced increase of ACTH in mice (131). Since the IL-6 receptor present in the human pituitary gland (48) and adrenal cortex (45), alternative pathways by which IL-6 can stimulate cortisol release in humans may exist. A dose-dependent relationship between the IL-6 and cortisol in humans has been demonstrated (184). In fact, a consistent increase of cortisol has been reported when plasma IL-6 is ~50 pg/ml or higher (Table 3). Conversely, the post-exercise increase of cortisol is attenuated if the release of IL-6 from the exercising leg is inhibited by supplementation with vitamins C and E (37). However, the increase of cortisol by IL-6 is abrogated during a euglycemic hyperinsulinemic clamp (19). Taken together, it seems likely that an exercise-induced systemic increase of IL-6 may reach concentrations capable of inducing cortisol secretion, although other factors contributing to an exercise-induced activation of the HPA axis not should be excluded. Of note, an increase of cortisol may contribute further to the increased lipolysis and hepatic glucose output induced by IL-6. Interestingly, the increase of cortisol may be involved in a negative feedback regulation of IL-6, at least when present in higher concentrations (124).

While cortisol is induced by even modest plasma IL-6 increases, somewhat higher plasma IL-6 concentrations appear to be necessary in order to increase plasma glucagon and growth hormone (GH) levels consistently (Table 3). During exercise, a low-level increase of IL-6 has no effect on either glucagon or GH (35). Plasma concentrations of both adrenaline and noradrenaline are increased when plasma IL-6 is ~300 pg/ml or higher (187). In healthy subjects, even very high IL-6 doses have no acute effect on fasting postabsorptive plasma insulin levels (Table 3). However, IL-6 infusion may decrease plasma insulin in subjects with type 2 diabetes without concomitant changes in glucose turnover (133). Of note, the increase of catecholamines and the decrease of insulin in response to exercise comprise two highly potent stimuli for lipolysis (28, 64), while GH and cortisol may further enhance the lipolysis (43, 151). Accordingly, IL-6 *per se* may induce lipolysis but more likely IL-6 may stimulate lipolysis in concert with catecholamines and cortisol. In type 2 diabetes, an additional decrease of plasma insulin may contribute to the lipolytic effect of IL-6 (133).

Immunoregulatory effects of exercise-induced IL-6. In humans, infusion of rhIL-6 increases plasma cortisol, IL-1 receptor antagonist (IL-1ra), IL-10, soluble TNF-α receptors (sTNF-R), and C-reactive protein (CRP) (149, 166, 180). Conversely, the increase of cortisol, IL-1ra and CRP after exercise is abrogated if the release of IL-6 from the contracting muscles is reduced by supplementation with antioxidants (37), suggesting that IL-6 from the contracting skeletal muscle in part accounts for the increase of cortisol, IL-Ira and CRP.

The anti-inflammatory properties of cortisol are well characterized (5). In response to rhIL-6 infusion, a significant increase of cortisol occurs within one hour (166). While moderate exercise increase number as well as antimicrobial capacity of the neutrophils in the circulation, intense exercise is associated with a reduced antimicrobial capacity of the neutrophils (126), which is likely to be

mediated by cortisol (91). In addition, cortisol may reduce the number of lymphocytes by enhancing the apoptosis. Thus, higher systemic increases of IL-6 – as observed after prolonged intense exercise – may in part be responsible for the changes in leukocyte subpopulations and antimicrobial capacity.

IL-1ra is a cytokine produced primarily by macrophages, but a further contribution may come from hepatocytes and monocytes (41, 180). IL-1ra attenuates the effect of the pro-inflammatory cytokine IL-1 by reducing the signal transduction through the IL-1 receptor (41). Plasma IL-1ra is increased after rhIL-6 infusion for one hour (166). In contrast to IL-1ra, IL-10 is capable of inhibiting the LPS-stimulated production of several pro-inflammatory cytokines including TNF-α, IL-1α and IL-1β (100, 140). The anti-inflammatory effect of IL-10 is exerted at both the transcriptional and posttranslational level (10, 191). Lymphocytes and monocytes are the primary sources of IL-10, which increases in plasma in response to rhIL-6 infusion for 2 hours (166).

IL-6 infusion also induces a delayed increase of CRP from the liver via activation of the STAT3 pathway (166, 196). CRP was originally characterized as an acute phase protein involved in precipitation of the somatic C-polysaccharide of *Streptococcus pneumoniae* (130). Whether CRP has pro-inflammatory effects or not is being debated (129). When purified adequately, even high doses of recombinant CRP do not induce a pro-inflammatory response (129). Rather, CRP may contribute to the increase of plasma IL-1ra during late recovery from exercise by enhancing the release of IL-1ra from monocytes (142).

Furthermore, while the pro-inflammatory cytokine TNF-α can stimulate IL-6 production (138), IL-6 does not stimulate the production of TNF-α (166). Rather, IL-6 attenuates the LPS-stimulated production of TNF-α in cultured monocytes (153) as well as *in vivo* in humans (161), while treatment with anti-IL-6 antibodies augment the TNF-α response following challenge with staphylococcal enterotoxin B in mice (94). In addition, IL-6 may attenuate the effect of TNF-α by induction of sTNF-R (180).

Taken together, the release of IL-6 from the contracting muscles may facilitate a broad anti-inflammatory response via effects on liver as well as on different leukocyte subpopulations.

IL-6 AND TRAINING ADAPTATION

Exercise training involves multiple adaptations including increased pre-exercise skeletal muscle glycogen content, enhanced activity of key enzymes involved in the beta-oxidation (152), increased sensitivity of adipose tissue to adrenaline-stimulated lipolysis (26), increased oxidation of intramuscular triglycerides (135), whereby the capacity to oxidize fat is increased (61, 150). As a consequence, the trained skeletal muscle is less dependent on plasma glucose and muscle glycogen as substrate during exercise (135).

Several epidemiological studies have reported a negative association between the amount of regular physical activity and the basal plasma IL-6 levels: the more physical active, the lower basal plasma IL-6 (23, 25, 123). Basal plasma IL-6 is closer associated with physical inactivity than other cytokines associated with the metabolic syndrome (36).

The epidemiological data are supported by findings from intervention studies, although these produce less consistent results. Basal levels of IL-6 are reduced after training in patients with coronary artery disease (44). Aerobic training of adults aged 64 ys or more for 10 months also decreases basal plasma IL-6 (79). In severely obese subjects, the combination of a hypocaloric diet and regular physical activity for 15 weeks reduces not only plasma IL-6, but also the IL-6 mRNA content in subcutaneous adipose tissue and in skeletal muscle (14). In addition, athlete skiers have lower basal plasma IL-6 during the training season than off-season (145). However, others have not observed changes in basal IL-6 levels in response to training (16, 85, 104).

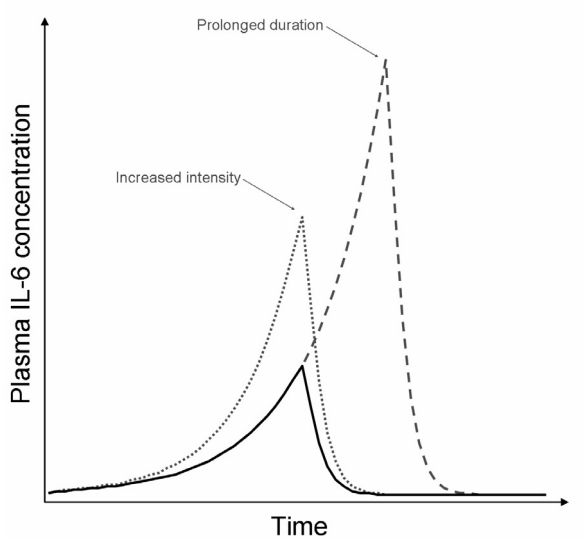

Fig. 3. The effect of exercise duration and intensity on the plasma IL-6 level.
Schematic presentation showing that in response to exercise, plasma IL-6 increases in a non-linear fashion over time (37, 119, 172) and peaks shortly after the cessation of the exercise (solid line). If the exercise intensity increases, plasma IL-6 is likely to increase faster resulting in a higher peak plasma IL-6 level (dotted line). If the exercise duration is extended, the peak plasma IL-6 occurs later but is also augmented (dashed line). From an "area under the curve" point of view, the cumulative systemic effect of IL-6 in response to exercise may accordingly be more prominent in response to prolonged exercise compared to an intense but shorter bout of exercise, even if the peak IL-6 values are similar.

At present, evidence that the exercise-induced increase of plasma IL-6 is affected by training is limited. Using knee-extensor exercise, 7 healthy men trained for 1 hour 5 times a week for 10 weeks (38). Before and after the training, the participants performed knee-extensor exercise for 3 h at 50% of the maximal workload. Due to a marked training response, the absolute workload was much higher after training compared to pre-training. Despite this, the increase in IL-6 mRNA content by acute exercise was 76-fold before training but only 8 fold after training. In addition, the exercise-induced increase of plasma IL-6 was similar before and after training, although the absolute workload was increased by 44% with training. Accordingly, it could be speculated that differences in training status may explain why elderly subjects release the same amount of IL-6 as young subjects from the leg during knee-extensor exercise at the exact same relative – but half the same absolute – workload (127).

Noteworthy, while IL-6 appears to be down-regulated by training, the IL-6 receptor appears to be up-regulated: In response to exercise training, the basal IL-

↑, increase; ↓, decrease; ↔ not affected by rhIL-6; GH, growth hormone; A, adrenaline; NA, noradrenaline.

[a] In response to rhIL-6 infusion, plasma insulin decreases in subjects with type 2 diabetes but not in healthy controls.

Plasma IL-6 level (pg/ml)	Insulin	Cortisol	Glucagon	GH	A, NA	References
< 50	↔	↔	↔	↔	↔	(35, 59, 184, 185)
~50	↔	↑				(82)
~100	↔			↑		(103)
~150	↔	↑	↔		↔	(166, 167, 187)
~200	↔/↓[a]	↑	↑	↑	↔	(133, 192)
~300	↔	↑	↔	↑	↑	(167, 184, 185, 187)
~500	↔	↑	↑		↑	(174)
~4000	↔	↑	↑	↔		(184, 185)

Table 3. Acute effects of rhIL-6 on hormone levels in humans.

6R mRNA content in trained skeletal muscle is increased by ~100% (70). Accordingly, it is possible that the downregulation of IL-6 is partially counteracted by enhanced expression of IL-6R, whereby the sensitivity to IL-6 is increased. However, it remains to be determined if the increased IL-6R mRNA content corresponds to an increased expression of the IL-6R protein. Furthermore, it is not known if the enhanced IL-6R expression following training occurs in several tissues or only locally within the trained skeletal muscle. In the circulation, the IL-6R concentration is affected neither by training nor acute exercise (70).

Thus, there is good evidence that low physical activity results in elevated basal IL-6 levels, while a high level of physical activity results in low basal IL-6 levels. Yet, there is limited evidence indicating that the exercise-induced increase of IL-6 in the contracting muscle as well as in the circulation is attenuated by training. Since training adaptation includes changes known to counteract potential stimuli for IL-6, it is, however, very likely that further studies will demonstrate alterations in the exercise-induced IL-6 response by training.

SUMMARY AND CONCLUSION

Clearly, exercise may increase synthesis and subsequent release of IL-6 from contracting muscles, and this release may induce multiple effects in multiple tissues. IL-6 possesses somewhat catabolic features, indicated by the ability to increase energy expenditure, increase lipolysis, increase fat oxidation, increase endogenous glucose output (in part via reducing insulin-signalling in fat and liver), and increase cortisol. On the other hand, this mobilization of glucose and FFA from liver and fat to the circulation may result in enhanced substrate uptake by other tissues, e.g., the contracting skeletal muscle. The apparent discrepancy between tissues regarding the response to IL-6 may be due differences in downstream IL-6 signalling in different tissues. In addition, the IL-6 released from the contracting muscles may induce an anti-inflammatory response reflected by increase of IL-1ra, IL-10, CRP, and cortisol without concomitant increases in pro-inflammatory mediators.

The time and intensity required in order to accumulate IL-6 protein within the contracting muscle are not well characterized. In contrast, duration of exercise is the single most important factor that determines the magnitude of the systemic IL-6 response. The longer duration of the exercise, the more pronounced the systemic IL-6 response will be. Accordingly, short bouts of exercise or exercise at low intensity are not likely to increase IL-6 to an extent where systemic effects of IL-6 are expected. Independent of mode, exercise for less than one hour induces a peak plasma IL-6 concentration below 10 pg/ml (< 10 fold increase from pre-exercise level, Fig. 1B), and this for only a short period of time (Fig. 2). Several studies have demonstrated that pre-exercise glycogen depletion accelerates the exercise-induced IL-6 response, while carbohydrate supplementation reduces the increase of plasma IL-6. Thus, reduced availability of substrates fuelling the mus-

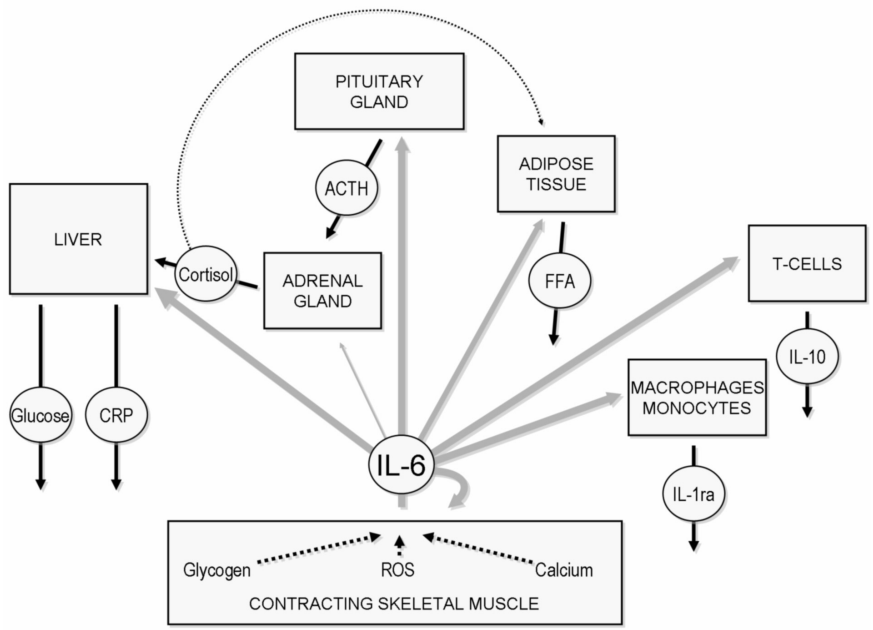

Fig. 4. Possible effects of IL-6 released from contracting skeletal muscle in response to exercise.
Several mechanisms may link muscle contractions to IL-6 synthesis. Changes in calcium homeostasis, impaired glucose availability, and increased formation of reactive oxygen species (ROS) are all capable of inducing transcription factors regulating IL-6 gene transcription. The synthesized IL-6 may act locally within the contracting skeletal muscle in a paracrine manner or be released into the circulation, thus able to induce systemic effects. In liver, the circulating IL-6 may increase hepatic glucose output and production of C-reactive protein (CRP). In adipose tissue, IL-6 produced locally and IL-6 from the circulation in concert may increase lipolysis. Via activation of the hypothalamic-pituitary-adrenal (HPA) axis, the circulating IL-6 may stimulate cortisol release, which may further enhance the lipolysis. In lymphocytes, macrophages, and monocytes, the circulating IL-6 may stimulate the production of IL-1ra and IL-10.

cle contractile activity appears to be one of the main triggers of IL-6 production. To reduce substrate availability, glycogen stores in liver and muscle have to be reduced markedly, which is process that takes time, although dependent on the intensity.

Low physical activity is associated with increased plasma IL-6 at rest. Exercise training dramatically reduces the exercise-induced accumulation of IL-6 mRNA within the contracting skeletal muscle. Training adaptation also includes increased glycogen content in the resting skeletal muscle and enhanced capacity to oxidize fat, whereby the contracting muscle becomes less dependent on plasma glucose as well as capable of performing more mechanical work before glycogen levels are reduced critically. Accordingly, exercise training may counteract several potential stimuli of IL-6 production. Therefore, a low plasma IL-6 concentration at rest as well as in response to exercise appears to characterize the IL-6 response after training adaptation. Interestingly, the training-induced downregulation of IL-6 may to some extent be compensated by an enhanced sensitivity to IL-6, at least within the trained skeletal muscle.

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Exercise and Toll-like receptors

Running Head: Exercise and TLRs

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Abstract

Toll-like receptors (TLRs) are highly conserved trans-membrane proteins that play an important role in the detection and recognition of microbial pathogens. The key product of TLR signalling in antigen presenting cells is the production of inflammatory cytokines and proteins. The TLR pathway plays an important role in mediating whole body inflammation, which has been implicated in the development of chronic disease. An accumulation of chronic, low-grade inflammation is common in individuals that live a sedentary lifestyle; however, the mechanism underlying this connection is not fully understood. There is evidence to show that TLRs may be involved in the link between a sedentary lifestyle, inflammation, and disease. Recent studies have shown that both acute aerobic and chronic resistance exercise resulted in decreased monocyte cell-surface expression of TLRs. Furthermore, a period of chronic exercise training decreases both inflammatory cytokine production and the cell-surface expression of TLR4 on monocytes. These effects may contribute to post-exercise immunodepression and the reported higher susceptibility to infection in athletes. However, over the long term, a decrease in TLR expression may represent a beneficial effect because it decreases the inflammatory capacity of leukocytes, thus altering whole body chronic inflammation. The precise physiological stimulus mediating an exercise-induced decrease in cell-surface TLR expression is not known; however, a number of possible signals have been implicated including anti-inflammatory cytokines, stress hormones and heat shock proteins.
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Keywords: Toll-like receptor, monocyte, infection, inflammation, training

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Introduction: The role of Toll-like receptors

Toll receptors are trans-membrane proteins that are highly conserved in animal phyla from insects to mammals, including humans (43, 49). A Toll protein was originally discovered in the fruit fly *(Drosophilia),* and was found to play an important role in ontogenesis and antifungal defence (43). In 1997, Medzhitov et al (49) identified and characterised a human homologue of the *Drosophila* Toll protein and named it a Toll-like receptor (TLR). Thus far 11 human TLRs (TLR1-11) have been identified (83), and they appear to play roles in pathogen detection and recognition and the induction of antimicrobial activity by both the innate and acquired immune system (82-84)

In mammals, TLRs function as pattern recognition receptors that recognise conserved pathogen-associated molecular patterns (PAMPs) expressed by a wide spectrum of infectious microorganisms. TLRs are central in the detection and recognition of pathogen subtypes including gram-positive and gram-negative bacteria, DNA and RNA viruses, fungi and protozoa (52). The specific PAMPs recognised by TLR family members have been well characterised: TLR2 homodimers and TLR2-TLR1 and TLR2-TLR6 heterodimers mediate responses to bacterial lipoproteins, peptidoglycan, lipoteichoic acid and zymosan (57, 73, 87); TLR3 to double-stranded RNA, a marker of viral infection (1); TLR4 to bacterial lipopolysaccharide (LPS) (29, 63); TLR5 to bacterial flagellin (25); TLR7 and 8 to imidazoquinolines and single stranded RNA (27, 34); and TLR9 to bacterial DNA (28). As PAMPs are not expressed by host cells, TLR recognition of PAMPs permits self-nonself discrimination. Nevertheless, there are several endogenous ligands of TLRs that may play a role in regulating the expression of the receptors (37) or allow the immune system to respond to damage or "danger signals."

As illustrated in Figure 1, the recognition of PAMPs by TLRs triggers intracellular signalling pathways (85) and results in induction of a conserved host defence programme which includes the production of inflammatory cytokines (1, 49) and the induction of antimicrobial activity (3), allowing the host to respond immediately to microbial invasion. It is now clear that TLRs on monocytes, macrophages, and dendritic cells contribute significantly to the development of adaptive immune responses (59). Activation of TLRs on these antigen presenting cells (APCs) results in both the activation of innate immune responses by inducing antimicrobial activity (3, 88), the production of inflammatory cytokines including IL-1β, IL-6, IL-8, and TNF-α (8, 48, 58). It also results in the generation of an adaptive immune response through the up-regulation of major histocompatibility complex class II (MHCII) and co-stimulatory molecule expression (CD80/86) (1, 8, 94) on APCs and the release of IL-12 from dendritic cells (DCs). Although the induction of MHCII and CD80/86 is critical to naïve T cell activation, the generation of adaptive immunity is also controlled by $CD4^+CD25^+$ suppressor or regulatory T (T_R) cells (8). A critical role for TLRs in regulating the suppressor activity of T_R cells has recently been reported. Specifically, Pasare & Medzhitov (58) demonstrated that IL-6 production by DCs following TLR activation is essential to the activation of pathogen-specific T cells by inhibiting the suppressive effects of $CD4^+CD25^+$ T_R cells.

Therefore, TLRs, through pathogen recognition and the control of innate and adaptive immune responses, play a pivotal role in the host defence response

against infection. Indeed, the importance of the TLR signalling pathway in mammalian immunity is evident from studies involving rodents with specific TLR deficiencies that have examined the role of the Toll family of receptors and their cognate downstream signalling molecules (2, 52, 70, 72, 86). Furthermore, human studies provide evidence that the expression and activation of TLRs *in vivo* contributes to host defence against microbial pathogens (40) and the effective generation of specific antibodies following vaccination (2).

	22°C	37°C	40°C
TLR1	20.5 ± 3.8	20.5 ± 7.2	18.9 ± 3.1
TLR2	109.5 ± 10.4	121.8 ± 14.3	99.9 ± 11.4
TLR4	33.8 ± 2.7	32.0 ± 4.0	28.8 ± 3.5
CD14	434 ± 15	401 ± 16	424 ± 22

Table 1. The effect of incubation for 1.5 h at room temperature (22°C), 37°C and 40°C on TLR and CD14 expression on CD14+ monocytes. All values are mean ± SEM geometric mean fluorescence intensity (GMFI). Previously unpublished data.

TLRs also interact with a variety of endogenous human ligands and influence the activity of a wide range of tissues and cell processes (35). TLRs are also known to play a role in asthma, coronary heart diseases, inflammatory bowel disease, rheumatoid arthritis and transplant rejection (15). Many important opportunities for disease modification through TLR manipulation can be imagined. The recent findings that both acute and chronic exercise result in lowered expression of some TLRs on APCs offers a new mechanism to explain some of the effects of exercise on immune function as well as the longer term health benefits of a more active lifestyle in reducing the risk of cardiovascular and metabolic diseases which are commonly associated with elevated levels of inflammation markers including cytokines.

Acute exercise and monocyte TLR expression

Only a small number of studies have examined the effect of exercise training on TLR expression (these are described later in this review), and the effect of acute exercise on TLR expression has received even less attention. Lancaster et al (42) were the first to report a decrease in monocyte (CD14+) TLR expression and function following a single bout of prolonged aerobic exercise. Specifically they investigated the influence of 1.5 h of strenuous cycling exercise (~65 % $VO_{2\ max}$) in the heat (34 ºC) on TLR expression and function in vivo. TLR1, TLR2 and TLR4 expression were significantly lower at post-exercise and after 2 h recovery compared to samples obtained at rest. In contrast, TLR9 expression was unaffected by exercise. Lancaster et al (42) exercised subjects in the heat (34 ºC) to enhance the exercise-induced stress response, and maximise the likelihood of observing an effect of exercise on TLR expression. Subjects in this study would have experienced much larger increases in body temperature than if they had exercised under normal conditions. As a result it is unclear whether the observed changes in TLR expression were the result of physiological changes induced by exercise, or physiological changes induced by sizable increases in body temperature comparable to those observed in febrile illness.

To determine if there is a direct effect of temperature on TLR expression, in the Loughborough laboratory we obtained peripheral blood samples from 8

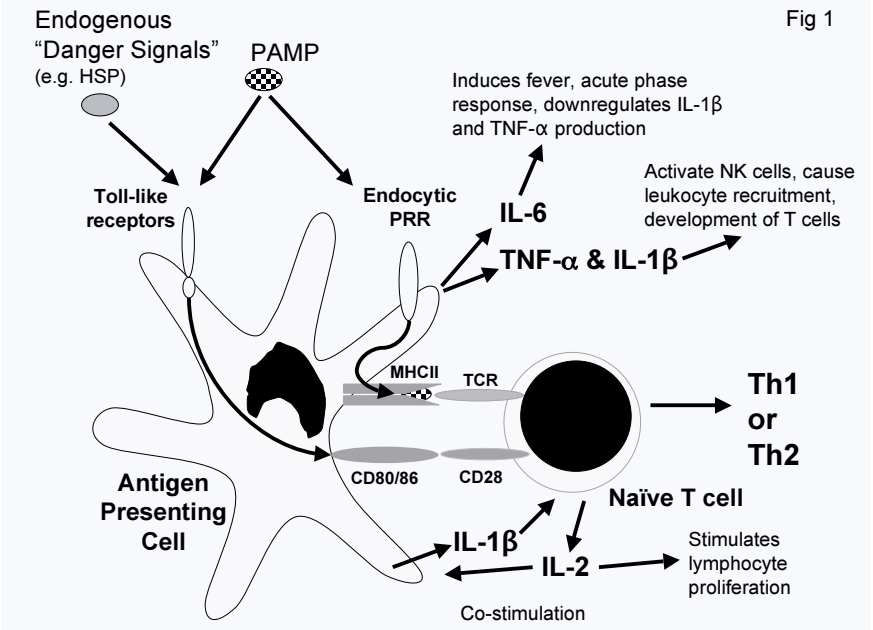

Figure 1: Binding of pathogen associated molecular patterns (PAMPs) and endogenous danger signal molecules such as heat shock proteins (HSP) to Toll-like receptors (TLRs) leads to activation of the antigen presenting cell (APC) and subsequent activation of T-helper (Th) cells that it interacts with. APCs take up antigen via endocytic pattern recognition receptors (PRRs) and process (degrade) it to immunogenic peptides which are displayed to T cell receptors (TCRs) in the polymorphic grove of MHC class II molecules after their appearance at the cell surface. An interaction occurs between the APC and the T cell as indicated, usually resulting in cellular activation. When naive CD4+ T helper (Th) cells are activated by APCs that provide appropriate co-stimulatory signals (cytokines and/or accessory binding molecules), they differentiate into Th1 or Th2 cells with polarised cytokine secretion. Cytokines produced by APCs and Th cells result in inflammation and proliferation and activation of other immune components.

healthy male volunteers (mean ± SD) (age 27 ± 11 yrs) at 09.00. Blood samples from each participant were incubated at room temperature (22 °C), 37 °C and 40 °C for 1.5 h and the expression of TLR1, 2 and 4 and CD14 on the cell surface of monocytes was measured as described by Lancaster et al. (42). Briefly, whole blood was surface stained with CD-14 FITC (Becton Dickinson Biosciences, Oxford, UK) and antihuman PE-conjugated TLR1 (clone GD2.F4), TLR2 (clone TL2.1) or TLR4 (clone HTA 125) antibody (e-Bioscience, San Diego, CA) or the appropriate isotype control for TLR1 (mouse IgG1-PE), TLR2 and TLR4 (both mouse IgG2a-PE). Samples were analyzed on a flow cytometer (BD FACSCalibur) equipped with CellQuest software package (BD Biosciences). Cells were gated according to side-scatter and CD14-FITC expression and the change in the geometric mean fluorescence intensity (GMFI) between TLR1, 2 and 4 antibodies and isotype controls was obtained to quantify TLR expression on CD14+ cells (5000 cells were analyzed). No significant effect of temperature was found for

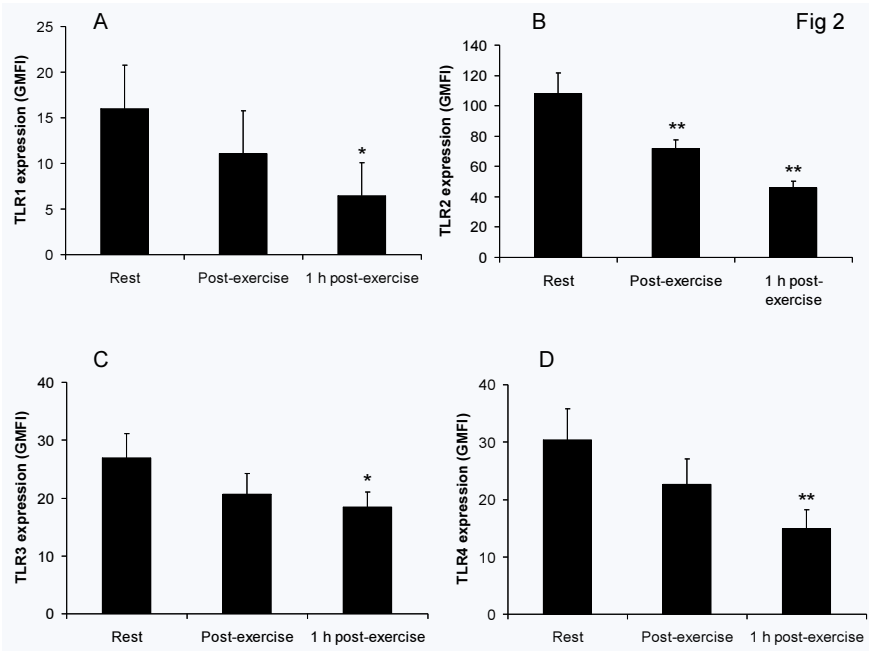

Figure 2: The effect of 2.5 h cycling at 60% VO2max on monocyte (CD14+) cell-surface expression of (A) TLR1, (B) TLR2, (C) TLR3 and (D) TLR4. Significantly less than pre-exercise (Rest) as determined by paired-samples t-test: * P<0.05, ** P<0.01. Data are mean ± SEM geometric mean fluorescence intensity (GMFI) from 11 recreationally active men. Previously unpublished data.

CD14, TLR1, TLR2 or TLR4 expression on CD14+ cells as illustrated in Table 1 Thus, an increase in blood temperature to febrile levels does not appear to alter monocyte TLR expression. These findings are in agreement with Zhou et al (96) who reported that one hour of exposure to 42 ºC followed by a further 6 h incubation at 42 ºC resulted in no change in cell-surface expression of CD14, TLR2 or TLR4 on cultured human monocytes despite an upregulation of mRNA for TLR2 and TLR4 that was associated with elevated cytoplasmic levels of HSP70.

Furthermore, we have recently examined the effects of prolonged exercise in temperate conditions on monocyte cell surface expression of TLRs 1, 2, 3 and 4. Peripheral blood samples were obtained from 11 endurance trained male cyclists (age 20 ± 2 yrs, maximal oxygen uptake ($VO_{2\,max}$) 57.2 ± 8.8 $ml.kg^{-1}.min^{-1}$) at rest and following 2.5 h cycling at 60 % $VO_{2\,max}$ in conditions of 20 ± 2 ºC and 40 ± 5 % relative humidity. Flow cytometry methods were the same as described above; for TLR3 an anti-human PE-conjugated antibody (clone TLR3.7) and mouse IgG1-PE isotype control were used (e-Bioscience). There was a trend for TLR1 expression to be lower following exercise compared with rest (P = 0.07; see Figure 2A) and a significant main effect of time was found for TLR2 (P = 0.004), TLR3 (P = 0.031) and TLR4 (P = 0.003; see Figures 2*B*, *C* and *D*). Core temperature assessed using an oral thermometer was not increased by more than 0.5 ºC in any of the subjects at the end of the exercise bout. Taken

together these findings suggest that acute exercise can result in decreased monocyte TLR expression but this effect is independent of changes in core temperature.

In contrast to these findings, McFarlin et al (47) found no change in monocyte (CD14+) cell surface expression of TLR4 following an acute bout of resistance exercise in trained and untrained elderly women. However, the exercise bout that involved 3 sets of 10 repetitions at 80 % of the 1-repetition maximum for each of 9 different muscle groups lasted only about 1 hour. The exercise used in this study may not have been of sufficient duration to induce a fall in monocyte TLR expression.

Implications for immunity and susceptibility to infection

TLR3 is expressed both intracellularly and on the cell surface. To our knowledge the study described above is the first study to demonstrate that cell-surface TLR3 expression is depressed by exercise. Since TLR3 detects double-stranded RNA—a molecular pattern associated with the presence of viral infection—this could be an important factor in the apparently increased risk of viral infection following very prolonged bouts of strenuous exercise. Although at

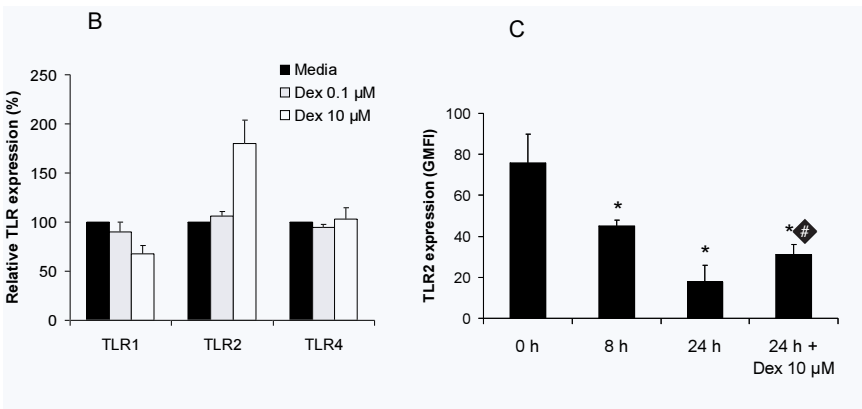

Figure 3: Effect of dexamethasone (Dex) on monocyte (CD14+) cell surface expression of TLR1, 2 and 4. Whole blood was incubated with either media or dexamethasone (10 µM or 0.1 µM) for either (A) 8 h or 24 h (B and C). The data in Figure A represent the mean ± SEM of samples performed in duplicate from 8 individual donors. Significant difference from media only as determined by paired-samples t-test: * $P<0.05$. The data in Figures B and C represent the mean ± SEM from 4 independent experiments performed in duplicate. The values for TLR expression in Figure C are expressed relative to samples incubated with media only. Significant difference from 0 h as determined by paired-samples t-test: * $P<0.05$. Significant difference from 24 h (without Dex) as determined by paired-samples t-test: # $P<0.05$. GMFI: geometric mean fluorescence intensity. Data from Lancaster (41).

present this is speculative there is some evidence that TLR3 is an essential component in innate immune defence against viral infection in mice (81). It is also known that activation of TLR3 induces the activation of NF-kappa-B, the production of type 1 interferons, and inflammatory cytokines (36) which are important mediators of host defence against viral infection. Exercise-induced down-regulation of TLR2 and TLR4 expression has been shown to be associated with reduced MHC II and co-stimulatory molecule (CD80 and CD86) expression and reduced IL-6 secretion by monocytes exposed to TLR2 and 4 ligands (42). These changes might similarly decrease host defence against bacterial infection.

We have used blood monocytes in these studies because it is not possible to collect a sufficient number of tissue macrophages when using a human model. Since monocytes (a less mature form of macrophage) and macrophages share a similar linear progression of their lifespan, it is possible that monocytes are good proxy measures of macrophage activity. As with other published immunological methods, we acknowledge that in vitro measurements may not be reflective of in vivo responses.

Mechanisms for exercise-induced changes in TLR expression

Although at present the mechanisms through which exercise suppresses TLR expression are unknown, altered cell populations, increased levels of circulating cytokines, heat shock proteins (HSPs), and glucocorticoids are obvious candidates. It could be argued that the effects of acute prolonged aerobic exercise on TLR expression could be due to the differential mobilisation of monocyte subsets to the circulation. Two monocyte populations have been identified in human blood, the $CD14^{++}CD16^{-}HLA\text{-}DR^{+}$ classical monocytes and the $CD14^{+}CD16^{+}HLA\text{-}DR^{++}$ pro-inflammatory monocytes (97). The $CD14^{+}CD16^{+}$ pro-inflammatory monocytes have a higher surface expression of TLR2 and produce a greater amount of TNF-α following treatment with TLR2 ligands compared with the $CD14^{+}CD16^{-}$ classical monocytes (7). However, $CD14^{+}CD16^{+}$ monocytes are released from the marginal pool and mobilised into the circulation to a greater extent than $CD14^{+}CD16^{-}$ monocytes during exercise (79) indicating that the effects of prolonged exercise on TLR expression that we have observed are not likely due to the mobilisation of phenotypically distinct monocyte subsets.

TLR activation is known to induce cytokine release (1, 49), but TLR expression appears to be modulated by cytokines. Staege et al (78) reported that in vitro TLR2 and TLR4 expression was down-regulated by interleukin-4 (IL-4) treatment to one-fifth and one-quarter of the level found in untreated cells, respectively. Other authors reported that TLR expression was influenced by cytokine concentrations both in vitro and in vivo (50, 74). It is widely accepted that the circulating concentrations of several cytokines are increased following exercise (56, 90), and it is possible that exercise-induced elevations in cytokines may suppress TLR expression.

Stress hormones, such as glucocorticoids (GCs), mediate many of the immunological changes associated with exercise (61). Lancaster (41) recently investigated the effects of *in vitro* treatment of human blood from 8 healthy donors with the synthetic GC dexamethasone (DEX). Incubation of whole blood for 8 h at 37 ºC (5 % CO_2), at DEX concentrations which resulted in a 50 % (0.1μM) and 100 % (10μM) inhibition of TLR2/TL6 dimer and TLR4 function (induction of IL-6 expression by LPS), resulted in a modest and concentration-dependent decrease in the surface expression of monocyte TLRs 1, 2 and 4 (Figure 3A). While DEX treatment

resulted in a highly reproducible decrease in the surface expression of TLR1, which was observed in all donors, 3 of the 8 donors showed no effect of DEX treatment on either TLR2 or TLR4 expression which could suggest a differential regulation of TLR expression by GCs. In support of this notion, peripheral blood mononuclear cells treated with GCs for 18 h showed a down-regulation of TLR3 gene expression, while TLR2 and TLR4 gene expression was markedly elevated (22). To further examine GC regulation of monocyte TLR expression, Lancaster (41) incubated whole blood with DEX for 24 h. Similar to the results obtained following 8 h of DEX treatment, 24 h of DEX treatment caused a moderate and concentration-dependent decrease in the surface expression of TLR1 (Figure 3B). Furthermore, and in agreement with the study by Galon et al (22), Lancaster (41) observed a significant increase in TLR2 expression in samples treated with 10 μM DEX for 24 h compared with the untreated controls (Figure 3C). However, in contrast to the study by Galon et al (22), there appeared to be no effect of 24 h DEX treatment on monocyte TLR4 expression (41). Galon et al (22) only examined TLR2 and TLR4 gene expression at a single time point. The Lancaster (41) data demonstrate that there is a time-dependent down-regulation of monocyte TLR2 expression that occurs *in vitro* (Figure 3C). Therefore, instead of up-regulating TLR expression *per se*, prolonged DEX treatment appears to attenuate the down-regulation of TLR2 that occurs over time when in culture. Taken together, these results suggest that GCs are able to modulate monocyte TLR expression, and that members of the TLR family are differentially sensitive to GC treatment. However, whether the effects of GCs are mediated directly or indirectly – possibly as a result of a modulation of the release of soluble factors capable of influencing TLR expression, e.g. cytokines – awaits further exploration. In addition, other researchers reporting that GCs altered TLR expression found that GCs induce rather than suppress TLR expression (22, 32). Therefore, it appears unlikely that GCs play a vital role in the down-regulation of TLR expression with exercise. GCs are also known to deplete the inflammatory monocyte population, which could influence TLR expression.

Given the central role of TLRs in innate and adaptive immunity, additional research is needed to identify the mechanisms by which acute exercise regulates and suppresses TLR expression. Another question to which we do not yet have the answer is what happens to the TLRs that disappear from the cell surface during exercise? Are the TLRs shed from the surface or are they internalised? If the latter is true, are they degraded? Other questions include the length of time required for TLR expression to recover following an acute bout of prolonged exercise and the dose of exercise required to induce a decrease in monocyte TLR expression?

Effects of exercise training on monocyte TLR expression

Only a handful of studies have examined the effect of chronic exercise training on TLR expression (20, 80) or compared trained and untrained individuals (46, 47). The results of these studies are summarised in Figures 4, 5 and 6. Flynn et al. (20) initially found that 10 weeks of resistance exercise training significantly lowered LPS-stimulated production of IL-6, IL-1β, and TNF-α (20). In an effort to identify a mechanism that may explain the initial findings, whole blood TLR4 mRNA content was measured. We found that resistance-trained older women (65-85

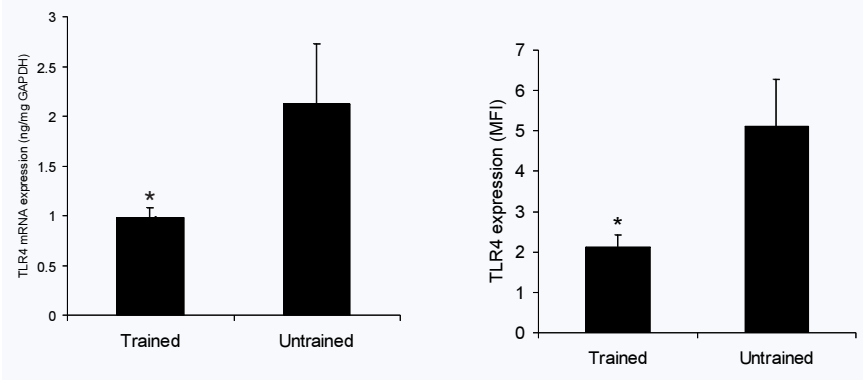

Figure 4: This figure summarizes the key findings with respect to TLR4 expression from the following studies: **A**) Cross-sectional comparison of elderly women following a 9-week resistance training programme (n=9) with untrained controls (n=6) (20); **B**) Cross-sectional comparison of elderly women examining the effects of activity status (n=10 trained and n=10 untrained) (47). * indicates trained significantly lower than untrained ($P<0.05$).

years old) had significantly lower TLR4 mRNA than sedentary untrained older women (20) (Figure 4A). The study design did not allow an analysis of the influence of an acute bout of resistance exercise on TLR expression. Therefore, a follow-up study was conducted in which the effect of a single bout of resistance exercise in trained and untrained older women on CD14+ cell-surface TLR expression (47) was compared. TLR4 expression was measured on CD14+ cells from venous blood prior to exercise, and immediately, 2 h, 6 h, and 24 h after an acute bout of resistance exercise. LPS stimulated whole blood cultures were run in parallel with the flow analysis (20). Exercise training status, but not the acute exercise session, influenced cell-surface TLR4 expression (47) (Figure 4B), such that TLR4 expression in trained subjects was roughly half of the untrained. We also found that high monocyte TLR4 expression was associated with high LPS-stimulated IL-6, TNF-α, and IL-1β production (47).

An early focus on older women (20, 47) prevented Flynn's group from determining whether training effects on TLR4 expression would be present in men and younger individuals. Follow-up studies were designed to address these gaps (46, 80). We recruited participants to fill one of the following groups (46): older, physically active (i.e. trained); older, physically inactive (i.e. untrained); younger, physically active; or younger, physically inactive. Physical activity status, but not age, influenced TLR4 cell-surface expression and LPS-stimulated inflammatory cytokine production (46). Physically active individuals had lower cell-surface TLR4 expression and lower LPS-stimulated inflammatory cytokine production than physically inactive individuals (46) (Figure 5). In a subsequent study, groups of older and younger physically inactive subjects, similar to those in the cross-sectional study, were endurance and resistance exercise trained for 12 weeks (80). Groups of age-, gender-, and health status-matched physically active individuals maintained habitual activity and served as controls. Exercise training (combined aerobic and resistance) significantly decreased cell-surface TLR4

expression (Figure 6) and LPS-stimulated inflammatory cytokine production (80). To our knowledge, these are the only published studies in which the TLR expression following a period of exercise training has been examined. These studies only provide descriptive evaluation of TLR4 and TLR2, making the next logical step to complete an evaluation of mechanisms to determine how exercise training suppresses TLR expression and alters inflammatory cytokine production capacity. Also, more descriptive research is needed to evaluate the effect of chronic exercise training on TLRs other than TLR4 and TLR2.

Implications for long-term health

The accumulation of chronic, low-grade inflammation has been linked to the development of a number of diseases, such as type 2 diabetes mellitus and cardiovascular diseases (13, 30, 77). Based on the literature, whole body chronic inflammation appears to be highest in individuals who are sedentary and/or obese (60). The most effective countermeasures against the accumulation of chronic inflammation appear to be a physically active lifestyle (3-5 days of exercise per week) and maintenance of a healthy body weight (i.e. a body mass index of less than 25 kg/m^2) (60). Physically active participants have significantly lower cell-surface TLR4 expression and monocyte inflammatory cytokine production capacity than physically inactive subjects (20, 46, 47, 80). Blood monocyte TLR cell-surface expression and inflammatory capacity may directly or indirectly affect an individual's level of whole body chronic inflammation. Direct effects are associated with production and release of inflammatory cytokines into the blood, while indirect effects are associated with the ability of monocyte/macrophage-derived inflammatory cytokines to stimulate the release of acute phase proteins from the liver and influence the activity of peripheral tissue macrophages.

In addition to assessing blood monocytes as a direct source of whole body inflammation, they may be a good proxy/convenience measure of the TLR expression and inflammatory capacity of macrophages found in adipose tissue, skeletal muscle, and other peripheral tissue compartments. In adipose tissue, the majority of the TNF-α and about half the IL-6 released comes from adipose tissue macrophages (19, 75). Weight loss has been reported to exert anti-inflammatory affects by decreasing the release of TNF-α from adipose tissue (39, 68) and based on the previous statement, it is clear that this occurs due to changes in macrophage TNF-α production (39, 53). The TLR4 pathway is responsible for

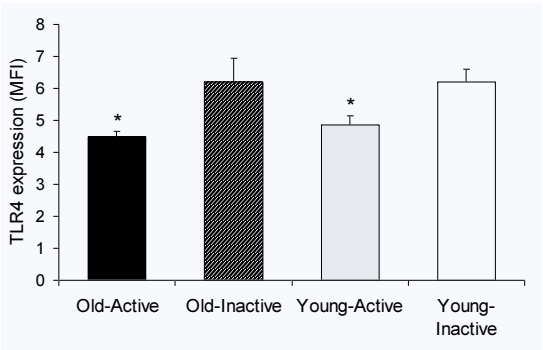

Figure 5: Cell surface (CD14+ cells) TLR4 expression (mean fluorescence intensity, MFI) in old (65-80 years) and young (18-35 years) physically active and inactive subjects (46). Subject numbers in each group were as follows: old, active n= 23; old, inactive n=21; young, active n=21; young, inactive n=19. * indicates active significantly lower than inactive ($P<0.05$).

macrophage production of TNF-α (92), which may be negatively influenced by weight loss. Macrophages from skeletal muscle and other peripheral tissue compartments may be similarly affected; however, to our knowledge this has not been confirmed.

The pathophysiologic link between whole body chronic inflammation and the development of inflammatory-related disease is well documented (13, 30, 77). Monocytes and peripheral tissue macrophages are responsible for an elevation in whole body chronic inflammation. According to the literature, TLR pathways are responsible for mediating the capacity of monocytes and macrophages to produce inflammation (8, 59, 82). More research is needed to address the possible mechanistic links between altered TLR expression and risk of chronic disease and other gaps in the literature.

Ageing, inflammation, exercise, and Toll-like receptors

It is not currently known whether inflammatory dysfunction is the cause or the result of the ageing process (69). Nevertheless, there is a considerable amount of published research to support the contention that ageing is associated with higher levels of inflammatory biomarkers (12, 17, 18, 66) In contrast, Beharka et al. (6) found that inflammatory markers were similar between younger and older subjects when subjects with chronic disease were excluded from the data set. Similarly, Flynn's group found similar LPS-stimulated inflammatory cytokine production and C-reactive protein (CRP) levels in healthy older and younger subjects—provided they were grouped by physical activity level. That is, physically active subjects had significantly lower biomarkers of inflammation, irrespective of age group, than physically inactive subjects. There is a growing consensus that exercise training or high levels of physical activity have anti-inflammatory effects (4, 21, 23, 55, 80, 89, 95). Ford et al. (21) for example, demonstrated a strong independent influence of physical activity on CRP with odds ratio for elevated CRP levels (95 % confidence intervals) of 0.98 (0.78-1.23), 0.85 (0.70-1.02), and 0.53 (0.40-0.71) for those who reported light, moderate, or vigorous physical activity, respectively. Some researchers concluded that body fat changes are responsible for the so-called anti-inflammatory effect of exercise (24, 53, 91), but the weight of evidence appears to be in favour of exercise exerting anti-inflammatory effects in the absence of changes in body fat (23, 55, 80).

Poorly regulated inflammation in the older population is linked to an increase in chronic diseases (11). Toll-like receptors or TLR signaling are linked to chronic diseases such as vascular disease (16, 93) and osteoporosis (33, 44). Nevertheless, there is uncertainty regarding the cause of the observed elevation in inflammatory biomarkers in older adults. It remains to be shown that physical inactivity plays a significant role in the elevated inflammatory status of the elderly cohort.

Ageing and Toll-like receptors

Few researchers have examined age-related differences in toll-like receptor expression. Much of what we currently know comes from animal research (9, 10, 65), but comparisons of human and murine toll-like receptor responses may be complicated

by substantial inter-species differences (26, 64). For example, LPS stimulation increased TLR4 expression in human monocytes and neutrophils (51), but LPS did not increase TLR4 expression in murine macrophages (45).

Boehmer et al (9) observed no effect of age on TLR4 expression in mice but explained an age-related defect in LPS-stimulated cytokine production with evidence of significantly lower mitogen-activated protein kinase (MAPK) signalling (10). Therefore, the TLR4 signaling pathway was defective, but the defect was attributed to MAPK activation and not a result of reduced receptor expression.

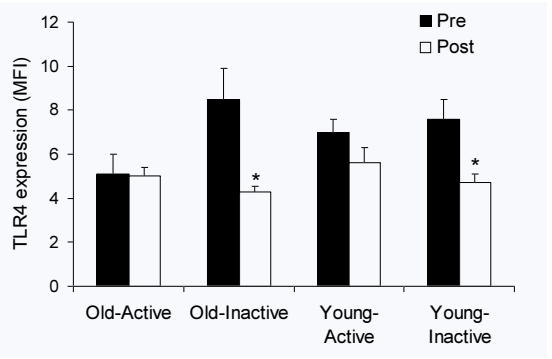

Figure 6: Cell surface (CD14+ cells) TLR4 expression (mean fluorescence intensity, MFI) in old (65-80 years) and young (18-35 years) physically active and inactive subjects before (Pre) and after (Post) 12 weeks of endurance and resistance training for the physically inactive subjects. The physically active subjects stayed physically active and served as controls (80). Subject numbers in each group were as follows: old, active n= 14; old, inactive n=17; young, active n=15; young, inactive n=14. * indicates post-training significantly lower than pre-training ($P<0.05$).

These findings were supported in a later paper by the same group and extended to show that impaired MAPK signaling reduced NF-κB activation in older mice (10). Renshaw et al. (65), on the other hand, observed substantially lower mRNA expression for TLR1-9 in both splenic macrophages and thioglycollate-stimulated peritoneal macrophages from older (18-24 months), compared to younger (2-3 months), C57BL/6 mice. These authors also reported significantly lower macrophage-surface expression of TLR4 in older mice (65).

There were no differences in TLR4 expression between younger (18-35 years of age) and older (65-80 years of age) humans (80) (Figure 5) who were screened to exclude several chronic diseases and drugs known to influence inflammatory processes. We are unaware of other published papers which have examined possible differences in TLR expression between young and old people.

Exercise, ageing and TLR4

Few studies have been conducted to examine the influence of physical activity or exercise training on TLR in older subjects, and these studies were all conducted by the same research group (20, 46 ,47, 80). Early research, undertaken to explain a training-induced lowering of LPS-stimulated inflammatory cytokine expression, showed significantly lower TLR4-mRNA in whole blood samples from older resistance trained, compared to age-matched untrained, subjects (20). In follow-up studies, physically active older adults had significantly lower CD14+ cell-surface TLR4 expression than physically inactive subjects of the same age and health status (4647).

As aforementioned, physical activity level appeared to be more important than age group (young: 18-35 years; old: 65-80 years) with respect to TLR4, CRP, and LPS-stimulated inflammatory cytokine production (46). Nevertheless, we found that 10-12 weeks of exercise training reduced TLR4 expression of both young and old sedentary people to the level found in physically active controls (80) (Figure 6). These changes occurred concomitantly with lower CRP (unpublished data), and marginally lower inflammatory cytokine production (80), but we are as yet unable to determine whether a training-induced blunting of TLR4 represents a positive change in terms of long-term health status.

TLR polymorphisms and age/disease

We assume that an exercise training-induced lowering of TLR4 signaling is a positive adaptation to training in older adults as it represents an adaptation that could lead to reduced systemic inflammation and improved overall health. Single nucleotide polymorphisms of TLR4, such as the Asp299Gly allele, provide an interesting model for comparison, since the Asp299Gly allele and high levels of physical activity are both associated with hyporesponsiveness to LPS (46, 47, 71). The Asp299Gly polymorphism is associated with a reduced incidence of cardiovascular disease (14, 38) and it has been shown that a higher proportion of centenarians possess this allele 5). Kolek et al. (38) suggested, after finding significantly lower CRP and lower incidence of myocardial infarction in subjects with the Asp299Gly allele, that "…down-regulation of innate immune responsiveness could beneficially modify CAD[CLA9] and diabetes risk and might provide a novel basis for genetic risk stratification and therapeutic targeting." Put into an antagonistic pleiotropy context, those with the wild type A allele (about 90 % of the Caucasian population) (5) are protected from early life bacterial infection but are at greater risk of death from diseases linked to inflammation, presumably from an overly aggressive inflammatory response, in later life (69).

TLR4 polymorphisms are also known to affect diabetic outcomes. For example, patients with type 2 diabetes possessing the Asp299Gly allele were less likely to have severe neuropathy than patients with the wild type allele (67). In contrast, these researchers found no differences in diabetic nephropathy between patients with the wild type and the Asp299Gly allele (67) and one group found no influence of TLR4 allele on diabetic outcomes (31).

Both TLR4 polymorphisms and exercise training are associated with hyporesponsiveness to LPS (20, 47, 71) and improvements in chronic disease outcomes (14, 23, 38, 76). Therefore, we speculate that exercise training-induced lowering of TLR4 is a positive adaptation in an older population.

Summary and conclusions

The purpose of this review was to summarize the research which has examined the effects of acute and chronic exercise on cell-surface TLR expression. The TLR pathway plays an important role in mediating whole body inflammation, which has been implicated in the development of chronic disease as well as acute disturbances of

immunity. TLRs have been reported to play roles in host defence against microbial pathogens (2, 40) and inhibition of the suppressor actions of CD4+25+ T-cells (58). The key product of TLR signaling is the production of inflammatory cytokines and proteins, which have been implicated in the pathophysiology of cardiovascular disease, type II diabetes mellitus, asthma, coronary heart disease, inflammatory bowel disease, and rheumatoid arthritis (15). An accumulation of chronic, low-grade inflammation is common in individuals that live a sedentary lifestyle; however, the mechanism underlying this connection is not fully understood. Based on the existing scientific literature, it appears that TLRs may be involved in the link between a sedentary lifestyle, inflammation, and disease.

Both acute aerobic and chronic resistance exercise have been reported to decrease monocyte cell-surface expression of TLRs (42, 80). Although another study found no effect of an acute bout of resistance exercise on monocyte cell-surface TLR4 expression (47), the most likely explanation for the difference in these findings is related to the severity/duration of the exercise stimulus and the age of the subject population. Others have reported that a period of chronic exercise training decreases inflammatory cytokine production and the study by Stewart et al. (80) was the first to report that cell-surface TLR4 expression decreased as well.

The exact physiological stimulus mediating an exercise-induced decrease in cell-surface TLR expression is not known; however, a number of possible signals have been implicated. In this review, we summarized the effects that have been attributed to anti-inflammatory cytokines (i.e. IL-4, etc.) (50, 74, 78) and stress hormones (i.e. glucocorticoids, etc.) (22, 32, 41, 42). The physiological stress associated with exercise directly affects the stress hormone release that occurs. These effects may contribute to post-exercise immunodepression and the reported higher susceptibility to infection in athletes. In the long-term, a decrease in TLR expression may represent a beneficial effect because it decreases the inflammatory capacity of leukocytes, thus altering whole body chronic inflammation.

Chronic inflammation has been implicated in the development of a number of different disease states (13, 30, 77) and leukocytes with high cell-surface TLR expression mostly account for this elevated inflammation. The exact stimulus by which exercise decreases cell-surface TLR expression is not known. More research is needed to identify and examine this response. Also more studies are needed to confirm previous studies from our laboratories. Future research in the area of exercise and TLR expression should develop mechanistic methodology to evaluate this pathway.

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The implication of alterations in leukocyte subset counts on immune function

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Short running head: Leukocyte counts and immune function

Abstract

Changes elicited by physical exercise in the numbers or the activity of blood T lymphocytes, NK cells and neutrophils are sometimes considered as indicators of altered immunocompetence. By comparing the pathological conditions, in which the changes in the numbers of cells resemble observations in the exercise studies, however, we notice large discrepancies in the clinical manifestations. Supplementary information regarding the differences in cytokines and mediators responsible for altered distributions helps to explain this difference and the significance of altered distribution of the cells for immunocompetence. Alterations in the numbers and activities of T lymphocytes, NK cells and neutrophils may serve as markers of sympathetic and glucocorticoid activities, which per se may influence immunocompetence not necessarily deterioration.
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Introduction

The immune system has various cellular components in different compartments of the body: blood and lymph, primary and secondary lymphoid organs, regional lymph nodes, gut associated lymphoid tissue, and most of the organs such as brain, liver, kidney, lung and skin. Among these compartments, cells in the blood compartment are the major targets of both clinical and physiological investigation because of their availability. Leukocyte and neutrophil numbers are commonly used clinical blood markers of acute inflammatory status. The number of blood leukocytes, however, is known also to alter in response to non-pathologic situations such as emotional or physical stressors including exercise. The kinetics of leukocyte fluctuation driven by both the duration and intensity of exercise are extensively documented in the review article by McCarthy (1).

Non-pathologic fluctuations of leukocytes as observed during and after exercise are often assumed to have certain significance for the individual's

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immunocompetence. These assumptions have often been made from the supposed role of each subset of leukocytes based on fundamental studies. However, such interpretations may not always be true. The aim of this article is to discuss the differences in the significance of increased or decreased blood leukocyte numbers in host defence mechanisms between pathological and physiological conditions.

A. Neutrophils

Clinical significance of neutropenia

Neutrophils are the major component of granulocytes in the blood. They differentiate in the bone marrow to highly potent bactericidal and fungicidal cells. Decreases in the number of blood neutrophils, namely, neutropenia, could be due to a variety of causes: auto-immune diseases such as systemic lupus erythematodes, hereditary diseases such as Chediak Higashi syndrome or glycogen storage disease type 1b, or non-hereditary drug toxicity (2). Various drugs, not only anti-neoplastic agents which directly affect myeloid precursor cells, may incidentally induce agranulocytosis or neutropenia because of myeloid suppression (3, 4). Various types of leukemia also cause dysregulation of myelopoiesis resulting in neutropenia.

Clinical manifestations of acute neutropenic patients depend on the underlying cause of neutropenia, but the common feature is the susceptibility to bacterial and fungal infections. Since neutropenia induced by non-anti neoplastic agents in various medical treatments is mostly specific to myeloid cells, it is in turn a good demonstration of the role of neutrophils in the host defence against bacterial and fungal infections (5). Elderly people are likely to present with decreased neutrophil count, but so-called benign idiopathic neutropenia subjects have been shown to respond properly to the granulocyte mobilization test induced by administration of hydrocortisone and adrenaline intravenously (6).

Clinical significance of neutrophilia and oxidative stress

While neutropenia is mostly caused in pathologic situations, neutrophilia is caused both in pathologic and non-pathologic situations. The major difference in the pathologic and non-pathologic neutrophilia is that the former mostly involves increased myelopoiesis induced by granulocyte colony-stimulating factor (G-CSF) (7, 8). Various factors contribute to non-pathologic neutrophilia. Neutrophilia observed during and after exercise mostly involves adrenaline and glucocorticoids (1). In contrast to neutropenia caused by myelosuppression, physiological neutrophilia per se does not lead to clinical manifestations.

Neutrophils play significant and essential roles in ischaemia -reperfusion injury of vascular endothelial cells (9). Blockade of neutrophil elastases or activation is effective in attenuating the clinical and pathological manifestations (10-13). A variety of pro-inflammatory cytokines, such as TNF-α, IL-1-β, and IL-6 are induced in ischaemia-reperfusion (I/R) injury. Pro-inflammatory cytokines promote expression of adhesion molecules such as integrins on

the vascular endothelial surface resulting in increased attachment of neutrophils (14, 15). Endothelial cells also produce IL-8 and macrophage inflammatory proteins (MIPs; CC chemokine) in responses to ischaemia, which effectively attract neutrophils and monocytes to the site of tissue injury/inflammation. Since the initial site of I/R injury is supposed to be at the endothelial surface, the responses reported are mostly common in various organs and tissues: lung (12, 16-18), liver (9, 19-21), heart (22-25), kidney (10, 11), brain (13, 26), and skeletal muscles (18, 27, 28).

Exercise and neutrophils

Exercise may involve certain oxidative damage as detected by carbonylated proteins in tissues such as skeletal muscles and lungs (29-31).

A series of pro- and anti- inflammatory cytokines is known to be induced during and after competitive endurance exercise (32). It is, however, unlikely that I/R injury, in which the neutrophil-endothelial interaction plays a significant role, causes the oxidative damage observed in such high-intensity endurance exercise unless complications such as severe dehydration or heat stroke are involved. In I/R injury, TNF-α plays a significant role in the promotion of endothelial damage (18, 20, 22, 27), but the increase in TNF-α production during or after exercise is rather modest or absent (33-36). Under inflammatory conditions, lysosomal enzymes such as elastases and myeloperoxidase are released in an explosive manner by degranulation. These enzymes also increase after prolonged endurance exercise, but the increase in the enzyme activity in the plasma was proportional to the increase in the number of neutrophils (37). The absence of amplification implies that the increase after exercise is not due to increased degranulation. Therefore, again it is not likely that neutrophil activation is involved in the oxidative stress of exercise, even when the number of neutrophils may rise several fold compared with the resting state. Claudicants with ischaemic vascular damage showed elevated plasma neutrophil elastase and up-regulated CD11b expression on neutrophils after exhaustive treadmill exercise. They also exhibited a neutrophilia, but the extent of the neutrophilia was similar to the control subjects (38). Thus, neutrophilia during and after exercise may not necessarily be related to oxidative tissue or endothelial damage due to neutrophil activation and degranulation.

B. Lymphocytes

Clinical significance of lymphopenia

Lymphopenia is caused by various pathological and physiological conditions. Viral infection is one of the frequent causes of lymphopenia. In one form of lymphopenia -inducing viral infection, human immunodeficiency syndrome virus (HIV) infects via CD4 molecules and chemokine receptor CXCR4 molecules. The host immune response targets and destroys infected CD4+ T cells, and an irreversible CD4 lymphopenia gradually develops (39-42). The manifestations of such immunodeficiency syndromes usually develop when the CD4+ T cell count drops below 200 per μL blood. Patients then usually suffer

	Pathological situation	Etiology	Immunological & clinical outcome	Non-pathological situation	Etiology	Immunological & clinical outcome
Neutrophil						
Neutropenia	drug-induced agranulocytosis	myeloid suppression	susceptible to bacterial and fungal infection			
Neutrophilia	acute infection/ inflammation	accelerated myelopoiesis (G-CSF)	–	exercise/stress	corticosteroid, catecholamines	healthy/fatigued
Lymphocyte						
Lymphopenia	HIV infection	selective CD4 destruction	susceptibility to fungal and viral infection	exercise/stress	chemokine receptor mediated	healthy/fatigued
	drug -induced (steroid)	chemokine receptor mediated	–			
Lymphocytosis	lymphopro-liferative diseases	neoplastic transformaton	disease progression			
NK cell						
NK lymphocytosis	–	genetic?	hypersesitivity to mosquito bites	exercise/stress/etc	catechola-mines	healthy/fatigued
NK deficiency	–	genetic?	recurrent HSV, CMV infection	intense exercise/stress	?	healthy/fatigued

Table Contrasting clinical outcome of pathological and non-pathological blood cell shifts

opportunistic infections such as pneumocystis carinii pneumonia, toxoplasmosis, mycobacterium infections, cytomegalovirus infection, mycosis, bacterial respiratory tract infection, hepatitis C virus infection, Kaposi's sarcoma and others. The profiles of the variety of complications in HIV positive patients suggest that the major impairment in the host defence caused by the absence of CD4+ T cells is the defective elimination of chronically infected intracellular microorganisms. Lederberger et al. (43) reported in their Swiss HIV Cohort Study with more than two thousand HIV infected subjects that the risk of opportunistic infection rose up to 2.5 fold when CD4+ T cell counts dropped below 200 per μL blood, and 5.8 fold when it dropped to below 50 per μL blood.

The decrease in circulating CD4+T cells from non-HIV causes such as common viral infections rarely leads to compromised immune functions as in HIV patients unless the host is elderly or infant, or under severe hyponutrition or dehydration. The difference in the clinical outcome is likely to be attributed to the nature of the lymphopenia. While loss of CD4+ T cells is the major cause of lymphopenia in HIV patients (39, 40, 42), lymphopenia or decreased circulating lymphocyte count of non-HIV causes is mostly due to the altered distribution of CD4+ T cells.

Exercise and lymphopenia

Circulating lymphocyte counts could be reduced by variety of factors that alter lymphocyte trafficking or distribution. Administration of G-CSF or pro-inflammatory cytokines such as IL-1 leads to a reduction in circulating lymphocyte counts (8). Exogenous glucocorticoid administration also elicits a fall in circulating lymphocyte counts (44). Endogenous glucocorticoid is suggested to be the major factor involved in transient lymphopenia after exercise, because post-exercise lymphopenia was only observed in the subjects whose plasma cortisol concentration was elevated during exercise (45). Elaborate regulation of chemokine receptor CXCR4 by endogenous cortisol was shown to be involved in post-exercise lymphopenia (46). In this study, we showed that incubation of lymphocytes with post-exercise plasma with varying concentrations of cortisol augmented CXCR4 on CD4+ T cells in a dose-dependent manner. Together with the dose-response of CXCR4 expression on CD4+ T cells to exogenous cortisol, the fluctuation of cortisol concentration in the physiological range is suggested to finely regulate the level of CXCR4 expression. The circadian fluctuation in the number of CD4+ T cells may correspond to the circadian fluctuation in the cortisol concentration due to the regulation of CXCR4 expression. Augmented expression of CXCR4 would drive T cells to tissues expressing CXCL12, the ligand for CXCR4, on endothelial cells. The lung, bone marrow, and liver are the potential candidates of the site of lymphocyte destination, because they express abundant transcripts of CXCL12 (47). The fact that administration of the CXCR4 antagonist AMD3100 to healthy subjects induces marked leukocytosis and lymphocytosis supports this assumption (48). Therefore, the major cause of glucocorticoid-induced lymphopenia is the altered distribution of lymphocytes among different compartments in the body.

Because glucocorticoids are generally accepted as immunosuppressive, such reduction in the number of lymphocytes is often considered to be an indicator of immunosuppression in general. In animal studies, however, Dhabhar et al (49, 50) showed endogenous glucocorticoid induced by acute restraint stress deploys lymphocytes to the skin, which resulted in an enhancement of the delayed type hypersensitivity (DTH) skin reaction. Interestingly, bronchial asthma patients who manifested acute exacerbation immediately after the withdrawal of steroid treatment were shown to have increased recruitment of T cells in the airways (51). Direct suppression of T cell activation by glucocorticoid administration might have prevented asthmatic symptoms during the therapy despite the accumulation of T cells in the airways. However, acute withdrawal of glucocorticoid administration might have activated the airway recruited T cells, which lead to acute asthmatic exacerbation.

In animal experiments, exercise training induced substantial thymic involution due to apoptosis of thymic cells by endogenous glucocorticoid (52). This result is likely to be interpreted as immunosuppressive. But in another study, steroid treatment only affected the naïve T cell population in the thymus without affecting peripheral memory resident T cells (53). Thymopoiesis recovered quickly after the cessation of steroid administration. Therefore, although pharmacological administration of glucocorticoid is generally immunosuppressive during the treatment, transient induction of endogenous glucocorticoid of a shorter duration as such in exercise or acute stress may not always be immunosuppressive.

It must be noted that true immunosuppression is similar to an anergy to certain pathogens. Hepatitis B virus carriers are anergy to hepatitis B virus (54). They don't have illness because their immune system neither reacts to the virus nor virus infected cells. Getting ill often means that the immune system is in action instead of suppression. Microbes are not always required to initiate an immune response. Cytokines may independently elicit a series of immune reactions. For example, interferon alpha/beta treatment for otherwise asymptomatic and apparently healthy hepatitis C patients elicits flu-like symptoms characterized by high fever, malaise and anorexia (55).

Milder decreases of unknown aetiology in circulating CD4+ T cell counts in the elderly may have certain clinical significance. Nakayama et al. (56) found that non-responders to the tuberculin PPD skin test had smaller numbers of circulating CD4+ T cells and Th1 cells compared with responders in immobile elderly subjects. They followed the subjects for 2 years and found that the odds of developing pneumonia was 2.57 fold greater in the tuberculin non-responders compared with the responders. This prospective study is a good example showing the significance of altered blood cell count in the actual host defence, no matter whether attenuated IFN-γ response is directly involved in the increased establishment of bacterial pneumonia.

The same group suggested, however, without denying the importance of immunocompetence, that older adults are at higher risk of pneumonia when their cough and swallowing reflexes are impaired (57). They found patients with basal ganglia infarction have higher incidence of pneumonia due to impairment in cough and swallowing reflexes (58). They found the impaired reflexes resulted in silent aspiration of oral contents including bacteria.

Accordingly, oral care to reduce the amount of oral bacteria was shown successfully to reduce the incidence of pneumonia (59). Amantadine or levodopa administration also improves the impaired swallowing reflex. Because both swallowing and cough reflexes are mediated by endogenous substance P (SP) released from vagal sensory nerves in the pharynx and upper airways, the addition of a low dose capsaicin to a liquid or food, which stimulates the release of SP, may help prevent aspiration pneumonia. Angiotensin-converting enzyme inhibitor decreases SP catabolism resulting in improvements in both reflexes (57, 60, 61). These results and facts clearly show the greater importance of defence mechanisms independent of the immune system.

C. Natural killer cells

Natural killer (NK) cells are a distinct subset of lymphocytes. They were one of the immune components that attracted the attention of exercise scientists, because their number and activity in the blood fluctuated greatly under the influence of exercise (62). Many have assumed that an increase in the numbers or the activity of blood NK cells is somewhat beneficial based on the experimental evidence that the mononuclear cell fraction containing NK cells lysed certain cancer cell lines or virus infected cells. But, if we carefully look at the clinical outcomes of NK deficient patients with variety of genetic defects, we realise that NK cells are not super-weapons. They only have a relatively narrow range of targets.

Target recognition of NK cells

To date, although the mechanism responsible for target recognition of NK cells is still a hot topic of research, we already have a good outline of the principles regarding which or what type of cells are targeted by NK cells. The key molecules in the recognition of target cells are both classical and non-classical major histocompatibility complex class I (MHC I) molecules on target cells and killer activating receptors such as NKG2D, and inhibiting receptors such as NKG2A on NK cells (63). Normal cells express classical MHC I molecules which are recognized by NK receptors with immunoreceptor tyrosine-based inhibitory motifs (ITIMs). The inhibitory recognition does not trigger NK cells to attack the recognized cells. On the other hand, stressed cells that express variant MHC I molecules such as MICA and MICB bind to killer activating receptors such as NKG2D and trigger the killing sequence (64, 65).

Murine paired immunoglubulin-like receptors (PIRs) are a pair of structurally similar receptors expressed concomitantly on NK cells, B cells and macrophages. The pair recognizes specific viral structures and transduces activating or inhibitory signals. PIR-A requires homodimeric Fc receptor common γ chain, which harbours an immunoreceptor tyrosine-based activation motif (ITAMs), for its efficient cell surface expression and for the delivery of activation signaling. PIR-B contains (ITIMs) in its cytoplasmic portion and inhibits receptor-mediated activation signaling *in vitro* (66).

Roles of NK cells in the host defence system

The ability of NK cells to lyse viral- or bacterial-infected cells is apparently limited. NK cells lyse herpes simplex virus (HSV), cytomegalovirus (CMV), EB virus, and papilloma virus infected cells (67-69), but not adenovirus infected cells (68, 70). Mycobacterium infected cells are also targeted by NK cells (71).

A more practical way to look at the role of NK cells *in vivo* is to see the clinical manifestations of NK deficient patients. A common feature of immunodeficiency manifested in NK cell deficient patients is the severe course of HSV or CMV infection, both viruses known to infect immunocompromised patients (72-75). NK cells, however, do not only lyse virus-infected cells but also to initiate adaptive T cell responses through a costimulatory pathway, OX40 and OX40 ligand interaction (76, 77). Therefore, clinical pictures observed in NK deficient patients may be affected by the absence of adaptive T cell responses resulting in a wider spectrum of viral infection.

Human NK cells destroy various human cancer cells of various origins such as melanoma, sarcoma, ovarian carcinoma, colon carcinoma, or leukemic cells *in vitro* (78-82). *Ex vivo* NK sensitivity of cancer cells or cancer cell-lines does not necessarily correspond to clinical outcomes. Although NK cells of a sarcoma patient can lyse freshly isolated sarcoma cells, cancer cells *in vivo* are not eliminated (79).

Contrary to our expectations based on *in vivo* studies, tumour associated findings are not reported in NK deficient patients except a case lacking CD3-CD16+ NKH+ NK cells but not NKT cells suffering recurrent condylomata, vulvar and cervical carcinoma *in situ*, pulmonary infiltrates of unknown significance, and a hypercoagulable state (83). Recurrent viral infection as in the other types of NK deficiency is not documented in this case. It is possible that NKT cells have a specialized role in tumour protection. Interestingly, a case, who has a profound deficiency of NKT cells and NKT cell activity, is susceptible to otherwise non-pathogenic attenuated vaccine strain of varicella virus (84).

Because tumour development needs a longer time for incubation, cohort studies better illustrate the role of NK activity. Natural cytotoxic activity of peripheral-blood mononuclear cells was assessed by isotope-release assay in 3625 residents of a Japanese population mostly older than 40 years of age, between 1986 and 1990 (85). The members of the cohort underwent an 11-year follow-up survey looking at cancer incidence and death from all causes, and the association between NK activity assessed at baseline and cancer incidence found in the subsequent follow-up was analyzed. They found 154 cancer cases in the follow-up. The relative risks of cancer incidence in those with NK activity in the highest and medium tertile compared with the lowest tertile were 0.72 and 0.62 respectively for men, 0.52 and 0.56 for women. They found, however, no association of questionnaire-based life style factors including physical activities. This study is a clear demonstration of NK activity in tumour surveillance. The fact that a result of a single baseline measurement is associated with cancer risks in an 11-year period has a more striking implication. The increase and decrease in the numbers and the cytotoxic activity of NK cells of a rather transient or acute manner observed in the majority of exer-

cise or stressor studies may not have significance in tumour surveillance. Future studies will reveal the cause of the varying ability of NK cells, which may arise from the genetic polymorphisms of NK function associated molecules such as killer cell immunoglobulin-like receptors (KIRs) (86-88). Association between the polymorphism and NK function will be elucidated in future studies.

Higher NK cell activity or larger number of circulating NK cells does not seem to be always favorable. Elevated activity and numbers of blood NK cells were detected in cases of patients with dermatological problems (89-91). They manifest skin lesions such as severe hypersensitivity to mosquito bites. Immunohistochemical study of the biopsy specimens taken from the lesional skin demonstrated an infiltrate of the cells bearing the natural killer cell phenotype, indicating a role of these cells in the development of the abnormal skin reactions to mosquito bites (90). Large granular lymphocytosis, namely increased number of circulating NK cells, in these patients does not seem to reflect acute reactive immune responses, because large granular lymphocytosis and enhanced NK activity remain even after the hypersensitivity skin reaction was cured. One of the case studies demonstrated that the increased NK cell activity was due to an abnormal expansion of large granular lymphocytes (89). They showed that the enhanced NK cell activity in this patient could be a compensatory adaptation to a primary T cell defect, because blood mononuclear cells from the patient showed a remarkable decrease in T cell mitogenic response, which was not restored by depletion of the NK cell subpopulation from the mononuclear cells (89). Therefore, an excess expansion of normal NK cells or an enhancement of NK cells activity may not always be beneficial.

Exercise-induced changes in NK cells

Both the number and the activity of circulating NK cells fluctuate greatly during and after exercise as well as in response to other stressors. There are several factors involved. The role of sympathetic activity and catecholamines in the distribution of NK cells in humans is reviewed in the previous article (92). Briefly, experimental results from quadriplegic and paraplegic spinal cord injury patients, splenectomized patients and finally a noradrenaline injection study clearly show that NK cells are mainly driven from the spleen into the circulation depending on the sympathetic outflow and catecholamines. The increase in blood NK cells after electrically stimulated exercise was lower in the quadriplegic spinal injury patients, who have spinal injuries at a higher level of the spinal cord affecting the sympathetic outflow compared with the paraplegic patients (93). Because the increase in circulating spleen cells is largely attenuated in the splenectomized patients compared with the healthy subjects, the spleen is assumed to be the major source of NK cells (94). Noradrenaline administration that raised plasma concentration to that observed at 75 % of maximal oxygen uptake induced a NK cell increase comparable to that elicited by the exercise load (95). β-endorphin is also involved in the regulation of NK activity. A series of animal studies by Hori et al. (96) showed intracerebroventricular IFNα or β-endorphin stimulates hypothalamic opioid receptors resulting in analgesia and decreased NK activity in the spleen.

Because surgical ablation of sympathetic outflow prevents this decrease, it is likely that β-endorphin through splenic sympathetic activation drives NK cells from the spleen into the circulation.

NK cells attached to the vascular endothelial cells *in vitro* are known to detach with only short incubations (as short as 5 minutes) with catecholamines in a dose dependent manner (97). Addition of a selective β-2 adrenoreceptor antagonist blocked the catecholamine dependent detachment of NK cells (98). Adrenaline as compared to noradrenaline was more effective in inducing the detachment, which is in line with the higher affinity of β-2 adrenoreceptors for adrenaline. We demonstrated *in vivo* that acute restraint stress expelled intraparenchymal NK cells from murine lung via β-adrenergic stimulation (99). The relationship between the amount of β-adrenergic receptors on the cell surface corresponds well with the circadian pattern of blood leukocytes including NK cells (100). NK cells, γδ-T cells, CD8+ T cells and neutrophils increase in the daytime and express higher amount of β-adrenergic receptors compared with CD4+ T cells and B cells.

The changes in NK activity independent of NK cell number can also be the result of altered NK cell distribution. Suzui et al. (101) showed that after high-intensity training sessions, the increase in CD56 (bright) NK cells was greater than the increase in CD56 (dim) NK cells. Since CD56 (bright) NK cells have lower cytoxicity, the overall NK cell activity appeared to be reduced. Therefore, blood NK cell activity may be a reflection of altered distribution in the different compartments of the body. The level of catecholamines was lower throughout the training session in the high-intensity group, although statistically not significant. If the density of β-adrenergic receptors expression on CD56 bright NK cells is higher than that of CD56 dim NK cells, such a difference may explain the altered distribution.

Recently "mirthful laughter" is suggested to be beneficial for the immune system because NK activity is enhanced (102-104). The enhanced blood NK activity in the results of these studies can be explained as a reflection of enhanced sympathetic activity by laughter, because Bennett et al. (103) reported a concomitant increase in circulating granulocytes with an increase in the NK cells in response to mirthful laughter. Laughter per se maybe beneficial in other ways, but possibly not through the "activation" of NK cell immunity.

Thus, experimental evidence supports the idea that NK cell distribution and activity are largely dependent upon sympathetic activity. In other words, the observed alterations of NK cell numbers and activities may reflect sympathetic activity independent of NK cell immunity. In addition, central opioid action may also be mediated by changes in sympathetic activity (96).

We have recently reported that there was no change in the serum level of granulysin, a cytotoxic protein produced and secreted by NK cells, during and after exhaustive treadmill exercise when there was a marked increase in the number of blood NK cells (105). Granulysin is a potential marker for the size of the whole-body NK cell population as suggested by the absence of granulysin in immunodeficiency patients lacking NK cells and the gradual increase in granulysin after bone marrow transplantation concomitant with the recovery of NK cells (106). Granulysin can be produced upon activation of NK cells and cytotoxic T cells in pathological conditions such as in viral infections, but are

produced constitutively mainly by NK cells in healthy humans (105, 106). Therefore, our observation supports the idea that fluctuation in the numbers of NK cells during and after exercise may reflect altered distribution of NK cells without substantial changes in the whole-body NK cell pool. Fluctuation of NK cells observed in other physical or psychological conditions may resemble the fluctuation observed in exhaustive physical exercise.

Conclusion

The fluctuations in the number of blood neutrophils, T lymphocytes, and NK cells during and after exercise are largely dependent on changes in either sympathetic activity or circulating glucocorticoids. However, the changes in the numbers of cells per se reflecting altered distribution in the body may not instantly affect the immune responses in which they become involved. The clinical or physiological significance of such changes may indirectly be involved in the host defence against microbes, but we still lack convincing epidemiological evidence. Alternatively, the changes may serve as useful measures of glucocorticoid and sympathetic activities.

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Systemic inflammatory responses to maximal versus submaximal lengthening contractions of the elbow flexors.

Muscle damage and systemic inflammation

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ABSTRACT

We compared changes in markers of muscle damage and systemic inflammation after submaximal and maximal lengthening muscle contractions of the elbow flexors. Using a cross-over design, 10 healthy young men not involved in resistance training completed a submaximal trial (10 sets of 60 lengthening contractions at 10 % maximum isometric strength, 1 min rest between sets), followed by a maximal trial (10 sets of three lengthening contractions at 100 % maximum isometric strength, 3 min rest between sets). Lengthening contractions were performed on an isokinetic dynamometer. Opposite arms were used for the submaximal and maximal trials, and the trials were separated by a minimum of two weeks. Blood was sampled before, immediately after, 1 h, 3 h, and 1-4 d after each trial. Total leukocyte and neutrophil numbers, and the serum concentration of soluble tumor necrosis factor-α receptor 1 were elevated after both trials (P<0.01), but there were no differences between the trials. Serum IL-6 concentration was elevated 3 h after the submaximal contractions (P<0.01). The concentrations of serum tumor necrosis factor-α, IL-1 receptor antagonist, IL-10, granulocyte-colony stimulating factor and plasma C-reactive protein remained unchanged following both trials. Maximum isometric strength and range of motion decreased significantly (P<0.001) after both trials, and were lower from 1–4 days after the maximal contractions compared to the submaximal contractions. Plasma myoglobin concentration and creatine kinase activity, muscle soreness and upper arm circumference all increased after both trials (P<0.01), but were not significantly different between the trials. Therefore, there were no differences in markers of systemic

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inflammation, despite evidence of greater muscle damage following maximal versus submaximal lengthening contractions of the elbow flexors.
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Keywords: muscle damage, cytokines, leukocytes, calprotectin, C-reactive protein

INTRODUCTION

Lengthening contractions (eccentric exercise) cause damage in skeletal muscle. This damage is demonstrated by histological evidence of transient alterations in the content and position of myofilaments within skeletal muscle (1, 7, 35, 36). Other markers of muscle damage that have been used include loss of muscular strength, reduced range of motion, muscle soreness and swelling, and elevated blood concentrations of muscle proteins (e.g., creatine kinase, myoglobin) (14, 16-19, 21, 31). Among these latter markers of muscle damage, muscular strength and range of motion are deemed to be the most valid and reliable (34). The extent of muscle damage after lengthening contractions appears to relate to mechanical loading of muscle during exercise. Compared with heavy loading (100% maximum voluntary force), light loading (≤ 50 % maximum voluntary force) causes a smaller loss of muscular strength (18, 19).

Lengthening muscle contractions elicit an inflammatory response. Within skeletal muscle, pro-inflammatory cytokines are produced, and phagocytic cells invade damaged muscle tissue (for review see ref. (22). Within the systemic circulation, the complement cascade is activated, leukocytes are mobilized, and cytokine concentrations increase (22). These inflammatory responses share some similarities with the responses to trauma injury (8). Many of the original studies that examined the relationship between contraction-induced muscle injury and inflammation were performed using animals. However, electrically-stimulated muscle contractions likely produce different contractile responses than do voluntary contractions. Consequently, the results of these animal studies cannot necessarily be applied to humans (15).

The inflammatory response to contraction-induced injury may be proportional to the severity of muscle damage, which in turn is dependent on mechanical loading of muscle during exercise. This relationship is important because the magnitude of the inflammatory response regulates adaptation to muscle injury (32). Few studies have directly examined the relationship between inflammation and the degree of muscle damage after lengthening contractions. There is indirect evidence from studies demonstrating that adaptation to repeated bouts of lengthening contractions is characterized by less muscle damage (as indicated by plasma creatine kinase activity), lower blood neutrophil counts, and reduced expression of cell surface adhesion molecules (27, 29). Furthermore, muscle damage after lengthening contractions (as indicated by loss of muscular strength) correlates with blood leukocyte counts and serum C-reactive protein concentration (21). More direct evidence of a relationship between inflammation and the degree of muscle damage comes from studies that modulated the degree of muscle damage by imposing different mechanical loads. For example, in a study of runners, running downhill caused greater muscle damage (as indicated by plasma creatine

	PRE	POST	1 h	3 h	1 d	2 d	3 d	4d
Total leukocytes (cells × 10^9/l)								
Submaximal	6.4 (1.6)	6.9 (1.7)	7.2 (2.6)	8.1 (2.7) *	6.8 (1.6)	6.8 (1.6)	7.4 (1.8)	5.9 (2.0)
Maximal	6.6 (1.3)	7.4 (2.3)	7.4 (1.9)	8.3 (2.0) *	7.0 (1.8)	6.6 (1.0)	7.0 (1.5)	6.8 (2.1)
Neutrophils (cells × 10^9/l)								
Submaximal	3.7 (1.3)	4.0 (1.4)	4.7 (2.6)	5.2 (2.7) *	4.2 (1.4)	4.2 (1.2)	4.6 (1.7)	3.3 (1.5)
Maximal	3.8 (1.1)	4.6 (2.1)	4.6 (1.9)	5.1 (1.8) *	4.2 (1.5)	4.0 (1.2)	4.2 (1.1)	4.1 (1.7)
Lymphocytes (cells × 10^9/l)								
Submaximal	1.9 (0.6)	2.0 (0.5)	2.1 (0.9)	2.0 (0.5)	1.9 (0.5)	1.9 (0.6)	2.0 (0.5)	1.8 (0.7)
Maximal	1.9 (0.4)	1.9 (0.4)	2.0 (0.4)	2.3 (0.4)	1.9 (0.5)	1.9 (0.5)	1.0 (0.5)	1.9 (0.5)
Monocytes (cells × 10^9/l)								
Submaximal	0.5 (0.2)	0.5 (0.3)	0.6 (0.5)	0.6 (0.3)	0.5 (0.3)	0.5 (0.3)	0.5 (0.3)	0.5 (0.3)
Maximal	0.5 (0.2)	0.6 (0.2)	0.6 (0.2)	0.7 (0.2)	0.6 (0.2)	0.5 (0.1)	0.6 (0.2)	0.6 (0.3)

Data are means (SD). * $P < 0.05$ versus pre-exercise values.

Table 1. Leukocyte counts before (PRE), immediately after (POST), 1 h, 3 h, and 1-4 days after submaximal and maximal eccentric exercise.

kinase activity), mobilization of neutrophils and lymphocytes, and activation of T-lymphocytes than did level running (30). In contrast, a more recent study found that although muscle damage was greater following downhill running at -8° versus -4°, the two exercise protocols did not differ in their effects on markers of inflammation, such as blood leukocyte counts, cytokine concentrations, and immunohistochemical staining for leukocytes and cytokines in skeletal muscle and epimysium (16). Therefore, evidence for a relationship between inflammation and the muscle degree of damage following exercise is equivocal.

Interleukin (IL)-6 plays an important role in mediating inflammatory responses to exercise. IL-6 is believed to exert anti-inflammatory effects during exercise by inhibiting the production of the pro-inflammatory cytokine tumor necrosis factor (TNF)-α, and stimulating the synthesis of other anti-inflammatory cytokines such as IL-1 receptor antagonist (IL-1ra), IL-10 and soluble TNF-α receptor 1 (sTNF-αR1) (25). IL-6 is produced within, and released from skeletal muscle during exercise in response to glycogen depletion, calcium signaling, changes in blood glucose availability, and to a lesser extent sympathetic activation (9, 11, 13). During concentric exercise, IL-6 release from contracting muscle is dependent on exercise intensity (9). Several studies have compared changes in IL-6 gene expression and plasma IL-6 concentration after shortening versus lengthening muscle contractions (2, 12, 24). However, data from Malm et al. (16) relating to the relationship between the extent of muscle damage and changes in serum IL-6 concentration after downhill running were inconclusive.

The aim of our study was to further investigate the relationship between inflammation and muscle damage after lengthening muscle contractions. We modulated the degree of muscle damage using two different protocols of lengthening muscle contractions using the elbow flexors (18, 19), and compared the inflammatory responses to each protocol. We hypothesized that if the magnitude of the inflammatory response is related to the degree of muscle damage, changes in markers of systemic inflammation such as systemic leukocyte numbers, cytokines, C-reactive protein and calprotectin (marker of neutrophil activation) would be greater after maximal than after submaximal lengthening contractions.

METHODS

Participants

Ten healthy young men volunteered to take part in this study. The mean ± SD age, body mass and height of the participants were 22.9 ± 4.7 yrs, 76.2 ± 11.8 kg and 1.80 ± 0.08 m, respectively. None of the participants took part in regular resistance training exercise, and all participants were told to avoid taking any anti-inflammatory or pain-killer medication during the study. All subjects completed a medical questionnaire and gave written informed consent prior to the study. The experimental procedure was approved by the Human Research Ethics Committee at Edith Cowan University.

Study design

The study involved a cross-over design. All participants completed the submaximal lengthening contractions before the maximal lengthening contractions. The two trials were separated by a minimum period of two weeks. The use of the dominant versus non-dominant arm for the first trial was randomized and counterbalanced among the 10 participants. After the submaximal exercise had been completed, the contralateral arm was used for the maximal exercise. We used this design for two reasons: (a) to avoid the prolonged adaptation that results from a single bout of lengthening contractions using one arm, and (b) to reduce the well known variability in markers of muscle damage between individuals. Furthermore, isolated muscle contractions have a low metabolic cost compared to other types of exercise such as downhill running (21). One week prior to any of the actual exercise trials, all participants took part in a familiarization session, during which they were introduced to the testing procedures. Height, body mass, maximal isometric strength, range of motion of the elbow joint, upper arm circumference, and muscle soreness and pain pressure threshold were measured. To establish reliability, the values recorded for maximal isometric strength, range of motion of the elbow joint, upper arm circumference, and muscle soreness and pain pressure threshold were compared with the values taken immediately before the actual exercise.

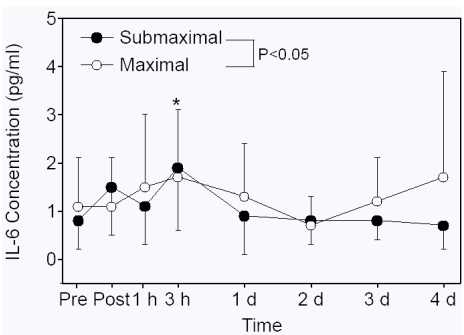

Figure 1: Serum interleukin (IL)-6 concentration before (PRE), immediately after (POST), 1 h, 3 h, and 1 – 4 days after exercise. * $P < 0.05$ significantly different versus pre-exercise values for submaximal exercise. Data are means (SD)

Exercise protocols

Two exercise protocols were conducted on a preacher curl bench placed alongside an isokinetic dynamometer (Cybex 6000, Lumex Inc., Ronkonkoma, NY, USA).

	PRE	POST	1 h	3 h	1 d	2 d	3 d	4d
IL-1ra (pg/ml)								
Submaximal	238 (88)	266 (80)	262 (170)	256 (130)	235 (81)	240 (105)	234 (72)	207 (61)
Maximal	259 (78)	311 (107)	257 (106)	275 (68)	246 (83)	306 (159)	239 (77)	267 (100)
IL-10 (pg/ml)								
Submaximal	1.7 (1.3)	2.1 (1.9)	2.1 (1.4)	2.0 (1.8)	1.7 (1.3)	1.7 (1.5)	1.7 (1.4)	1.7 (1.5)
Maximal	1.5 (1.4)	1.6 (1.8)	1.7 (1.6)	2.0 (1.4)	2.4 (1.7)	2.3 (1.6)	1.3 (1.0)	2.0 (1.4)
TNF-α (pg/ml)								
Submaximal	1.1 (0.4)	1.3 (0.7)	1.0 (0.4)	1.0 (0.4)	0.9 (0.5)	1.0 (0.3)	0.9 (0.3)	1.3 (0.5)
Maximal	1.5 (1.6)	0.9 (0.3)	1.0 (0.3)	1.0 (0.4)	1.2 (0.4)	1.0 (0.3)	1.1 (0.6)	1.2 (0.7)
G-CSF (pg/ml)								
Submaximal	2.0 (1.5)	2.2 (1.1)	2.5 (1.4)	2.2 (1.0)	2.3 (0.9)	1.7 (0.9)	2.2 (1.0)	1.6 (0.7)
Maximal	2.7 (1.9)	2.4 (1.8)	2.7 (1.7)	3.0 (1.2)	3.3 (1.6)	2.6 (1.3)	2.4 (1.6)	2.8 (2.2)

Data are means (SD).

Table 2. Serum concentrations of interleukin-1 receptor antagonist (IL-1ra), IL-10, tumor necrosis factor (TNF)-a and granulocyte-colony stimulating factor (G-CSF) before (PRE), immediately after (POST), 1 h, 3 h, and 1-4 days after submaximal and maximal eccentric exercise.

The protocol for the submaximal contractions involved 10 sets of 60 lengthening contractions of the elbow flexors of one arm at 10 % of maximal isometric strength. Participants watched a visual representation of their strength output on a computer screen, and were told to match their strength against a line on the screen that corresponded to 10 % of their maximal isometric strength. The protocol for the maximal contractions involved 10 sets of three lengthening contractions of the contralateral arm, in which subjects were asked to maximally resist against the lengthening motion of the dynamometer. The muscle actions in both trials involved extending the elbow joint from a starting angle of 120° down to a fully extended angle of 180°. The angular velocity for both protocols was 30°/s. The rest intervals between sets for the submaximal and maximal trials were 1 min and 3 min, respectively. Two seconds of passive recovery were allowed between each contraction as the mechanical arm of the dynamometer returned the participants' arm to 120°. The total muscle activation time for the submaximal contractions was 20 min (2 s x 60 contractions x 10 sets), whereas the total muscle activation time for the maximal contractions was 2 min (2 s x 3 contractions x 10 sets). The total work completed during the submaximal and maximal contractions was 6791 ± 187 J and 1288 ± 36 J, respectively.

Markers of muscle damage

The following variables were measured before exercise, immediately after, 3 h, 1 d, 2 d, 3 d and 4 d after exercise: maximal isokinetic torque at 30, 180 and 300°/s, isometric torque at 90°, range of motion, upper arm circumference (swelling), muscle soreness on palpation and pain pressure threshold. The test-retest reliability of these measures was established by an intraclass correlation coefficient (R) comparing the values from familiarization session and pre-exercise measurements. R-value for maximal isometric strength, range of motion, upper arm circumference, muscle soreness and pain pressure threshold was 0.94, 0.90, 0.89, 0.95, and 0.88, respectively.

Maximal isometric strength was used, as opposed to maximal eccentric strength because testing of eccentric strength itself causes some muscle damage. The changes in maximal isokinetic strength at all three angular velocities (30, 180 and 300°/s) were very similar to the change in isometric torque at 90°. Therefore,

we chose to represent changes in muscular strength only by reporting data for isometric torque at 90°. Range of motion was calculated as the difference between flexed and extended elbow joint angles. Upper arm circumference was measured at a point 11 cm beyond the elbow joint. To assess muscle soreness, the elbow flexors were palpated at the mid-belly of biceps brachii. Participants were asked to rate the soreness on a visual analog scale that had a 100-mm line with"no pain" on one end and"extremely painful" on the other. Pain pressure threshold was assessed using a pressure algometer (Type II, Somedic Production AB, Sollentuna, Sweden). For this assessment, the probe head (1 cm^2) of the algometer was placed perpendicular to the mid-belly of biceps brachii, and force was gradually applied at a rate of 50 kPa per second until the participant reported the first feeling of noticeable pain. The value (in kPa) corresponding to the amount of force applied was then recorded. All measurements of strength, range of motion, upper arm circumference, muscle soreness and pain pressure threshold were performed twice. The data presented in the results represent the average of the two numbers.

Blood sampling

Venous blood samples were drawn from a forearm vein before exercise, immediately after, 1 h, 3 h, 1 d, 2 d, 3 d and 4 d after exercise. Due to demands on the participants' time on the day of exercise, it was not possible to collect blood samples between 3 h and 1 d after exercise. Blood was collected into sterile vacutainers containing K_2-EDTA (Becton Dickinson, Franklin Lakes, NJ, USA) or serum separation tubes. Before the K_2-EDTA tube was centrifuged, 1 ml blood was removed to obtain a complete blood cell count. The K_2-EDTA tube was then centrifuged for 10 min at 1000 × g at 4°C to obtain plasma. The serum separation tube was left at room temperature for the blood to clot, and then centrifuged for 10 min at 1000 × g at 4°C. The plasma and serum samples were stored in 0.7-ml aliquots at –80°C until the day of analysis.

Blood analysis

EDTA-treated whole blood was analysed to obtain a complete blood cell count using a Beckman Coulter-Counter, Gen-S (France SA, Villepinte, France). Plasma samples were analyzed for myoglobin, calprotectin and C-reactive protein concentrations, in addition to CK activity. Myoglobin was measured using a commercially available enzyme-linked immunorsorbent assay (ELISA) kit (BioCheck, Foster City, CA, USA). Calprotectin was also measured by ELISA (HyCult Biotechnology, Uden, The Netherlands). CK was measured using an automated analyser (Model 7450 or Model 7170, Hitachi, Japan). Plasma C-reactive protein concentration was measured using an immunoturbidimetric assay (Kamaya Biomedical Company, Seattle, WA, USA) on an automated analyzer (Cobas Mira, Roche Diagnostics, Indianapolis, IN, USA). Serum samples were analyzed by ELISA for IL-1ra, IL-6, TNF-α (Quantikine® High Sensitivity ELISA, R&D Systems, Minneapolis, MN, USA), sTNF-αR1, IL-10 (Opt EIA, Becton Dickinson, San Diego, CA, USA) and granulocyte-colony stimulating factor (G-CSF) (Immuno Biological Laboratories, Gunma, Japan). ELISA measurements were performed using a microplate reader (VERSAmax, Molecular Devices, Sunnyvale, CA, USA). The coefficient of variation for duplicate measurements using ELISA was <6 %. Leukocyte counts were

adjusted for percentage changes in blood volume, whereas plasma and serum variables were adjusted according to percentage changes in plasma volume, as calculated from hemoglobin and hematocrit (5).

Statistical analysis

Data are presented means ± SD. All data were checked for normal distribution, and when necessary the data were log transformed to obtain a normal distribution before further statistical analysis. The data requiring log transformation included leukocyte and neutrophil counts, IL-1ra, IL-6, IL-10, C-reactive protein and CK. The data for upper arm circumference and muscle soreness could not be log transformed to follow a normal distribution. Therefore, these data were analyzed non-parametrically using Friedman's repeated measures analysis of variance and sign ranked t-tests. The data for strength, range of motion and pain pressure threshold were analyzed using a 2 (trials) × 7 (time points) repeated measures analysis of variance. The blood data were analyzed using a 2 (trials) × 8 (time points) repeated measures analysis of variance. Statistical significance was set at $P < 0.05$ for the main effects of time and time × trial interactions. Student's paired t-tests were used to compare differences between the trials and individual time points. The false discovery rate procedure was used for these multiple comparisons (4). Statistical analysis was performed using SigmaStat 3.1 software (Systat, Point Richmond, CA, USA).

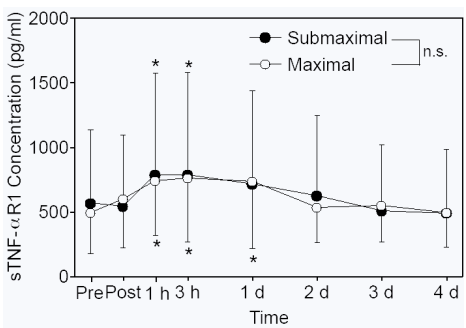

Figure 2: Serum soluble tumor necrosis factor (TNF)-α receptor 1 (sTNF-αR1) concentration before (PRE), immediately after (POST), 3 h, and 1 – 4 days after exercise. * $P < 0.05$ significantly different versus pre-exercise values for the maximal trial. Data are means (SD).

RESULTS

Leukocyte numbers

The number of total leukocytes and neutrophils increased after both trials (time effect $P = 0.0005$), reaching a peak at 3 h after exercise (Table 1). There were no differences between the trials. There were no significant changes in either lymphocyte (time effect $P = 0.23$) or monocyte numbers (time effect $P = 0.17$) after either trial.

Serum cytokine concentrations

Serum IL-6 concentration increased after exercise (Figure 1). The pattern of changes in serum IL-6 was different between the trials (interaction effect $P = 0.044$); serum IL-6 was elevated 3 h after the submaximal contractions, but not after the maximal contractions. The serum concentration of sTNF-αR1 also increased significantly after exercise (time effect $P = 0.0001$), but there were no

differences between the trials (Figure 2). Serum IL-1ra concentration tended to increase after exercise ($P = 0.068$) (Table 2), while the serum concentrations of IL-10 (time effect $P = 0.36$), TNF-α (time effect $P = 0.48$) and G-CSF (time effect $P = 0.36$) remained unchanged after exercise (Table 2).

Plasma CK activity, myoglobin calprotectin and C-reactive protein concentrations
Plasma myoglobin concentration and CK activity increased after exercise (time effect $P = 0.001$) (Table 3), but there were no differences between the trials. There were no significant changes in plasma calprotectin or C-reactive protein concentrations after either trial (Table 3).

	PRE	POST	1 h	3 h	1 d	2 d	3 d	4d
Myoglobin (ng/ml)								
Submaximal	27 (10)	28 (13)	55 (26) *	55 (31) *	49 (52)	54 (68)	28 (13)	43 (51)
Maximal	37 (21)	45 (31)	80 (50) *	60 (31) *	43 (21)	41 (27)	57 (21) *	65 (45) *
CK (IU/l)								
Submaximal	248 (165)	269 (174)	258 (168)	276 (164)	337 (220)	364 (419)	492 (818)	836 (1546) *
Maximal	342 (310)	352 (326)	355 (334)	356 (295)	389 (286)	420 (295)	469 (248) *	770 (589) *
CRP (mg/l)								
Submaximal	0.2 (0.3)	–	–	–	0.2 (0.1)	0.2 (0.2)	0.5 (1.0)	0.5 (1.3)
Maximal	0.5 (0.7)	–	–	–	0.7 (0.8)	0.7 (0.9)	0.5 (0.5)	0.5 (0.8)
Calprotectin (ng/l)								
Submaximal	3.9 (2.7)	4.9 (2.7)	3.8 (1.8)	4.4 (3.1)	4.7 (3.2)	4.2 (2.2)	2.7 (1.5)	3.3 (2.0)
Maximal	2.6 (1.9)	2.8 (1.8)	2.9 (1.8)	2.6 (2.6)	4.0 (2.6)	3.5 (2.5)	2.8 (2.2)	2.3 (1.5)

Data are means (SD). * $P < 0.05$ versus pre-exercise values.

Table 3. Plasma myoglobin concentration, plasma creatine kinase (CK) activity, C-reactive protein (CRP) and calprotectin concentrations before (PRE), immediately after (POST), 1 h, 3 h, and 1-4 days after submaximal and maximal eccentric exercise.

	PRE	POST	3 h	1 d	2 d	3 d	4 d
Range of motion (degrees)							
Submaximal	133 (5)	118 (9) *	124 (10) *	126 (5) *	129 (5)	130 (6)	131 (5)
Maximal	138 (4)	126 (5) *	130 (6) *	128 (8) *	127 (9) *	129 (11) *	128 (16) *
Upper arm circumference (mm)							
Submaximal	279 (32)	287 (32) *	282 (31)	285 (32)	287 (33) *	286 (32)	286 (32)
Maximal	285 (32)	289 (31)	288 (33)	291 (32) *	292 (32) *	291 (31) *	291 (31)
Muscle soreness (mm)							
Submaximal	0 (0)	0 (0)	9 (16)	15 (8) *	15 (7) *	7 (9)	3 (4)
Maximal	0 (0)	0 (0)	0 (0)	14 (11)	21 (17) *	25 (21) *	18 (19) *
Pain pressure threshold (kPa)							
Submaximal	394 (81)	410 (132)	392 (112)	238 (91) *	248 (110) *	292 (93) *	362 (126)
Maximal	339 (94)	367 (82)	376 (80)	242 (87) *	232 (82) *	278 (138)	316 (146)

Data are means (SD). * $P < 0.05$ versus pre-exercise values. N.B. Participants rated their muscle soreness on a 100-mm visual analog scale ranging from “no pain” to “extremely painful”.

Table 4. Range of motion, upper arm circumference (swelling), muscle soreness assessed by palpation of the biceps and pain pressure threshold before (PRE), immediately after (POST), 3 h, and 1-4 days after submaximal and maximal eccentric exercise.

Strength and range of motion

Maximal isometric strength decreased after exercise, and the pattern of changes was different between the trials (interaction effect $P < 0.0001$) (Figure 3). Strength decreased by 30–40 % immediately after both trials. No further strength decrement was evident 3 h after the submaximal exercise, whereas strength continued to decrease at this time after the maximal exercise. At 1 d after exercise, strength remained below pre-exercise values for both trials; however, strength was lower at this time following the maximal exercise when compared to the submaximal exercise. Whereas strength had almost returned to the baseline 2 d after the submaximal exercise, strength remained ~30 % below pre-exercise values at 4 d after the maximal exercise. Range of motion at the elbow joint decreased by ~10 % immediately after exercise, and the pattern of changes was different between the trials (interaction effect $P = 0.002$) (Table 4). Whereas range of motion had returned towards normal 2 d after the submaximal exercise, it remained lower than normal at 4 d after the maximal exercise.

Upper arm circumference, soreness and pain pressure threshold

As an indicator of swelling, upper arm circumference increased after exercise (time effect $P < 0.0001$) (Table 4), but there were no differences between the two trials. When biceps brachii was palpated, the participants reported greater muscle soreness after exercise (time effect $P < 0.0001$) (Table 4). Participants were also more sensitive to pain when force was applied against their biceps brachii at 1, 2 and 3 d after both trials (Table 4). Neither muscle soreness nor pain pressure threshold was different between the trials.

DISCUSSION

The aim of this study was to compare systemic inflammatory responses to submaximal versus maximal lengthening contractions. Our data indicate that there were no significant differences in systemic markers of inflammation, despite evidence of greater muscle damage (as indicated by impaired muscular strength) following the maximal versus submaximal contractions. The magnitude of systemic inflammation was relatively small compared to lengthening contractions involving multiple and/or larger muscle groups. This factor may account for the lack of differences between submaximal and maximal contractions. It is also possible the maximal contractions caused greater local inflammation within skeletal muscle that were not reflected by the systemic changes.

Elevated blood neutrophil counts are a consistent finding after lengthening contractions of the elbow flexors (17, 27-29). Neutrophils are most likely mobilized from endothelial surfaces into the circulation in response to tissue injury such as exercise-induced muscle damage. Once in the circulation, neutrophils travel to the site of injury, and then bind and break down damaged tissue fragments. In our study, neutrophil numbers were highest 3 h after exercise. Unfortunately, due to constraints on the time availability of the participants we could not obtain blood samples to assess neutrophil numbers in the period between 3 h and 1 d after exercise. Others have reported that neutrophil numbers peak up to 12 h after lengthening contractions of the elbow flexors (27-29). In contrast

to our findings, some studies have also found a secondary increase in blood neutrophils 2 d after exercise (17, 29). We used untrained participants and a similar protocol to these studies for the maximal lengthening contractions. Therefore, it is difficult to explain why this secondary increase did not occur in our study.

We hypothesized that blood neutrophil or total leukocyte numbers would be higher after the maximal contractions. This hypothesis was based on data from several studies indicating that (a) a reduction in muscle damage (plasma CK activity) was accompanied by lower blood neutrophil counts (27, 29, 30), and (b) muscle damage (decline in muscular strength) correlated with blood leukocyte counts (21). However, Malm et al. (16) also reported no significant differences in blood leukocyte numbers after downhill running at –8° versus –4°. Taken together, these findings suggest that the leukocytosis following lengthening contractions is not always proportional to the degree of muscle damage.

Compared with studies of endurance exercise, relatively few studies have investigated changes in systemic cytokine concentrations after lengthening contractions using the elbow flexors. Two studies have reported that plasma IL-6 concentration increased after three sets of 10 maximal lengthening contractions of the elbow flexors (3, 26). We previously found no significant change in plasma IL-6 concentration following six sets of five lengthening contractions of the elbow flexors at 40 % maximum voluntary force, despite evidence of muscle damage (10). The changes in serum IL-6 concentration that we observed in the present study do not appear to be related to muscle damage. In their comparison of downhill running at different gradients, Malm et al. (16) found no significant change in serum IL-6 concentration. However, this result may have been due to the low sensitivity of the assay used to measure IL-6. The small increase in serum IL-6 concentration after the submaximal contractions could represent the release of IL-6 from muscle in response to muscle glycogen depletion (13). The greater amount of work completed during the submaximal versus the maximal contractions (6791 ± 187 J versus 1288 ± 36 J, respectively) likely caused greater muscle glycogen depletion. Others have reported that the systemic concentration of IL-6 is significantly elevated at 6 h (14, 21), 12 h, 1 d and 3 d (31) after lengthening contractions using larger muscle groups. If we had been able to take blood samples between 3 h and 1 d after exercise in the present study, it is possible that we may have seen similar responses. The use of larger muscle groups may also partially explain these different findings. Other factors such as muscle glycogen depletion, calcium sig-

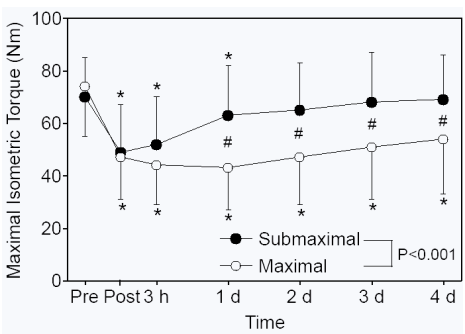

Figure 3: Maximal isometric strength before (PRE), immediately after (POST), 3 h, and 1–4 days after exercise. * $P < 0.05$ significantly different versus pre-exercise values for both trials. # $P < 0.05$ significant difference between submaximal and maximal eccentric exercise. Data are means (SD)

naling and blood glucose availability also influence changes in plasma IL-6 concentration following exercise (9, 11, 13). But it is unlikely that these factors can explain delayed changes in plasma IL-6 concentration in the hours and days following lengthening muscle contractions.

The delayed increase in the serum concentration of sTNF-αR1 after the maximal lengthening contractions was similar to that seen after marathon running (20) and eccentric cycling (33). sTNF-αR1 may be produced as an anti-inflammatory response (25). Our data suggest that the production of sTNF-αR1 is not dependent on the extent of muscle damage. There were no significant changes in the serum concentrations of IL-1ra, G-CSF, TNF-α or IL-10 after exercise in the present study. These findings contrast with our own study described previously (10), and findings from other groups who have also examined lengthening muscle contractions (14, 21, 31). Because we used maximal lengthening contractions and untrained subjects, it is difficult to explain these differences.

The plasma concentration of calprotectin remained unchanged after exercise. This result contrasts with the findings of another recent study which reported that plasma calprotectin concentration increases after aerobic exercise (6). Calprotectin is a marker of neutrophil activation. The factors affecting the release of calprotectin from neutrophils during exercise are unknown, but our data suggest that muscle damage is not a strong stimulus for its release. Other neutrophil functions, such as oxidative burst activity, are stimulated by growth hormone and cytokines such as IL-6 and IL-8 (23). Therefore, growth hormone and cytokines may also stimulate the release of calprotectin after strenuous endurance exercise, independently of muscle damage. We and others have also found little or no change in plasma myeloperoxidase concentration (another marker of neutrophil activation) following maximal lengthening contractions of the elbow flexors (3, 10). Taken together, these data suggest that damage resulting from lengthening contractions of small muscle groups does not activate neutrophils, at least in the systemic circulation.

Debate exists regarding the best method for assessing muscle damage. Warren et al. (34) contend that changes in muscular strength and range of motion are valid and reliable indicators of the extent of muscle damage. Our data indicated that from 1-4 d after exercise, muscular strength was significantly lower after the maximal versus submaximal contractions. We interpret this difference in muscular strength as evidence of greater muscle damage following the maximal contractions. The finding that strength decreased to a similar extent immediately after both submaximal and maximal lengthening contractions may reflect a greater contribution of muscle fatigue during the submaximal contractions (19). This idea is supported by the greater total amount of work completed in the submaximal than in the maximal trial.

The alterations in plasma myoglobin concentration and CK activity in our study were smaller than those reported in other studies involving lengthening contractions of the elbow flexors (17-19, 28, 29). We can only speculate about the reasons for this. One possibility is that although the participants in our study were not regularly involved in resistance-type exercise, they could have been more physically active in general than participants in other studies that reported larger changes in myoglobin and CK after lengthening contractions. Participation in general physical activity may prevent large changes in myoglobin and CK follow-

ing lengthening muscle contractions. The small changes in myoglobin and CK in our study may raise the question of whether (a) muscle damage did in fact occur and (b) there were any differences in muscle damage between the submaximal and maximal contractions. However, the minor changes in myoglobin and CK in our study should not be over-emphasized, because changes in these proteins do not always correlate with, or follow the pattern of changes in muscle function following lengthening contractions (34).

In conclusion, the present data indicate that although maximal lengthening contractions of the elbow flexors may result in greater muscle damage, this is not accompanied by a greater systemic inflammatory response. The magnitude of the systemic inflammatory response may differ when comparing lengthening contractions of small muscle groups such as the elbow flexors with larger muscle groups. Furthermore, although we could not detect differences in systemic inflammation, differences could exist locally within skeletal muscle.

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Physiological improvements and health benefits during an exercise-based comprehensive rehabilitation program in medically complex patients

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Running title: Therapeutic exercise, aging, and chronic illness

Abstract

Objectives: *To determine the effects of an exercise-based comprehensive rehabilitation program on the physiological, health, and cost benefit in medically complex patients.*
Design: *Case series*
Setting: *Comprehensive rehabilitation centers.*
Participants: *Elderly chronically ill men (n = 39, age = 75.3 ± 1.4) and women (n = 74, age = 76.5 ± 0.9 years)*
Intervention: *Patients participated in individualized physical therapy with therapeutic exercises (stretching, strengthening, endurance, balance, sitting and standing dynamic exercises) three times/week for three months under the supervision of a physician.*
Measurements: *Upper (back) and lower (leg flexors) extremity strength, aerobic power as measured by metabolic equivalents (METS) at 80% of age predicted maximal heart rate (APMHR), physical functioning and mental health as assessed by the Short Form-36 (SF-36) questionnaire, and medical events (falls, physician visits, and hospitalizations) questionnaire was collected at baseline and after three* months of the program.
Results: *Strength measures improved by ~30% ($P < 0.05$) as well as aerobic power improved by ~25% ($P < 0.05$) over the three-month period. There were significant improvements in two of the SF-36 Physical Component Scales: Physical Functioning ($P < 0.05$) and Role Physical ($P < 0.05$); plus, there were significant improvements in all four of the Mental Component Scales: Vitality ($P <$*

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0.05), Social Functioning (P < 0.05), Role Emotional (P < 0.05), and Mental Health (P < 0.05). There were significant reductions in fall rate (P < 0.05), physician visits (P < 0.05), and hospitalizations (P < 0.05).
Conclusion: *Patients improve physical capacity, which result in improvements in health status with concurrent reductions in healthcare utilization during a comprehensive rehabilitation program. (Exerc. Immunol. Rev. 12, 2006: 86-96)*

Key words: Exercise; rehabilitation; aging; chronic illness

Introduction

The elderly population in the U.S. is growing rapidly and is currently estimated at ~35 million people over the age 65 [1]. Increases in the aging population pose some severe consequences. Eighty percent of people >65 years is living with a chronic illness (e.g., Parkinson's, balance deficits, chronic pain, coronary artery disease, hypertension, diabetes, etc.) [2]. The chronically ill population suffers from decreased function, endurance, mental health, ability to perform activities of daily living (ADL), independence, and balance, along with an additional myriad of problems.

The increase in the elderly population, with its concurrent elevation in chronic illness and pain and subsequent utilization of health services, will result in an exponential increase of health care expenditures. For example, falls are a foremost health care cost for the over 65 population, presumably due to balance deficits and weakness associated with either aging or secondary to a illness or disease state (e.g., stroke, Parkinson's) [3]. The high incidence of injury from falls can be attributed in part to low bone mass in the elderly (higher occurrence of fractures) [4] and low muscle mass (since soft tissue can attenuate fall impact); furthermore, greater than 90% of hip fractures may result from falls [5]. Other rising costs include the increased need for hospitalizations, medications, and physician office visits. Therefore, it is important to find ways to counter the decline in health status with advancing age.

The answer to these health problems may lie in the investigation of comprehensive rehabilitation programs that utilize active-based physical therapy protocols involving therapeutic exercise. However, clinical programs, other than cardiac and pulmonary rehabilitation and preventative programs, have been slow to emerge as a standard treatment due to limitations in medical direction and supervision to handle the complex medical problems that those with chronic illnesses present. These patient populations need a carefully planned exercise prescription with regular assessments to monitor modes and intensities of work, medication interaction, disease interaction, and medical events [6]. Many older patients who have an orthopedic malady may have one or more diagnoses of chronic illness. Of all the treatment options, such as hospice care, medications, and surgical intervention, only active treatment options address recovery in terms of function, overall medical status, and future prevention.

Older adults who undergo regular physical training have marked improvements in strength and aerobic power [7]. Physically frail adults who

are placed in a physical therapy program focus on improving strength improve functional ability [8]. Older people that exercise on a regular basis are more resistant to chronic illness. As such, there are clear recommendations that the elderly maintain regular physical activity to help offset functional decline and offset risk for illness [9].

The association of mental health and chronic illness is a complex issue, as medical conditions are a risk factor for mental disorders such as depression and conversely depression is a risk factor for medical illness[10]. For example, frequency of negative emotions are a significant predictor of coronary heart disease [11] and depressive symptoms are greater in patients with chronic illness [12]. Physical exercise interventions may lead to improved mental health in chronically ill patients, as evidenced by improved state anxiety scores in cardiac rehabilitation patients [12] and improved mental health, as assessed by the mental health inventory of the Short Form-36 (SF-36) health questionnaire, in chronic low back patients following a 12 week cycle ergometry program [13].

The evidence in the medical literature clearly indicates the utility of active interventions in the chronically ill; furthermore, many patients are afflicted with multiple diagnoses and orthopedic limitations. However, due to the diverse complications with frail elderly, such as of falls, a multidisciplinary team is necessary to address various areas such as gait training, strengthening, and coordination [14]. Therefore, the purpose of the present article is to describe the changes in outcomes following a medically-directed comprehensive rehabilitation program utilizing a team of physicians, physical therapists, and exercise physiologists to supervise therapeutic exercises in medically complex elderly patients. Specifically, our aim is to quantify the changes in both mental and physiological outcomes. Strength and exercise workload, as they relate to health status (physical functioning and mental health status), as measured by the Short Form – 36 questionnaire (SF-36), as well as changes in health care utilization, as measured by fall rates, hospitalizations, and physician visits during the program.

Methods

Subjects:

The following protocol was approved by the Human Research Committee at the University of Colorado - Boulder. Medical data from 113 chronically ill elderly men (n = 39, age = 75.3 ± 1.4) and women (n = 74, age = 76.5 ± 0.9 years) were retrospectively analyzed for changes in response to therapy. Subjects were diagnosed with three or more of the following: hypertension, coronary artery disease (CAD), chronic obstructive pulmonary disease (COPD), type II diabetes, muscle disuse/atrophy, severe deconditioning, osteoporosis, gait abnormality, stroke, obesity, balance deficit, Parkinson's Disease, degenerative joint disease, and/or orthopedic issue. Patients were living independently either in their own home or in independent living communities. Each patient met monthly with a medical doctor (M.D.) to ensure that the patient did not have any serious changes in medical condition that would adversely affect the patient during any therapeutic exercises. The M.D. would also screen for any

contraindications to exercise that the patient might have, and give guidelines to address any limitations to ensure patient safety.

Intervention:
All patients underwent three months of therapeutic exercise under the direction of an M.D. and physical therapist, and under the daily direct supervision of the medical doctor, physical therapist, and clinical exercise physiologist in a comprehensive rehabilitation center. The M.D. was available in case of medical emergency and to provide general medical support. The physical therapist would work with the patients who had an orthopedic barrier to perform the therapeutic exercise program. The physiologist would monitor the patient's blood pressure, oxygen saturation, heart rate, and perceived exertion on a daily basis. The physical therapists and physiologists would also monitor the therapeutic exercise progress and modify the daily intensity of the program as needed, as well as monthly following physiological testing.

Therapeutic Exercises:
The subjects performed therapeutic exercises three times/week for 60 minutes per session via a combination of cardiorespiratory equipment, strength equipment, balance training, dynamic stabilization, and functional movements. Strength testing on resistance machines were performed at baseline, then monthly, for the following body parts: knee extensors via leg extension, knee flexors via seated hamstring curl, biceps via seated biceps machine, and back via lat pull down. Testing consisted of a ten repetition maximum test where each patient would progressively have the load increased with each exercise until they could no longer complete ten repetitions (reps) without break in proper biomechanics. Data were recorded as weight in pounds for the ten repetitions.

On strengthening modalities (leg flexion, etc.) patients would complete three sets of ten repetitions of each exercise at 70-90 % of their ten-repetition maximum. Patients were carefully directed to ensure that orthopedic limitations were addressed during the program, as well as other guidelines to ensure safety for other diagnoses (e.g. osteoporosis and hypertension). Functional exercises included working with gym balls, balance activities, therapeutic bands in attempt to increase function with activities of daily living.

All subjects performed work on either a treadmill or cycle ergometer two to three times per week for three months to develop functional endurance and balance. Testing was performed at baseline, repeated each month, and consisted of a graded submaximal exercise to 80 % of age predicted maximal heart rate (APMHR) on treadmill; however, subjects with balance deficits or gait abnormalities were tested on cycle ergometer. Cardiorespiratory fitness was recorded in metabolic equivalents (METS) attained at 80 % of APMHR. Daily sessions would work subjects between 70-90 % of their tested value. Exercise intensity was increased as each testing session demonstrated improvement.

Assessment of physical functioning and mental health:
The SF-36 questionnaire is a widely used and previously validated [15] health questionnaire and was utilized to assess physical functioning and mental health. All subjects completed the SF-36 health questionnaire at baseline and

repeated at three months. Forms contain 36 questions that consist of four categories pertaining to physical functioning and the remaining pertaining to mental health. The forms were then scored using the standard SF-36 formula, with 0 being the lowest score and 100 being the best score for each category.

Medical Events:
All patients completed a medical events questionnaire at the initial visit and at three months. The number of hospitalizations, falls, and M.D. visits for the prior three month period were recorded for each individual patient. Visits to the M.D. or hospitalization for any reason was included (e.g., pre-planned visits, procedures, etc.).

Statistics:
Strength and cardiorespiratory data were analyzed using repeated measures ANOVA with gender as an independent variable ($P < 0.05$). Gender groups were pooled for SF-36 and health care utilization analysis to increase the power for subjective questionnaires and were analyzed using repeated measures ANOVA ($P < 0.05$).

	Age (years)	Height (in)	Weight (lbs)
Men (n=39)	75.3 ± 1.4	67.6 ± 0.3	193.7 ± 8.6
Women (n = 74)	76.5 ± 0.9	62.9 ± 0.3	157.0 ± 4.6

Table 1. Subject Characteristics
Mean ± Standard Error of Measurement (SEM)

Diagnosis	% of Patients
Back Pain	18%
Balance Deficits	36%
Coronary Artery Disease	15%
Neck Pain	6%
Chronic pain	13%
Congestive Heart Failure	4%
Chronic Obstructive Pulmonary Disease	7%
Stroke	6%
Degenerative Joint Disease	20%
Gait abnormalities	7%
Hypertension	39%
Multiple Sclerosis	2%
Muscle Disuse	33%
Osteoarthritis	4%
Osteoporosis	11%
Parkinson's Disease	2%
Severe Deconditioning	58%
Morbid Obesity	4%
Type-I Diabetes	3%
Type-II Diabetes	7%
Other	21%

Table 2. Subject Problem List
All subjects were diagnosed with a minimum of three diagnoses

Results

Subjects
Select subject characteristics are in Table 1. No adverse events were reported in subjects in conjunction with this exercise program. Percentage of subjects with primary diagnoses are presented in Table 2.

Strength
Both male and female subjects had a significant increase in leg flexor strength [$F(1,110) = 165.87$; $P < 0.0001$; Fig. 1A] and back strength [$F(1,111) = 108.54$; $P < 0.0001$ Fig. 2A] over time. There was a difference in strength between men and women for leg flexors [$F(1,110) = 19.14$; $P < 0.0001$) and back strength [$F(1, 111) = 26.07$; $P < 0.0001$].

Figure 1 A

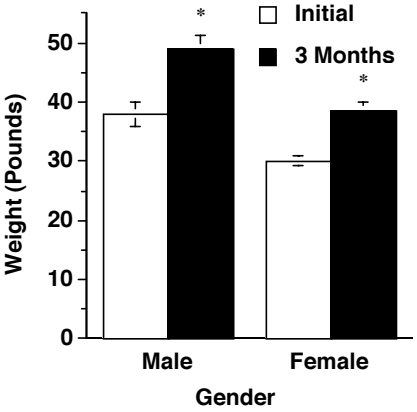

Figure 1 B

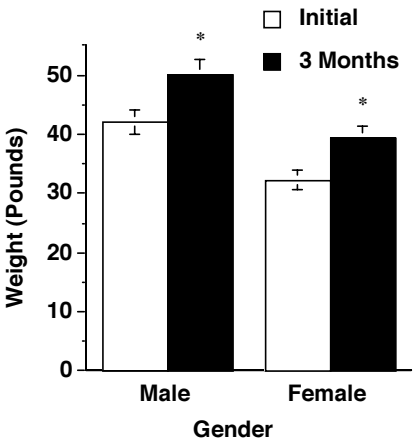

Figure 1 Improvements in Leg Strength Both male and female patients had significant improvements in lower (1A) and upper (1B) extremity strength over a three-month period. *P < 0.05 compared to baseline.

Figure 2

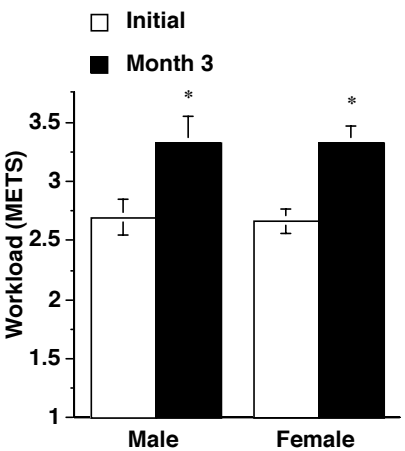

Figure 2 Improvement in Workload Both male and female patients had significant improvements in workload attained at 80% of APMHR over a three-month period. *P < 0.05 compared to baseline.

Aerobic Capacity

Both male and female subjects had significant increase in METs at 80% of APMHR from baseline [F(1,99) = 96.96; $P < 0.0001$; Fig. 2]. However, there were no differences between men and women [F(1,99) = 0.0054; $P = 0.09418$].

SF-36 Questionnaire

There was an improvement in two of the physical components of the SF-36 questionnaire over time (Table 3): Physical Functioning [F (1,107) = 6.84; $P < 0.05$] and Role Physical [F(1,105) = 8.0035; $P < 0.01$]. However, there were no differences in Bodily Pain [F(1,108) = 1.7410; $P = 0.1898$] and General Health [F(1,106) = 0.7753; $P = 0.3806$].

There was an improvement in all mental components of the SF-36 questionnaire over time: Mental Health [F (1,105) = 4.8339; $P < 0.05$], Role Emotional [F(1,102) = 6.56; $P < 0.05$] Social Functioning [F(1,107) = 12.3756; $P < 0.001$], and Vitality [F (1,105) = 12.5013; $P < 0.001$].

SF-36 Category	Initial	Month 3
Physical Component Scales:		
Physical Functioning	41.4 ± 2.3	45.7 ± 2.4*
Role Physical	26.2 ± 3.3	34.5 ± 3.5*
Bodily Pain	55.7 ± 2.3	58.1 ± 2.2
General Health	57.8 ± 1.8	59.1 ± 1.8
Mental Component Scales:		
Vitality	43.2 ± 2.0	49.3 ± 2.0*
Social Functioning	66.8 ± 2.6	75.2 ± 2.5*
Role Emotional	59.2 ± 4.2	69.1 ± 3.7*
Mental Health	72.0 ± 1.7	75.2 ± 1.6*

Table 3. SF-36 Scores
Mean ± SEM. *Denotes significantly different than baseline (P < 0.05)

Medical Events

There were significant reductions in hospitalization rate [F (1, 94) = 4.1814; $P < 0.05$; Fig. 3A], fall rate [F (1, 98) = 16.0353; $P < 0.001$; Fig. 3B], and physician visits [F (1, 90) = 13.3043; $P < 0.001$; Fig. 3C].

Figure 3 A

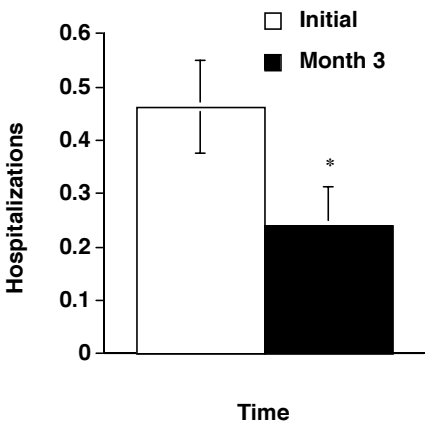

Figure 3 B

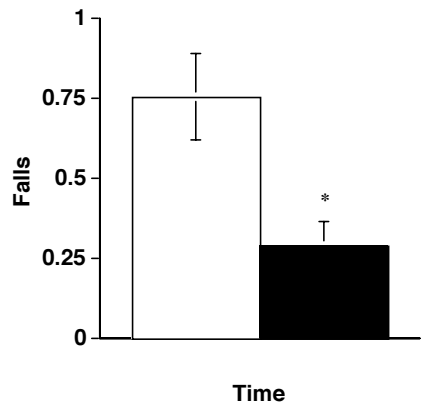

Figure 3 C

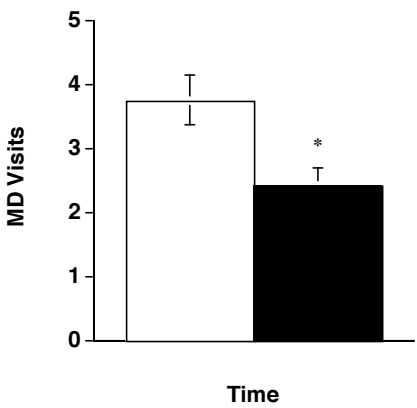

Figure 3 Reduction of medical event
During the program, patients significantly decreased the rate of hospitalizations (3A), falls (3B), and physician office visits (3C) as assessed by number of events over the prior three-month period. *P < 0.05 compared to baseline.

Discussion

The present findings of this paper demonstrate marked improvements in strength, workload, SF-36 measures (physical functioning and mental health), and importantly a concurrent reduction in medical utilization and events. The goal of this program was aimed at optimizing gains in function and health status. Both cardiorespiratory and strengthening modes of exercise were utilized since concurrent training optimizes many aspects of functional fitness [16]. Furthermore, therapeutic exercises, aimed at improving balance and function, were also utilized.

The ~30 % improvement in strength found in this patient population translates to improved functional capacity. Our results are in agreement with Fiatarone et al. [17] who demonstrated that severe weakness in older adults is reversed through exercise training over a ten-week period. This would indicate that frailty is in part due to the muscle inactivity associated with chronic illness, pain, and/or bedrest, and this frailty is treatable. Brown et al. [18] demonstrated that elderly subjects who performed strengthening exercises for the elbow flexors had a significant gain in muscle strength (48 %) and muscle cross sectional area (30.2 %), demonstrating the ability for elders to overcome muscle atrophy. In addition, elderly subjects improve treadmill endurance, dynamic strength, and peak cycle workloads in a resistance training only program, indicating that improved strength is a key factor for improving function[19].

The improvement in workload as measured by a ~25 % increase in METs in our patients may be functionally significant as others have demonstrated that a 15 % improvement in aerobic capacity is of functional importance [20]. In addition to increased workload, cardiorespiratory exercises lead to positive adaptations in nervous and endocrine system regulation, metabolic and functional economy, functional stability, and has anti-atherosclerotic effects [21]. Improvements in workload in these patients may be of further importance, as achieved workload (METs) during exercise testing provides prognostic value for predicting mortality in the elderly [22, 23].

The SF-36 questionnaire indicated that the patients had a concurrent improvement in two out of four on the physical functioning and four out of four on the mental health components. The SF-36 has been used extensively as a quality of life measure to assess health [24, 25] and is an indicator of physical performance in older adults [26]. As such, our results reinforce the self-perception of health with these patients with actual objective improvements in workloads attained during the intervention. Improvement in mental health or psychological affect following the intervention is of particular importance in the medically complex patient population as depressed mood state serves as a predictor of strength decline in the elderly [27].

The reductions in fall, physician visits, and hospitalization rates in response to the program may be significant. For example, fall-related injuries result in an average of $19,440/fall in healthcare costs [28] and falls are now the eighth leading cause of death in the elderly [29]. The high susceptibility to injury and mortality with falling may be attributed to decrements in strength. Both muscle strength (grip strength) [30] and muscle mass (corrected arm muscle area) [31], are important predictors of mortality in the elderly, as well

as muscle strength serves as a predictor of mortality following a bone fracture in older people [32]. Others have been successful in reducing the risk of falling with multidimensional exercise programs [33]. While we did not measure the risk of falling per se we did demonstrate a ~60 % reduction in the rate of falls during the program, presumably due to the improvement in strength in the patients.

Clearly, the medical community supports the use of active interventions to address the needs of the chronically ill population. For instance, clear guidelines are presented for both heart disease and cardiopulmonary disease by the American Heart Association [34] and the American Thoracic Society/American College of Chest Physicians [35]. The U.S. Preventive Task Force recommends that high fall risk elderly patients receive multi-factorial interventions where adequate resources are available [36]. This is important, as it should be noted that many of the chronically ill have multiple diagnoses including orthopedic maladies; therefore, careful restrictions and goals must be outlined for each individual patient to provide proper intervention prescription. In the present study, a team of physicians, physical therapists, and clinical exercise physiologists treated the patients to address the multiple problems presented.

Conclusion

The results of the current study apply previous research in individual chronic disease states (e.g., heart disease, cancer, etc.) to a genuine clinical setting and in a large number of medically complex (multiple diagnoses) patients. Strong functional outcomes are imperative in the medically complex populations as there is a robust relationship between functional health status and mortality [37]. Paffenbarger et al. [38] demonstrated that all-cause mortality was significantly lower among physically active subjects, suggesting that physical activity is an important factor for longevity. The present study works to apply these functional and exercise principles to chronically ill patients. In conclusion, integrated therapy interventions that aim to increase the functional strength and functional capacity result in an improvement in ability to perform activities of daily living, psychological state, and a reduction in health care utilization.

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Moderate Exercise Early After Influenza Virus Infection Reduces the Th1 Inflammatory Response in Lungs of Mice

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Running Head: Exercise and immune response to influenza virus infection in mice

Abstract

We have previously shown that moderate exercise significantly increased survival after influenza virus (A/PR/8/34) infection in mice. We hypothesized that this brief duration of exercise would either increase innate immune defences and/or shift the immune response from a Th1 inflammatory to a Th2 anti-inflammatory response resulting in decreased lung pathology. Adult male BALB/cByJ mice (5-6 months old) were infected with 50 μL of A/PR/8/34 virus (40HAU) intranasally and randomized to either an exercise (EX) or sedentary (SED) group. EX mice performed 20-30 min of moderate exercise (8-12 m/min) on a motorized treadmill 4 hr post-infection and then exercised similarly for 4 consecutive days. SED mice were exposed to similar environmental conditions but did not exercise. Mice from both EX and SED groups were sacrificed 1, 3, or 5 days post-infection (p.i.) and lungs, mediastinal lymph nodes (MLNs) and spleens were harvested. EX significantly reduced total cellular infiltration and IFN-γ gene expression in lungs at Days 3 and 5 p.i. and there was a qualitative shift in the expression of cytokines in the lung from a Th1 to a Th2 response. There was also a tendency toward a reduction in influenza M1 protein mRNA expression. There was no difference in IFN-β protein levels between groups. These data suggest that moderate exercise when applied early after infection shifts the immune response away from a Th1 profile in mice infected with influenza virus. This exercise-induced shift in immune response may be responsible for improved survival after influenza virus infection. (Exerc. Immunol. Rev. 12, 2006: 97-111)

Keywords: influenza, exercise, inflammation, immunity, Th1, interferon-γ

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Introduction

Many laboratories, including ours, have conducted research examining the influence of exercise on immune responses. While far from certainty, the prevailing data suggest that moderate exercise improves immune function (43), whereas strenuous, prolonged exercise decreases many aspects of immune functioning (18, 19, 29). Aside from a few recent studies with herpes simplex-1 virus and lymphocytic choriomeningitis virus (LCMV) (6, 19, 43), the majority of research evidence comes from studies examining in vitro immune responses using pathogen non-specific immune challenges. For instance, several studies have examined the response of isolated peripheral blood mononuclear cells to polyclonal mitogens or examined natural killer cell activity from cells obtained before and after exercise (14, 24, 33). Examination of the effects of exercise on the immune response to clinically relevant challenges is lacking.

Each year, diseases associated with the influenza virus are responsible for an estimated 36,000 deaths and up to 150,000 hospitalizations in the United States making it a significant public health problem (4). Both the innate and the adaptive immune systems are activated following primary infection with influenza virus. Early innate detection of influenza virus in the respiratory tract occurs through plasmacytoid dendritic cell (pDC) toll-like receptor (TLR)-7 leading to the induction of type 1 interferons (IFNs), NF-κB activation, and pro-inflammatory cytokine expression (26). TLR-7-triggered production of IFN-α and IFN-β induce an anti-viral state important to limit the initial spread of the virus, and these Type I interferons also begin to drive the innate immune response to influenza (5, 32). Perhaps more importantly, the initial triggering of TLR-7 makes the pDCs key players driving the early events that ultimately lead to the development of Th1-dominated cell-mediated immune responses. The pDCs secrete multiple pro-inflammatory cytokines (e.g. TNF-α, IL-1β, IL-6 and IL-18) and chemokines (e.g. RANTES, MCP-1) that directly and indirectly lead to increased antigen processing and presentation by macrophages and increased cytotoxicity by natural killer (NK) cells. Ultimately, TLR-7 signaling can be linked to activation of antigen-specific immune responses by the production of IFN-γ, which is the principal mediator of Th1 responses. That link is further manifested in the subsequent proliferation and recruitment of antigen-specific $CD8^+$ T lymphocytes (the expansion of which depends on $CD4^+$ Th1 cells) in the draining mediastinal lymph nodes (MLNs). These antigen-specific $CD8^+$ T cells play a significant role in viral clearance and recovery from influenza infection (42). Interestingly, while a strong Th1 response is important in influenza viral clearance, such an inflammatory response may also lead to tissue damage and death (38). It has been suggested that Th2 cytokines, such as IL-4 and IL-10, counteract potentially harmful high levels of Th1 cytokines (and their induced responses) in influenza infection (1).

We have previously determined that 4 days of moderate exercise applied early after infection with influenza virus significantly increases survival rate in BALB/c mice (25). In contrast, prolonged exercise led to higher mortality rates. In this study, we explored moderate exercise-induced changes in immune responses of mice infected with influenza virus in an attempt to begin to understand why it is that exercise protected mice from death due to influenza virus challenge. We hypothesized that this brief duration of exercise would result in

decreased lung pathology and shift the immune response from a Th1 inflammatory response to a Th2 anti-inflammatory response.

Materials and Methods

Animals. Specific pathogen-free inbred male BALB/c mice aged 5-6 months (n=110) were bred in our facility and used in all experiments. Mice were housed individually in micro-isolated shoe box cages in facilities maintained at a temperature of 23 ºC. All mice were kept on a 12:12-h light-dark cycle (0700-1900 dark) and given autoclaved food (8640 Harlan Teklad 22-5 Harlan, Madison, WI) and water *ad libitum*. All animal treatments were approved by the Institutional Animal Care and Use Committee at the University of Illinois @ Urbana/Champaign and within the guidelines set by the NIH for the care and use of laboratory animals.

Influenza Infection. We challenged BALB/c mice intranasally (i.n.) with a dose of virus (40 hemmaglutinating units [HAU]) that resulted in ~50% lethality of sedentary control mice as in our previous study (25). In this study, 50 μL of influenza virus (40 HAU of A/Puerto Rico/8/34) was delivered i.n. into mice lightly anesthetized with Isoflurane.

Exercise Paradigm. Mice were infected 2-3 hr into their dark cycle on Day 0. Four hr post-infection (p.i.) (1300-1400, Day 0) mice were exercised using a motorized treadmill at a speed of 8-12 $m.min^{-1}$ and 5 % grade for 30 min. This treadmill speed was approximately 55-65 % of their maximal oxygen uptake (VO_2max) (23). Our own studies measuring VO_2max in BALB/c mice of various ages have confirmed these relative exercise intensities (data not shown). Mice then exercised similarly for 3 more consecutive days (Days 1-3), with each exercise session separated by 22-24 hr. We expected the effects of exercise to be greatest when exercise was performed in the early stages of infection. Mice were not exercised past Day 3 because they exhibited illness symptoms. Mice ran without electric shock or prodding. Control mice were exposed to the treadmill environment for a similar time but did not exercise. All mice were denied access to food and water during the exercise period.

Tissue Collection and Processing. Lungs isolated for mRNA or protein analysis were excised and immediately flash-frozen in liquid nitrogen in 2.5 mL micro-centrifuge tubes and then stored at -80 °C until analysis by real-time reverse transcriptase (RT)-PCR. Lungs isolated for cellular analysis were placed in 5 mL of media (RPMI 1640) and homogenized. The homogenate was added to 25 mL of media for a total volume of 30 mL. This was followed by the addition of 1,500 units of Type I collagenase per sample (Worthington Biochemical Corporation, Lakewood, New Jersey). This mixture was placed in a beaker and stirred using a magnetic stir plate for 45 min at 4°C. Following this digestion, the mixture was centrifuged at 200 x *g* for 5 min. The supernatant was removed, residual red blood cells were lysed with ammonium chloride solution, the cells were resuspended, counted, and single-cell suspensions were

adjusted for antibody staining and flow cytometric analysis. In addition to isolating lungs, we also examined cell content and phenotype of the draining MLNs and spleens. Single cell suspensions from MLNs and spleens were obtained by passing these tissues through a wire mesh screen (Sigma-Aldrich, St. Louis, MO). Residual red blood cells were lysed with ammonium chloride solution, cells were resuspended, counted, and single-cell suspensions were concentration adjusted for antibody staining and flow cytometric analysis.

Cytospin Preparation. Following sacrifice, lungs were lavaged with 3-5 mL of PBS. Cells were centrifuged at 250 x *g* for 5 min, resuspended in PBS, counted and adjusted to 0.5 x 10^6 cells per mL. Cells were kept on ice, and 100-200 µL of this solution was used for Wright's staining on microscope slides. Cell identification was performed at 40X in triplicate in 3 previously determined fields in a blinded fashion, with the three cell count (minimum of 200 cells counted per field) identifications averaged for each mouse. Neutrophils, lymphocytes, and macrophages are presented as a percentage of the total number of cells counted.

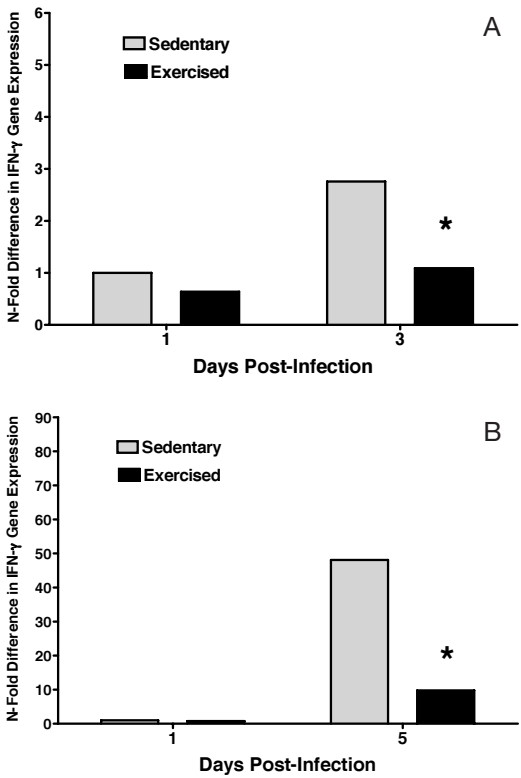

Figure 1. IFN-γ gene expression in lungs of mice on Day 3 (Figure 1a; n = 8 and 7 per group at Days 1 and 3, respectively) and Day 5 (Figure 1b; n = 8 and 10 per group at Days 1 and 5, respectively) following infection with A/PR/8/34. Note the differences in the y-axis scale between Figure 1a and 1b. *Significantly ($p < 0.05$) lower than SED group on same day.

Flow Cytometry. Cells (0.5 x 10^6) were added to 1 mL of PBS/2 % FBS/0.1% sodium azide and centrifuged at 250 x *g*. Cells were agitated following removal of supernatant and 100uL of PBS/2 % FBS/0.1 % sodium azide was added along with appropriate labeled antibodies (0.5 µg phycoerythrin-conjugated anti-CD8, Pharmingen, San Diego, CA; or 0.5 µg Rat Anti-Mouse CD49b/pan–RPE, Southern Biotechnology, Birmingham, AL) for 30 min at room temperature. Cells were then washed and fixed in PBS/1 % buffered Formalin (Sigma) and stored at 4°C until analysis using a Becton Dickinson LSR Benchtop Flow Cytometer with a 488 nm argon-ion laser. A minimum of 10,000

events were analyzed in each sample. The autofluorescence gate was set using an unstained sample. All autofluorescent samples fell within the first log, with stained samples falling between the first and third log.

Gene Expression. Lung tissue samples were homogenized using a standard single-step TRIzol RNA isolation procedure (GIBCO BRL, Gaithersburg, MD). After isolation, total RNA was quantified spectrophotometrically at 260 nm and 280 nm. RNA was reverse transcribed into cDNA according to manufacturer's protocol (Promega, Madison, WI). The reverse transcription system consisted of 25 mM $MgCl_2$, 10X reverse transcription buffer, 10 mM dNTP mixture, rRNase in ribonuclease inhibitor, avian myeloblastosis virus (AMV) reverse transcriptase, and random primers. Samples were incubated at 42˚C for 60 min, 100˚C for 5 min, and then cooled to 4˚C. All samples underwent real-time polymerase chain reaction (PCR), in order to quantify the nucleotide sequences of interest. 18S RNA was used as an internal control in all samples. Primers were purchased from Invitrogen Life Technologies (Carlsbad, CA), and probes were purchased from Applied Biosystems (Foster City, CA). Primer sequence information can be found in Table 1. The master mix for real time PCR consisted of 2X Universal Master Mix (PE Biosystems, Roche Molecular Systems, Branchburg, New Jersey), 0.9 μM of cDNA 5'primer, 0.9μM of cDNA 3'primer, and 0.25μM of cDNA probe. Samples were subjected to 40 cycles of amplification each consisting of 2 min at 50˚C, 10 min at 95˚C, 15 s at 95˚C, and 1 min at 60˚C. Real-time PCR was performed using a Prism 7000 Sequence Detection System Software (Applied Biosystems, Foster City, CA). Samples obtained from sedentary animals 24 hr post-infection were used as the reference control.

M1 Protein	**Sequence**
Forward	5'-GGACTGCAGCGTAGACGCTT-3'
Reverse	5'-CATCCTGTTGTATATGAGGCCCAT-3'
Probe	5'-CTCAGTTATTCTGCTGGTGCACTTGCCA-3'
IFN-γ	**Sequence**
Forward	5'-AGCAACAGCAAGGCGAAAA-3'
Reverse	5'-CTGGACCTGTGGGTTGTTGA-3'
Probe	5'-CCTCAAACTTGGCAATACTCATGAATGCATCC-3'
IL-10	**Sequence**
Forward	5'-TTTGAATTCCCTGGGTGAGAA-3'
Reverse	5'-ACAGGGGAGAAATCGATGACA-3'
Probe	5'-TGAAGACCCTCAGGATGCGGCTG-3'

Table 1. Primer sequences used for real-time RT-PCR.

Qualitative Protein Analysis. We utilized an inflammatory cytokine antibody array (RayBiotech, Lakewood, New Jersey) to probe for qualitative differences in protein expression in lung homogenates pooled from the two different groups. As this was a costly assay, only mice from Day 3 were used (n=6 SED and n=8 EX). Ten mg of lung was removed and added to 250 μL of lysis buffer, 250 μL PBS, and 50 μL of protease inhibitor cocktail (Sigma, St. Louis, MO). This mixture was homogenized and centrifuged at 2500 x *g* for 5 min. The resulting super-

natant was frozen at -80 °C. Samples from individual mice were then pooled (equal amounts of protein from each mouse) and analyzed according to manufacturer's specifications (RayBiotech, Lakewood, New Jersey).

Lung IFN-β ELISA. Analysis of lung IFN-β protein expression was performed using a commercially available (PBL Biomedical Laboratories, Piscataway, NJ) ELISA kit.

Data Analysis. Cell counts and percentages and lung IFN-β concentration are reported as mean ± standard error of the mean (SEM). For these variables, significant treatment effects (e.g. EX vs. SED) were determined using General Linear Model univariate analysis of variance (SPSS 14.0, Chicago, IL). Significant effects were followed up by a series of Bonferroni-adjusted post hoc tests. To assess the influence of exercise on gene expression during influenza infection, the quantity of specific RNA (e.g. IFN-γ, IL-10, or influenza M1 protein) obtained from a lung sample was compared to the quantity of the same RNA species present in sedentary lungs Day 1 post-infection. EX treatment did not affect baseline gene expression in uninfected lungs (data not shown). Briefly, real-time PCR generates a C_t value, which is the PCR cycle where amplification of the cDNA of interest begins exponential expansion. For analysis, the C_t value for the internal standard (i.e., 18S RNA) was first subtracted from the C_t value for the cDNA of interest. This subtraction controls for differences in reverse transcription and sample loading, and the value is denoted as the ΔC_t. Next, the ΔC_t value generated from referent tissue samples were then subtracted from the ΔC_t for each experimental sample. This equation sets the control sample to a reference value of 0 and generates a $\Delta\Delta C_t$for each unknown. And finally, these values were averaged for each treatment group; these mean values were used to generate the N-fold difference in RNA expression relative to the control using the equation: $2^{(-\Delta\Delta C_t)}$; using this equation the control = 1. Although this data transformation accurately illustrates the logarithmic amplification following each PCR cycle and is used in each of the figures, statistical evaluation was performed using the $\Delta\Delta C_t$ value. Ranked sum tests with a Student's *t-test* were performed using Sigma Stat statistical software version 2.0. p values of less than 0.05 were considered significant. Due to the logarithmic transformation of the

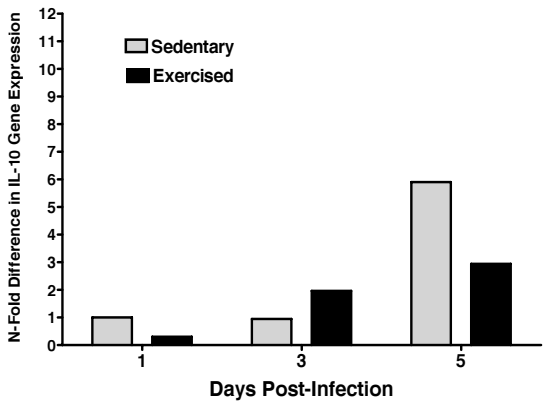

Figure 2. IL-10 gene expression in lungs of mice on Days 3 and 5 following infection with A/PR/8/34. (n = 12 and 10 in each group for Days 1 and 5 post-infection, respectively).

data, it is visually misleading to depict a standard deviation or standard error of the mean; thus the figures that illustrate real-time PCR data do not include such error bars.

Results

Exercise reduces cell influx into the lungs and secondary lymphoid organs of influenza infected mice. Infection resulted in significant cellular infiltration into the lung when compared to non-infected SED and EX mice (data not shown). Importantly, there was a significant reduction of total cells infiltrating the lung on Day 5, but not Day 3 (2.5 ± 0.6 vs. 2.5 ± 0.4x 10^6 for SED and EX, respectively; p = 0.98), p.i. in the EX when compared to the SED group (Table 2). We prepared cytospin slides of cells obtained from the lungs to determine whether exercise preferentially reduced specific phenotypes of cells including neutrophils, lymphocytes or macrophages. Despite the reduction in total cell number, there were no significant differences in the percentage of neutrophils (34 ± 4 % vs. 36 ± 5 %, for SED and EX, respectively, p = 0.78), lymphocytes (42 ± 4.7 vs. 42.5 ± 4.2 %, for SED and EX, respectively, p = 0.93) or macrophages (24 ± 3.7 vs. 21.6 ± 3.5 %, for SED and EX, respectively, p = 0.64) between EX and SED mice at Day 5 post-infection indicating that the anti-inflammatory effect of exercise was cell non-specific, at least as grossly defined by cell morphology. Furthermore, we found no significant differences in the percentage of cells positive for the cell specific surface markers CD8 (cytotoxic T lymphocyte) (Table 2) or CD49 (12.8 ± 3.6 and 15.9 ± 2.1, for SED and EX, respectively). EX mice exhibited significantly reduced numbers of $CD8^+$ cells in the spleen and a tendency for reduced $CD8^+$ cells in the MLNs and lung (Table 2).

Tissue	Total Cells ($x10^6$)		Percent $CD8^+$		$CD8^+$ ($x10^6$)	
	SED	EX	SED	EX	SED	EX
Lung	5.4 ± 0.44	4.1 ± 0.34*	12.6 ± 0.8	14.5 ± 2.7	0.68 ± 0.06	0.59 ± 0.1
MLN	4.8 ± 0.64	2.8 ± 0.66+	12.7 ± 2.9	11.7 ± 2.2	0.65 ± 0.19	0.33 ± 0.08+
Spleen	20.1 ± 5.7	12.9 ± 2.3	16.8 ± 3.7	15.9 ± 2.4	2.8 ± 0.4	1.7 ± 0.17*

Table 2. Effects of exercise on cellularity and CD8 phenotype in lung, MLN, and spleens of influenza infected mice 5d post-infection (N=5-8 per group). *p<0.05; +p<0.08

Exercise reduces IFN-γ gene expression in lungs of influenza infected mice. We measured lung cytokine gene expression to determine whether exercise modulated the Th1 (IFN-γ) or Th2 (IL-10) immune response to influenza infection. EX mice exhibited a significantly reduced expression of IFN-γ mRNA on both Day 3 (Figure 1a) and Day 5 (Figure 1b) post-infection. Irrespective of group, IFN-γ mRNA levels were significantly increased on Day 3 and even more so on Day 5 when compared to Day 1. Note the large y-axis scale difference between Figures 1a and 1b. While influenza infection increased IL-10 gene expression significant-

Cytokine	EX	SED
BLC	2,988.29 △	1,366.63
CD30L	1,282.62 △	0.00
Eotaxin	1,897.55 △	0.00
Eotaxin-2	1,503.41 △	0.00
FAS ligand	305.27 △	0.00
Fracktaline	4.08 △	0.00
G-CSF	0.00	0.00
GM-CSF	4,023.74	4,153.74
IFN-γ	43.34 ▽	2,165.44
IL-1α	4,312.05	2,578.38
IL-1β	2,554.57 △	243.31
IL-2	591.69	0.00
IL-3	0.00	0.00
IL-4	255.96 △	0.00
IL-6	0.00	0.00
IL-9	0.00	0.00
IL-10	0.00	0.00
IL-12p40/p70	338.24 △	0.00
IL-12p40	0.00	0.00
IL-13	1,679.28	2,988.31
IL-17	0.00 ▽	765.12
I-TAC	0.00 ▽	1,379.57
KC	0.00 ▽	834.00
Leptin	0.00 ▽	93.23
LIX	1,841.02 ▽	3,732.08
Lymphotactin	0.00 ▽	1,076.71
MCP-1	2,661.98	3,004.55
M-CSF	404.20 ▽	894.15
MIG	0.00	0.00
MIP-1-α	0.00	0.00
MIP-1-γ	10,887.22	6,527.00
RANTES	7,405.86	9,855.47
SDF-1	0.00 ▽	2,017.47
TCA-3	4,489.18	3,879.45
TECK	637.86	1,037.31i
TIMP-1	5,689.52	4,475.25
TIMP-2	1,271.95	1,801.83
TNF-α	1,776.01	1,728.44
sTNFrI	2,522.22	1,542.28
sTNFrII	1,968.53 △	743.47

Table 3. Qualitative protein expression (expressed as arbitrary units) in lungs 3 days post-infection. Numbers in green or △ indicate a > 2-fold increase in expression compared to SED. Numbers in red or ▽ indicate a > 2-fold decrease in expression compared to SED.

ly by Day 5 p.i., we found no significant differences between EX and SED in IL-10 gene expression on Day 3 or 5 p.i. (Figure 2); although there was a tendency ($p = 0.10$) for IL-10 gene expression to be lower in EX mice on Day 5 p.i. We also compared IFN-γ and IL-10 gene expression in the lungs of exercised (for either 1 or 4 days and sacrificed 24 hr after the last exercise bout), but non-infected, mice with that of sedentary non-infected mice. We found that exercise alone did not appreciably affect IFN-γ or IL-10 gene expression in the lung when compared to sedentary mice. Moreover, the level of IFN-γ and IL10 gene expression in lungs of non-infected sedentary or exercised mice was very low compared to infected mice.

Exercise causes a qualitative reduction in Th1 and an increase in Th2 proteins in the lungs of influenza infected mice. We used an antibody array to qualitatively assess inflammatory cytokine protein expression in pooled (equal amounts of protein per mouse) lung samples from SED and EX mice at Day 3 p.i.. As can be seen in Table 3, the levels of many inflammatory proteins were altered by exercise. Most notably, when compared with SED mice, exercise resulted in a 2-fold reduction in IFN-γ, RANTES, IL-13, IL-17, I-TAC, SDF-1, KC, LIX, M-CSF and lymphotactin. Two-fold increases in eotaxin, eotaxin-2, IL-2, IL-4, BLC, sTNFrI and II and CD30L were seen in exercised mice, as well as IL-1α and IL-1β. However, the pro-inflammatory cytokine TNF-α was similar in both groups and IL-6 was undetectable in either.

Exercise does not affect lung IFN-β expression. IFN-β aids in the early antiviral response through transcriptional activation of many genes, including the double-stranded RNA activated protein kinase (PKR) and Mx proteins, which in turn disrupt the replication of influenza virus (32). While IFN-β expression in infected lungs tended ($p = 0.11$) to be higher Day 1 post-infection in the EX mice there were no statistical differences between groups (Figure 3).

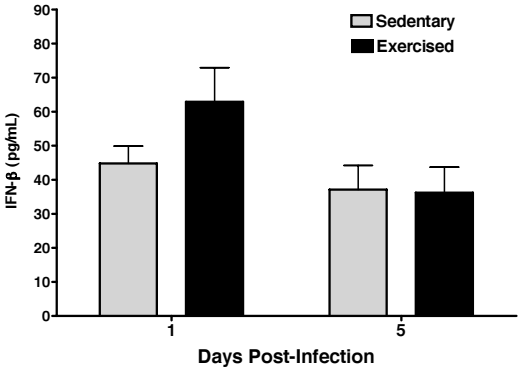

Figure 3. Effects of exercise on IFN-β protein expression in lungs of mice following infection with A/PR/8/34. (n = 8 per group).

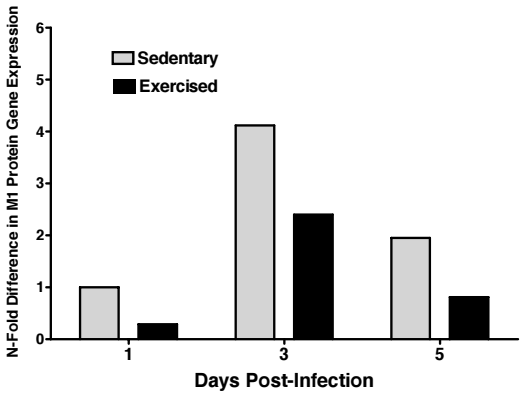

Figure 4. Effects of exercise on influenza virus M1 gene expression. Sedentary 24 hr post-infection sample was used as referent. While not statistically significant, there was a tendency towards lower viral replication in exercise when compared to sedentary mice. M1 gene expression in lungs of mice following infection with A/PR/8/34 (n = 11, 8, and 10 in each group at Days 1, 3, and 5 post-infection, respectively).

Exercise tends to reduce early viral replication in lungs of influenza infected mice. Influenza A/PR/8/34 infection in lung was measured using real-time PCR with a primer/probe set that amplified the virus-encoded matrix (M1) gene. Using the expression of this late viral gene product as a surrogate marker for virus replication (11), the data demonstrated a tendency for exercise to reduce viral M1 gene expression in the lung (Figure 4). While there were no significant treatment effects between EX and SED groups, at Days 1, 3 and 5 M1 protein gene expression was 40-60 % of that of SED mice. Not surprisingly, there was a significant increase in viral mRNA on Day 3 and Day 5 p.i. when compared to Day 1.

Discussion

After intranasal inoculation with A/Puerto Rico/8/34 (H1N1) virus, mice develop a lower respiratory tract infection with lung consolidation resulting in death starting on Day 6 after infection (38). In a previous study (25), we found

that moderate exercise, when performed during the initial stages of a mounting primary immune response to influenza infection, improved survival in mice. In this follow-up study, we hypothesized that exercise would shift the immune response from a Th1 inflammatory phenotype to a Th2 anti-inflammatory response. We hypothesized that such a shift would result in a decrease in immune-mediated lung pathology and would thus explain why exercised mice had higher survival rates as seen in our earlier study. To address this hypothesis, we examined the lungs and secondary lymphoid organs (MLNs and spleen) of exercised and sedentary mice at Days 1, 3, or 5 post-infection with influenza A virus.

In support of the hypothesis, the data illustrated that moderate exercise, performed in the early stages of a mounting primary immune response to influenza virus infection, resulted in a significant reduction in the total number of cells infiltrating the lung. This suppressive effect of exercise was without regard to cell type. In addition, the data showed that exercise significantly reduced the expression of the potent Th1 inflammatory cytokine IFN-γ, while not altering gene expression of the Th2 cytokine IL-10. While it is well known that Th1 mediated immune responses are responsible for influenza virus clearance and recovery from infection (42), there is some evidence to suggest that an exaggerated Th1 inflammatory response to influenza virus can cause lung pathology and increase mortality (38). Indeed, injection of neutralizing anti-TNF-α antibodies at the time of influenza infection reduced lung lesion severity and prolonged survival by 24 hr (31). Moreover, treatment with IL-1 receptor antagonist produced a small but statistically significant increase in food intake and survival rates after influenza infection in mice (37). We also found that exercise had no effect on lung expression of the early anti-viral protein IFN-β. This, coupled with a failure to detect a reduction in viral replication (although there was a trend), suggests that moderate exercise did not affect early innate immune defences in this influenza model that could contribute to reduced mortality.

In agreement with our gene expression data, qualitative antibody array protein analysis of lung homogenate obtained on Day 3 post-infection revealed an exercise-induced shift from a Th1 towards a Th2-type immune response. Exercise resulted in a greater than two-fold reduction in protein levels of IFN-γ, lymphotactin, IL-17, IL-13, I-TAC, KC, leptin, SDF-1, and LIX and a greater than two-fold increase in eotaxin, eotaxin-2, IL-2, IL-4, BLC, FasL, IL-12p40/p70, and CD30L. While not entirely consistent with our hypothesis due to elevated IL-12 (although expression of this protein was low), it is noteworthy that exercise led to an increase in many cytokines that are characterized as Th2-like and a decrease in those that are indicative of a Th1 response. For example, in addition to the reduction in IFN-γ, exercise also reduced the LPS-induced CXC neutrophil chemoattractant LIX which has been shown to be involved in tissue pathogenesis in the dextran sodium salt-induced model of colitis (22). Moreover, exercise reduced interferon-inducible T cell α chemoattractant (ITAC) which plays a pivotal role in attracting effector T cells into the sites of Th1-type inflammation and is critically involved in the development of multiple Th1-type inflammatory diseases (9, 27) and allograft rejection (17).

Conversely, exercise increased expression of IL-4 (the prototypical Th2 cytokine) and the eotaxins; which act as chemoattractants for eosinophils and are induced by the Th2 cytokines IL-5 (34). While the role of eosinophils in

defence against viral infection is controversial, eosinophil granules contain abundant ribonucleases that degrade single-stranded RNA containing viruses (34). Interestingly, exercise also resulted in an increased expression of IL-1α and IL-1β in lungs of infected mice which appears at odds with an anti-inflammatory or Th1 reducing effect of exercise. However, while IL-1 is responsible for acute lung immunopathology it also leads to increased survival in response to A/PR/8/34 (35). Lastly, lungs from the exercised group displayed higher levels of soluble TNF receptors with no change in TNF-α indicating induction of anti-inflammation. The results of this qualitative antibody array analysis should be interpreted with caution (although the analysis verified that IFN-γ protein was lower, in agreement with our gene expression data) and future studies will need to statistically verify changes in protein expression using data from individual animals. We predicted an increase in lung histopathology, although in the few animals that we examined it did not appear that exercise had a marked effect on lung tissue damage, these studies were far from conclusive because histopathology was only examined on Days 1, 3, and 5 post-infection, likely too early to distinguish differences between groups.

There is precedent that exercise and other stressors can cause shifts away from Th1 toward Th2 immune responses (2, 36) and it has long been known that glucocorticoids and prostaglandins of the E series (PGE_2 in particular) can decrease Th1 and increase Th2-mediated immune responses (8, 15). While catecholamines may also play a role, Kohut et al. (2004) found that β blockade exacerbated exercise training-induced increases in HSV-induced *in vitro* IFN-γ production in young mice, but decreased this same response in old mice (21). Neuroendocrine hormones are clearly involved in acute and chronic stress-induced alterations in leukocyte trafficking and increases, decreases, or changes in the composition of the cellular influx could have beneficial or detrimental consequences dependent on the immunologic context (39). Unfortunately, a definitive role for specific neuroendocrine molecules as being responsible for exercise or stress-induced shifts in the Th1/Th2 balance awaits further study. It is interesting to note that while complete shifts away from Th1-mediated immune responses would be detrimental in virally infected animals a more subtle counter-regulation of Th1 responses may be beneficial.

Exercise dosage and the timing of exercise relative to infection appear critical in determining whether exercise reduces or increases morbidity and/or mortality to infectious diseases. The data suggest a hormetic effect where single bouts of prolonged exercise or intense exercise during symptoms exacerbates disease (6, 10, 12, 13, 16, 18, 29), whereas prior moderate exercise training or moderate exercise during early times after infection (but before overt symptoms) lessens disease severity (3, 7, 16, 25, 30) or is without effect (40, 41).

Only a few studies have examined exercise-induced alterations in the immune response that could potentially explain changes in morbidity or mortality. Kohut et al. (2001) examined the *ex vivo* cytokine responses to i.n. HSV-1 infection after a single bout of prolonged exercise (19), which they had found previously increases mortality to such a challenge (6). This ~2.5 hr run resulted in reduced ex vivo splenocyte production of both Th1 (IL-2, IFN-γ and IL-12) and Th2 (IL-10) cytokines on Day 2, but not Day 7 (except for IL-12) post-infection (19). Moreover, Kapasi et al (2004) found that either single or multi-

ple bouts of exhaustive exercise decreased LCMV-specific $CD8^+$ T cell numbers and their production of IFN-γ in response to LCMV infection in young, but not old mice (18). The clinical consequences of this, however, were not examined. These data are consistent with our data examining *in vivo* responses to influenza in the lung except that we did not document a decrease in IL-10. It is interesting to note the different dose-response effects of moderate exercise (increased survival) vs. prolonged exercise (decreased survival) on mortality despite similar changes in cytokine production.

Employing a different exercise paradigm, Davis et al. (2004) found that 1 hr of moderate exercise performed daily for 6 days prior to HSV-1 infection resulted in a decrease of 45 % in morbidity and 38 % in mortality when compared to sedentary mice (7). A mechanistic role for exercise-induced changes in alveolar macrophage function was recently confirmed when this group demonstrated that depletion of alveolar macrophages with clodronate encapsulated liposomes could abrogate the moderate exercise-induced increase in morbidity and mortality (28). Seemingly at odds with the exercise-induced reduction in IFN-γ seen in our study, Kohut et al. (2004) found that 8 weeks of moderate exercise training prior to HSV-1 infection increased IFN-γ and IL-2 production in splenocyte cultures stimulated with HSV-1 one week post-infection in aged, but not in young mice (20). This effect was likely mediated by exercise-induced increases in catecholamines acting through β-adrenergic receptors, at least in old mice (21). The different results in their study when compared to ours can likely be attributed to different aged mice, exercise protocols (training vs. acute) and virus challenge.

While the mechanistic underpinnings of the differential dose response to exercise are difficult to reconcile, it may be that moderate exercise and prior training reduce immune-mediated damage without severely affecting immune effector functions. Strenuous exercise, on the other hand, may be of sufficient intensity to reduce effector functions allowing infectious disease progression. In support of this, we have found that moderate exercise, when applied during the initial stages of a mounting immune response to influenza, causes a shift in the immune response away from a Th1 and toward a Th2 response without altering early anti-viral defences. This may be responsible for the increased survival rates in exercised mice because a strong Th1 response may lead to immune mediated pathology and death.

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Regulation of immediate early gene expression by exercise: Short cuts for the adaptation of immune function

Running head: Immediate early genes in exercise immunology

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Abstract

Onset of physical activity induces a wide variety of rapid biophysical and biochemical responses that act on cells and lead to a wide range of phenotypic adaptations. Here we elucidate the role of immediate early gene (IEG) expression as a first line of transcriptional response that mediates protein neosynthesis and leads to adaptation of immune function. New posttranscriptional mechanisms have been described that speed up transcriptional responses. These include RNA-RNA interactions such as those exploited by miRNAs and stimulus-dependent cytoplasmic polyadenylation. We describe these shortcuts that modulate expression and discuss the challenges of accurately measuring them using various transcriptomic screening and quantification approaches.

Although there is high complexity of the upstream as well as the downstream pathways that lead to IEG expression, IEG expression itself may only show a limited number of response patterns. Focusing transcriptomic approaches in exercise immunology at the IEG-level may facilitate the discovery of exercise-specific transcriptional signatures. (Exerc. Immunol. Rev. 12, 2006: 112-131)

Key Words: Immediate early genes, posttranscriptional regulation, cytokines, ncRNA, cisNAT

1. Immediate early genes: The "sprinters" among our genes.

The term immediate early gene (IEG) was originally coined during the mid 1970s and early 1980s in reference to viral genes that were rapidly transcribed following invasion of a host (12;62;68). The main features of the IEG consist of a fast and

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transient transcriptional response after stimulation and several minutes well before the first expression of stimuli-related proteins. It has been shown that expression of IEGs is the key trigger leading to the initiation of protein neosynthesis and the subsequent expression of so-called"late response genes" that can only be activated following the translation of the small cohort of IEG transcripts (78).

With the discovery of the human homologs to retroviral IEG oncogenes the concept of IEGs was transferred to humans (15;50;75). In the beginning most of the IEGs, for instance the immediate early growth response gene 1 (Egr1), were characterized as transcription factors and key regulators of cell growth and differentiation (70). During the past 20 years it has been shown that 1. IEG function is not limited to transcription factor activity, 2. adaptation to a variety of stimuli including cytokines, mechanical stress, and somatosensory perception requires IEG expression, and 3. the subset of IEGs activated by a certain stimulus can be specific to a cell type and condition.

Here we propose a central role for IEGs in the adaptation of the immune system to physical stress and exercise. Transcriptomic approaches and improvements in RNA quantification have greatly facilitated the systematic analysis of IEG expression (25). Here we review results of some of the first studies using such approaches to study effects of physical exercise on expression in immune competent cells. We then describe how IEGs may exploit posttranscriptional processes such as cytoplasmic polyadenylation, degradation by Adenosine- and Uracil-rich element binding factors, and even expression of related non-coding RNAs (ncRNAs) to further accelerate their tightly controlled expression. These atypical mechanisms affect and perturb conventional mRNA expression assays.

2. The activation of immediate early genes in immune competent cells by physical exercise and stress

The majority of studies of IEG function have involved the central nervous system and rapid responses to activity and other environmental and experimental stimuli. Among roughly 180 reviews dealing with the term"immediate early gene/s" during the past 20 years more than 90 % are related to CNS function.

This is due to the fact that neuronal responses to stimuli – neuronal plasticity and learning – are processes physiologists agree depend on rapid molecular adaptations (54). However, there is nothing as crucial for the viability as the fast and robust responses to physical stress and exercise. Speed matters, not only for the immediate action itself, but also for the processes of rapid adaptations that inevitably require protein neosynthesis.

Immediate and effective upstream (earlier and causal) events have been identified which are known to induce effects in the various cell types of peripheral blood in response to exercise. Here we discuss the most prominent of these upstream events regarding their ability to induce IEG expression.

Upstream events can be subdivided into biophysical and biochemical events (see Fig. 1 for illustration). The former may principally act directly on peripheral blood cells or indirectly via induction of biochemical pathways anywhere within

the body. Accordingly, it is difficult to determine the source of many important immune modulators altered by exercise. This situation has already led to controversies in exercise physiology, as for instance in the case of the source for the increase in plasma IL6. The current opinion is now that it is rather the muscle cells than leukocytes which contribute to this effect (51, 67). This discussion can also be extended to alterations of any other kind of cytokines, including the extracellular heat shock proteins (eHSPs) with known cytokine function (3, 43). Clarification of the respective situation *in vivo* is not trivial given that just any organ or cell type, or even certain cell types neighbouring muscle cells, such as adipocytes or endothelial cells, could outrange production by peripheral blood cells or the muscle cells depending on type, duration and intensity of exercise. In terms of the question of IEG activation in leukocytes we can treat this complex of still to be addressed questions as a black box and look at the stimuli acting on leukocytes regardless of their origin.

Both biophysical and biochemical activation of cells rapidly elicits transcription of IEGs (38, 8, 74, 32). The upper part of Figure 1 gives an overview about biophysical and biochemical stimuli acting on cells following exercise. Despite the very diverse nature and a high interactivity of these stimuli, a striking observation is that they all activate MAP kinase cascades (8, 65, 18, 79, see Fig.1 middle). Induction of IEGs in a specific way is achieved, since the exact extent and time-course of activation of each of the ERK, JNK/SAPK and p38 MAP kinase cascades by a particular stimulus is highly distinctive and characteristic for that stimulus (see 7, 32, and references therein).

Events upstream of IEG expression in Leukocytes

Muscular activity is always combined with at least an increase of intramuscular temperature or even whole body temperature, which in turn activates expression of heat shock proteins (HSPs) (19, 23). Expression of HSPs is rapidly induced in an IEG fashion by the nuclear translocation of the heat shock transcription factor 1 upon posttranslational modification (60). The various functional aspects of HSPs on and in leukocytes include the protection from free radical induced DNA damage, induction of proinflamatory cytokine release, the direct activation of NK cells and a more general stimulation of adaptive, as well as innate immune responses. It is well known that these effects depend on intra- / extra-cellular localization, peptide loading status, origin and route of application (61).

HSPA1A (also termed HSP70, HSP70-1, HSP72, HSPA1 with alias) is known to be an IEG that is induced by heat and also by ischemia as another biophysical factor associated with exercise (13, 48). Elevated levels of extracellular HSPA1A, for instance, are found in plasma directly following exercise (21, 45). It has been implicated *in vivo* and *in vitro* that the ability of HSPA1A to activate the innate immune response by the release of NO and cytokines seemed to be improved in trained animals (6, 27). Interestingly, it has been shown that HSPA1A cooperates with heat shock factor 1 to repress expression of the immediate early gene c-fos; a central transcription factor for cell growth and differentiation (33). Repression of certain IEGs combined with activation of specific MAP-kinases therefore contributes to a stimuli specific IEG expression signa-

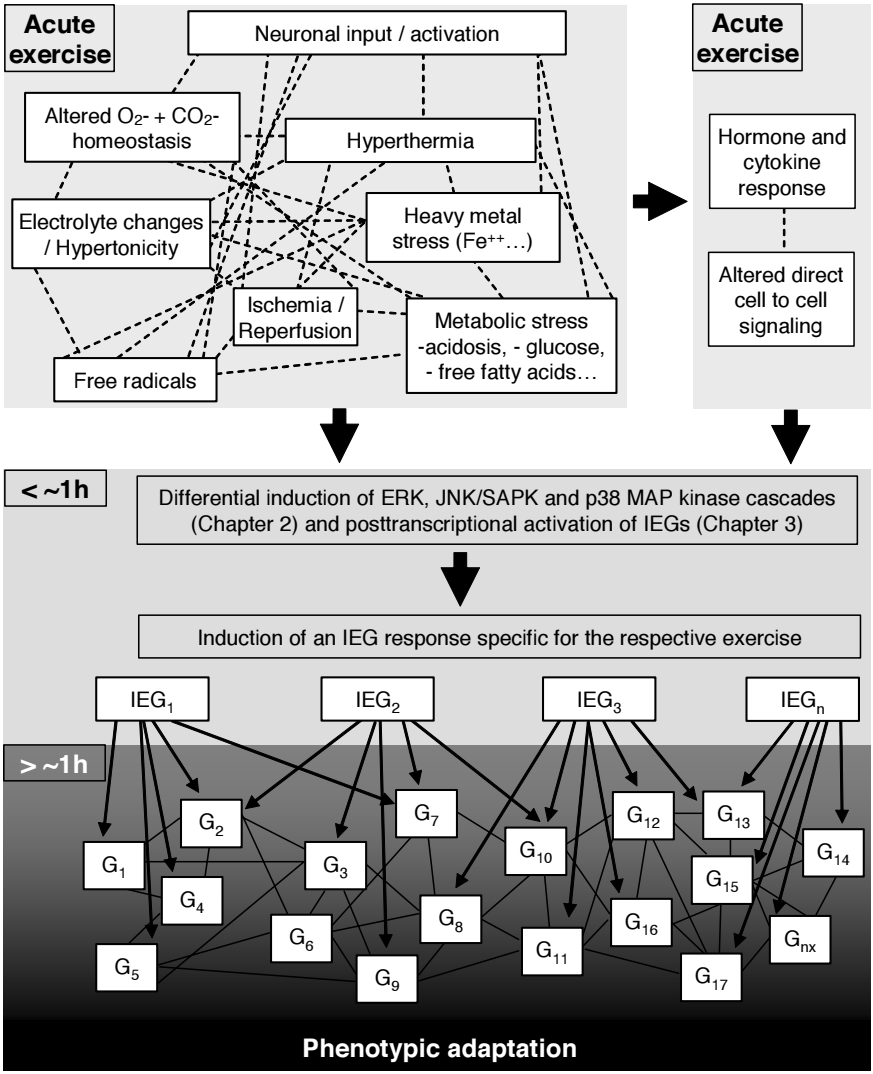


Fig.1: The upper part illustrates the main events upstream of the induction of IEG expression following exercise. Biophysical stimuli along with neuronal activation build up a complex interactive network (upper left) that generates a humoral response (upper right). The complex upper part leads to an induction of an IEG response specific for the respective exercise as described in chapters 2 and 3. The IEG response typically initialized 5min – 1h following exercise leads to the secondary induction of a more and more complex network of gene expression changes (G1-nx as arbitrary genes). As explained at the end of chapter 2, measuring gene expression at the level of the IEGs should enable us to get a limited number of gene expression signatures for a whole variety of different exercise regimes.

ture. As a consequence the complex transcriptional responses and the following protein neosynthesis are well adjusted to the intensity, duration and type of exercise (Fig.1 lower part).

Adaptation to increased temperatures *in vivo* is not readily distinguishable from the adaptation to mechanical forces that act directly on leukocytes, muscle cells, and endothelial cells during exercise. The process of mechanoperception is rather complex, since stretch-activated ion channels, caveolae, integrins, cadherins, growth factor receptors, myosin motors, cytoskeletal filaments, nuclei, extracellular matrix, and numerous other structures and signalling molecules have all been shown to contribute to the mechanotransduction response (37). Accordingly, there is a wide variety of IEGs that respond to mechanical force. Well characterized are for instance the induction of the IEGs c-fos, c-jun and PU1 in monocytes / macrophages (85) and the induction of Egr1 in endothelial cells (84). Especially the induction of Egr1 and c-fos is described for many different cell types and known to occur as quickly as 10 min following the stimulus (69).

Mechanical forces, in combination with heat and ischemia / reperfusion induce further biophysical stimuli, such as hyperosmosis and heavy metal stress (Fe^{++}). All of these changes are known to induce IEG expression via MAP-kinase signalling (63, 30). Hyperosmosis / hypertonicity induce expression of a variety of IEGs that carry a tonicity / osmotic response element (TonE / ORE) in their promoter region that is activated via MAP-kinase signalling (41, 77). HSPA1A is among the candidates that possess functional TonEs / OREs, along with other HSPs (34).

Another important biophysical stimulus related with exercise is the generation of free radicals (reviewed in 22 and 55). Free radicals activate the consensus sequence CC(A/T)6GG, known as a CArG box within the promoters of IEGs via serum response factor (16). Egr1 is a typical IEG well known to be activated through this pathway (16). Just recently, it has been shown that many genes of the immunoglobulin family have CArG-box elements, which provides an indirect link between free radicals and B-cell function (2).

In addition to the biophysical paths, there is a multitude of immediate hormonal and cytokine responses (reviewed in 71, 28, 72) as well as substrate and ion changes observed in the blood that add an additional level of complexity to the regulation of IEGs in leukocytes in response to exercise. The pathways leading to IEG expression again involve MAP-kinases and their action on respective enhancers and suppressors in the promoter regions of IEGs (53).

It should be pointed out that secretion of hormones and cytokines due to exercise can principally act as fast or even faster on leukocyte IEG expression than the biophysical stimuli itself. For both routes of IEG induction, there is a feature additional to MAP-kinase activation which is necessary for successful activation of an IEG. This is chromosomal remodelling necessary for the initiation of transcription (66, 73, 80). There is clear evidence of enhanced histone H3 and H4 acetylation, both at the promoters and within the body of genes upon transcriptional activation of IEGs (66).

If we summarize the upstream site to IEG expression in leukocytes due to physical activity, the following conclusions can be drawn (see Fig. 1 for illustration).

1. Many different biophysical and biochemical stimuli act together in order to induce IEG expression.
2. The initial stimuli acting on leukocytes to generate IEG expression are known to vary extensively depending on type, intensity and duration of exercise.
3. All stimuli act through the pathways of MAP-kinase activation in order to exhibit IEG expression.
4. Activation IEGs can be limited by a) the activation of other IEGs with direct or indirect repressor function as it is shown for HSPA1A and b) by the absence of a nucleosomal complex.
5. Every single IEG, once expressed, controls the expression of a variety of related late response genes.

Complexity of IEG response is limited by point 3 and 4. This leads to the situation that even though the upstream site to IEG expression, as indicated by points 1 and 2 and the downstream site (point 5), are rather complex there should be a much more simplified response on the IEG level (Fig.1). Various forms of physical exercise may all lead to a very limited number of different expression profiles on the IEG level. If this was the case, looking at IEG expression profiles following exercise would help us to categorize the different exercise regimes with regard to their effect on immune function.

First data about IEG expression in Leukocytes following exercise

Since many different biophysical and biochemical stimuli act together in order to induce IEG expression, data obtained by studying leukocyte expression following certain stimuli in vitro may be of very limited value for the field of exercise physiology. In addition, contact with foreign matter is a severe stimulus to leukocytes limiting the significance of in vitro studies. Approaches on the transcriptome level have already studied this effect (36).

The advantages of screening approaches on the transcriptome level for studying the effects of exercise on leukocytes *in vivo* have already been described and first results have been published (14, 25, 90). Comparison and interpretation of the results of such screening attempts may enable the deduction of specific transcriptomic signatures associated with certain exercise regimes (91).

Connolly et al. used Affymetrix HU133A GeneChips and investigated the expression differences in peripheral blood derived mononuclear cells (PBMCs) directly after and 1hour after a 30 min run at 80 % of the participants predefined VO_2max (14). This setting is particularly helpful to study IEG expression for two reasons. First, 30 min of exercise just enables the expression of IEGs and avoids differential expression of late response genes (see also Fig.1). Second, a typical IEG response is transient and would therefore rather be detected 30 min following the onset of exercise but not necessarily 1 hour after the end of exercise anymore. Therefore, this point in time helps to further categorize the IEG response found directly after exercise into a typical transient IEG expression and an atypical prolonged IEG expression. It has to be considered that the points in time chosen by

Connolly et al. are particularly suited for endurance exercise. More intense exercises and also weight lifting may require even shorter measuring intervals, since it is well known that many important IEGs show prominent gene expression changes as early as 10 min following the stimulus. In the case of endurance exercise, however, biophysical stimuli - such as heat – may take some time to reach a critical threshold to induce IEG expression. Since this delay might be specific for one or another biophysical or humoral stimulus, this situation may offer a chance to unravel the effect of certain stimuli on gene expression *in vivo*. Prerequisite is of course that studies are conducted with measuring intervals adjusted in this respect.

In the study by Connolly et al., Affymetrix HU133A GeneChips were used. Multiple single probe measurements are summarized on these chips to calculate a representative expression of a so called "probe set" that belongs to one mRNA. Quite often, more than one probe set belongs to one reference mRNA of a gene. 433 probe sets belonging to 337 different reference RNAs showed significant gene expression changes directly or 1 hour following exercise in the study of Connolly at all. Among these candidates 66 probe sets representing 55 different reference RNAs were altered more than 2-fold directly after 30 min of exercise (for > 3-fold change: 15 or 12 candidates respectively). All of these candidates showed a reduced level of expression 1 hour later and accordingly would meet the criteria of typical exercise induced IEGs in PBMCs. Among these candidates is HSPA1B encoding for a protein variant of HSP70 and HSPA1A as the classical candidate for the protein HSP70. HSPA1B showed a 1.96-fold increase 30 min following exercise.

Important for our first impression about exercise induced gene expression changes is the observation that 1 hour after exercise only 15 candidates showed a significantly increased expression ratio compared to the baseline level, which is just a fourth of the number seen directly following exercise. Given that we would principally expect an even higher number of early and late response genes being induced as a consequence of IEG expression, this finding needs to be explained. One reason could be the bias of the expression changes due to blood cell shifts. Connolly et al. showed that the ratio of lymphocytes to monocytes was on average 25 % elevated and 40 % decreased compared to baseline directly after and 1 hour after exercise. Expression of particular candidates varies between the various cell types among the PBMCs from not expressed to highly expressed and often shows a difference of more than a factor of 100 between cell types (56). Therefore the above mentioned cell shifts could principally be the cause of any of the significant gene expression changes observed regardless of the height of the respective fold-change ratio (10, 31).

A second study that investigated gene expression changes in PBMCs following exercise in a systematic way was done by Fehrenbach and Zieker et al. (25, 90). This study used a cDNA array centred on inflammation and looked for expression changes directly after and 24 hours after a half marathon competition. It could be shown that expression analysis of cell type specific surface molecules reflects the observed individual cellular shifts in peripheral blood cells with high statistical significance. Accordingly, Zieker et al. could focus their evaluation on those candidates which showed differential regulation that could not be explained by the cell shifts found. Directly following exercise an upregulation of MAPKAP-

K2, L-selectin and IL1-ra was reported to occur and this agrees with previous observations in exercise physiology studies based on different methods (90). Novel findings were a down-regulation of CD81 and GSTM3, as well as an up-regulation of thioredoxin. Since the average running time during this study was more than 1.5 hours, one cannot conclude whether these candidates can be considered as IEGs or as early response genes transcriptionally activated by the first IEGs.

A very interesting IEG candidate is MAPKAP-K2. So far, MAPKAP-K2 has only been described to be elevated in muscle cells following exercise (86). The increase in MAPKAP-K2 in PBMCs following exercise is of particular importance since it has been shown that this induces rapid induction of the protein expression of many inflammatory genes by posttranscriptional mechanisms. These will be discussed in the following section (47, 83).

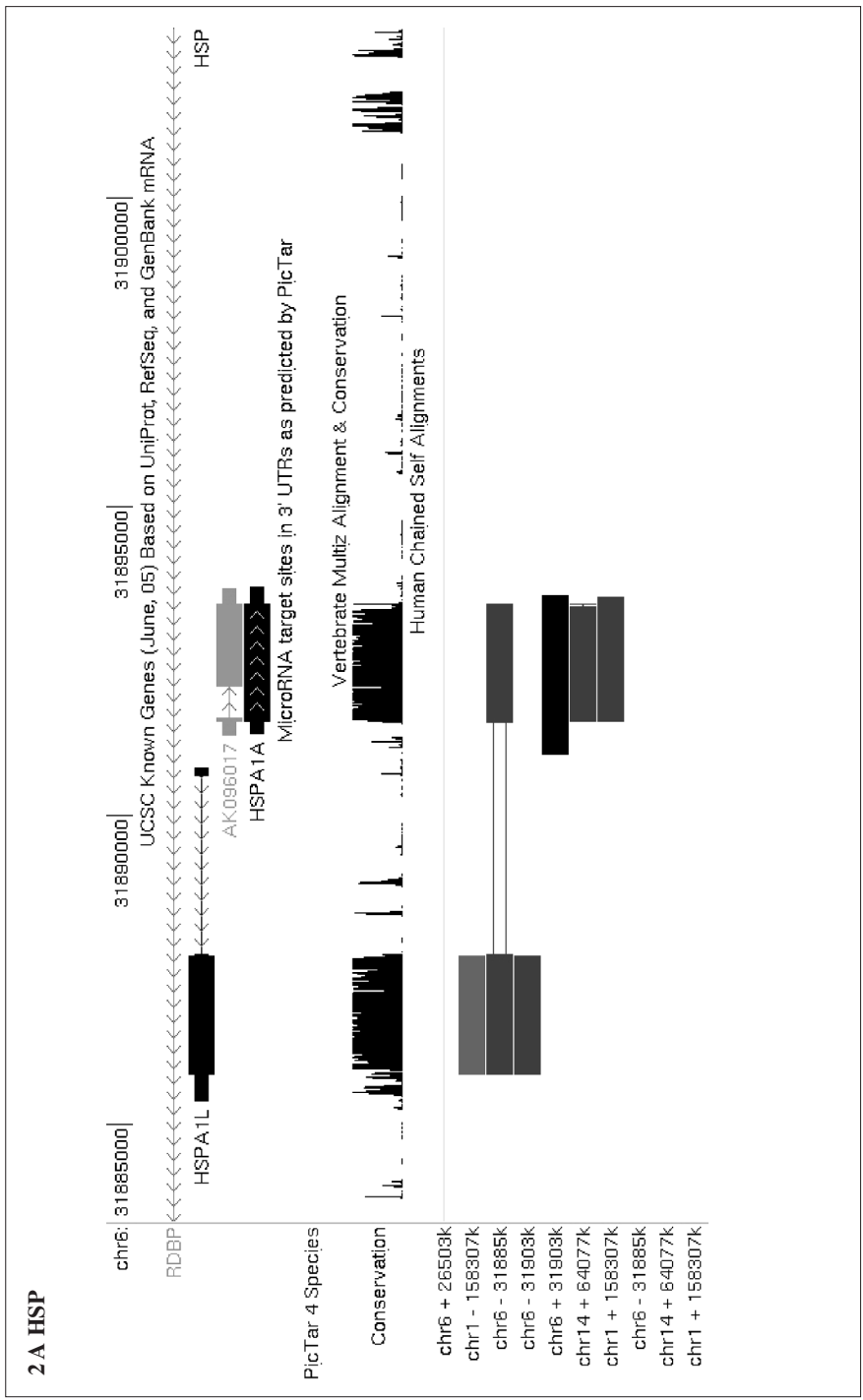
Future studies that aim at detecting IEG expression changes following exercise could combine the advantages of the two studies which used screening techniques so far. It would be informative to look at a timeframe between 30-40 min following onset of exercise and compare this to a second sampling point about 1-3 hours later. A classical IEG would show increased expression levels at the first point in time but already decreased values at the second point in time. It would also be important to control for the cell shifts occurring during the particular periods of exercise or recovery from exercise. Ideally one could integrate the just recently generated knowledge on the basal expression differences between the various cell types among the PBMCs in order to exclude that expression changes can be explained by cell shifts only (10, 31, 56).

3. New insights into the modulation of IEG expression: Pitfalls and potentials for future transcriptome oriented exercise immunology studies

The functional aspect of non-coding RNAs

This years Nobel prize in Physiology or Medicine was awarded for the discovery of RNA interference (RNAi) in 1998 (26). RNAi by the use of so-called micro RNAs (miRNAs) is one among many other mechanisms that act posttranscriptionally to control gene expression. miRNA belong to the class of non-coding RNAs (ncRNAs) that are not translated into proteins. Recently the magnitude of the expression of many different kinds of ncRNAs including miRNAs in the mammalian genome has been elucidated (9, 39). ncRNAs influence transcription, RNA editing including heterogeneous nuclear RNA (hnRNA) splicing, RNA transport, RNA stability, and RNA translation of many important protein coding genes (35, 52, 59, 81). Accordingly, ncRNAs can influence the expression level of many other coding RNAs and there is increasing evidence for their importance to modulate phenotypes (11, 35, 46, 76, 87).

Once, the intergenic spaces and introns were referred to as"genetic junk" and it is still true that only 1-2 percent of a mammalian genome contributes to exons of known genes that are expressed. However, there is evidence that ~60 % of the mouse genome is transcribed and that half of the roughly 181,000 independent transcripts represent ncRNAs (9, 39). 70 % of the mapped transcription


2 A HSP
chr6: 31885000
31890000
31895000
31900000
UCSC Known Genes (June, 05) Based on UniProt, RefSeq, and GenBank mRNA
RDBP
HSPA1L
AK096017
HSPA1A
HSP
MicroRNA target sites in 3' UTRs as predicted by PicTar
PicTar 4 Species
Vertebrate Multiz Alignment & Conservation
Conservation
Human Chained Self Alignments
chr6 + 26503k
chr1 - 158307k
chr6 - 31885k
chr6 - 31903k
chr6 + 31903k
chr14 + 64077k
chr1 + 158307k
chr6 - 31885k
chr14 + 64077k
chr1 + 158307k

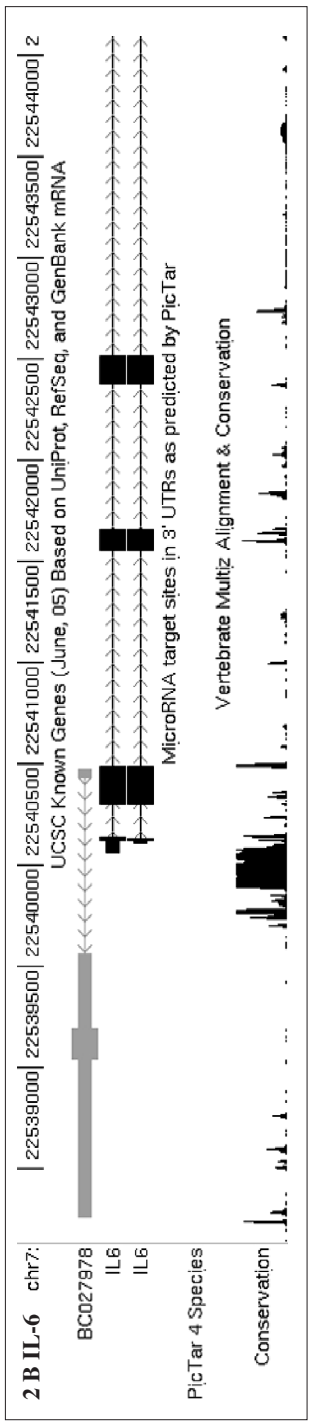


Fig. 2: The figure was generated by using the Genome Browser and its integrated bioinformatics software tools that are described in the following. It shows an alignment of four paralogs that code for HSPA1A (HSP70) protein variants to a locus on chromosome 6 (a) as well as an alignment of the two reference mRNA sequences to a locus on chromosome 7 (b). Lines represent introns, arrows are directed from 5' to 3' of the respective transcripts, thin boxes represent untranslated regions and thick boxes the coding sequence of a transcript. Target sites for miRNA were calculated using the PicTar algorithm and their localization is indicated as a thin line. In the"Conservation" diagram the maximum height of the line indicates complete phylogenetic conservation among 17 different vertebrate species. Below"Human Chained Self Alignments" the presence of the respective box indicates the existence of a complementary sequence to the respective box-locus at a different chromosomal location that is given on the left hand site. The darker the box, the higher is the sequence homology to the other chromosomal location. Further explanations are given in the text.

units seem to overlap to some extent with a transcript from the opposite strand (9, 39). This has led to the question "Transcription will never be simple again, but how complex will it get?"(11).

The complexity of the transcriptome is therefore another reason for concentrating on immediate early effects. It can be expected that the more time we wait following a stimulus the more complex the transcriptional response is going to appear (Fig. 1). In the next paragraphs we review how posttranscriptional regulation influences expression levels of IEGs.

Posttranscriptional regulation of IEG expression via recognition motives

An important issue influencing level of expression and detection of IEGs is the modification of RNA-stability and -translatability depending on stimulus-dependent posttranscriptional interactions at recognition motives located within an mRNA. Most of these recognition motives are located in the 3'- or to a less extent in the 5'-untranslated region (UTR) of an mRNA. Well characterized among these motives are cytoplasmic polyadenylation elements (CPEs) and Adenosin- and Uracil- rich elements (AREs). mRNAs that possess a CPE in their 3' UTR are polyadenylated in the

cytoplasm following stimulation, and the addition of the polyA tail leads to increased mRNA stability and translatability (5, 57). mRNAs with an ARE have been shown to be either stabilized or destabilized by binding of the protein HuR or AUF1 to the ARE, respectively (17, 58).

This situation is important for an understanding of gene expression regulation in exercise immunology, since many cytokines contain ARE elements in their 3' UTRs (4). The mRNAs of IL2, IL6, IL8, and TNFα all have been shown to be inherently unstable in unstimulated PBMCs due to the presence of AREs within their 3' UTRs. Following stimulation, IL6 and IL8 are stabilized by the activation of the MAPK-pathway. Therefore the MAPK-pathway is of central importance for the transcriptional, as well as post-transcriptional regulation of gene expression. Interestingly, MAPKAP-K2 has been shown to be effective to achieve the full stabilizing effect on IL6 and IL8 mRNA (83). Both cytokines are known to rise in the plasma following exercise (71). The discovery that MAPKAP-K2 is up-regulated in skeletal muscle and in PBMCs following exhaustive endurance exercise is therefore in line with a posttranscriptional regulation of IL6 and IL8 expression following exercise by MAPKAP-K2. In contrast to this situation the stabilization of IL2 and TNFα mRNA following T-cell activation is known to be independent of MAPKAP-K2 (82). Interestingly, these cytokine candidates are not reported to increase in plasma following exercise (71). Future studies may therefore elucidate the importance of MAPKAP-K2 expression and activity for mediating cytokine responses following exercise by taking posttranscriptional regulation of cytokines via AREs into account.

Posttranscriptional regulation and its impact on "detectable" IEG expression

Regulation of RNA expression via ncRNAs and recognition motives generally either stabilizes or destabilizes an mRNA that has already been transcribed. Both mechanisms are potential short cuts that modulate levels of a protein without requiring changes in basal transcriptional state. Mature and nearly mature mRNAs can be stored when and where they may be most needed for quick up-regulation of transcription.

In the following we will use information about posttranscriptional gene regulation and sequence alignment tools in order to illustrate the chances as well as the difficulties to detect differential regulation of IEGs by looking at HSPA1A and IL6 as an example.

Figure 2 illustrates prominent bioinformatic features of these two candidates that are well known to play a central role in exercise immunology. Concerning the phylogenetic conservation HSPA1A is a typical mRNA. Conservation across 17 species is very high for all the protein coding regions (thick boxes) ("Conservation" line in figure 2a), while the 5' UTR and 3' UTR (thin boxes) are not well conserved. IL6 shows the opposite pattern. Here the promoter region and especially the 3' UTR are highly conserved. How could it be that IL6 shows a much higher level of conservation in its UTRs than in the protein coding region, given that a high level of conservation implies functional relevance? One explanation is that the genetic code is redundant and therefore the IL6 proteins show a much higher level of conservation across species than

the respective coding sequences. In contrast, the recognition motives in UTRs have to be almost 100% conserved in order to maintain functionality across species.

Even though four HuR binding ARE-motives are located in the 3' UTR of IL6, the high level of conservation for almost the entire 500 bp 3' UTR can not be explained by the roughly 68–80 bp that are important for the four functional AREs (44). A more likely scenario is that there are additional recognition motives within this 3' UTR. The PicTar 4 Species track in Fig 2b uses a recently developed miRNA target site detection algorithm and highlights target sites for the miRNAs let-7, mir-26, mir-98, and mir-149 in the 3' UTR of IL6 (29, 42). Accordingly, targeted destruction of IL6 mRNA by miRNAs could be an additional way to suppress IL6 translation in the presence of ongoing transcription. This would enable almost immediate translation after stimulation.

Last but not least, alignment of reference mRNA sequences using the UCSC Genome Browser (40) reveals a cis natural antisense transcript (cis-NAT) for IL6 with a 5' UTR overlap. CisNATs are antisense transcripts derived from transcription of the opposite DNA strand that overlap the sense transcript. Note that the promoter region of this antisense transcript is again highly conserved. Although the IL6 cis-NAT is listed in the recently released cis-NAT database, its functional or regulatory significance has not yet been investigated (89).

In contrast to IL6, where we already know that posttranscriptional regulation is a major factor contributing to its mRNA expression, the four mRNAs that code for paralogs of the HSPA1A gene located in proximity to each other on chromosome 6 do not show evidence for regulation by miRNA or recognition motives. Their promoter regions and UTRs are poorly conserved. Instead they are very short genes with 1-2 exons only. As shown in Figure 2a below"Human Chained Self Alignments" there are even more paralogs located in different chromosomal positions that are highly similar and do all code for HSP70 protein variants. This leads to the situation in which a protein can be generated quickly after stimulation by 1. transcriptional activation of two or more closely related HSPA1A genes or 2. by fast transcription of a small primary transcript (hnRNA). Interestingly, both HSPA1A coding gene and a number of other small genes are located within an intron of the RD RNA binding protein (RDBP). RDBP is known to be part of a multi-protein complex that negatively regulates the transcription of IEGs (89). It is possible that concomitant expression of RDBP and the intronic HSP70 variants occurs. If this is the case, it would lead to the situation that once the RDBP protein (long hnRNA) is translated, further transcription of HSP70 mRNA variants would be attenuated (1). As a consequence we would have a negative feedback loop, possibly important in preventing excessive production of HSP70 in those situations – such as during a marathon - where body temperature increases and is elevated for more than two hours. This leads to the prediction that the known down-regulation of HSP70 by regular endurance exercise (20, 24) may at least partially be mediated through elevated levels of RGDB.

Taken together IL6 mRNA and the HSP70 coding mRNAs appear to be two very different types of mRNAs known to be altered by exercise. While for

IL6 many different posttranscriptional mechanisms may act together to achieve expression in an IEG manner, the HSPA1A paralogs coding for HSP70 protein variants provide evidence for transcriptional regulation. Importantly, the diversity of transcriptional and posttranscriptional regulatory features shown for these two mRNAs has an impact on the detlectability of these candidates by different transcriptomic approaches and subsequent real-time RT-PCR.

The HSPA1A paralogs impose one main problem: Which of the paralogs or how many of them with what different kind of sensitivity for each one of them are we measuring with our procedure? When we apply real-time RT-PCR we could use primers that prime all of the different variants, specific primers that prime only one variant, or we could neglect this issue and just choose any kind of primer within one of the transcripts by chance. The estimates of expression will differ substantially. When we use short oligonucleotide microarrays for expression analysis like Connolly et al. (14) a similar problem may occur. These chips use so called mis-match probes, where just one base is exchanged compared to the perfect match probe. Mis-matches to one variant may accidentally be a good match for another HSP70 variant. Accordingly, the standard quantification software, MAS5, which takes match to mis-match detection ratios into account to calculate expression values, may end up with results that are relatively noisy in situations where a lot of paralogs are expressed.

It has already been shown that using the match signals only by applying the quantification procedure PDNN reduces measuring noise (88). Depending on the method used for data processing (MAS5, dChip PMMM, dChip PM, RMA, GC-RMA and PDNN) different genes will be identified as differentially regulated. In a recent study, there was only 27 to 36 % overlap of differentially regulated transcripts if the same raw data were processed by the different quantification procedures and differential regulation of most of the candidates could not be verified by real-time RT-PCR (49). In case cDNA microarrays are used for quantification the data processing procedure is not problematic, since there is in most cases only one probe to look at. Additionally, in the study of Fehrenbach and colleagues most of the candidates could be verified by real-time RT-PCR (25, 90). However, there is no evidence that the proportion of verifiable differentially regulated candidates is significantly higher in cDNA microarray studies than in studies using short oligonucleotide microarrays. Also a reduced false positive rate may come along with a severely reduced sensitivity.

Two questions arise when we consider the differential expression of IL6. 1. It has to be taken into account that there is a second reference mRNA for IL6 that has neither the known AREs nor the miRNA binding sites (Fig. 2b). It may well be that these two variants show differential regulation and all of the different quantification procedures may want to take this circumstance into account. 2. What happens with the detection ratio on a microarray or in a real-time PCR assay in those cases in which antisense sequences or miRNAs to the transcript are present? So far this issue has not yet been investigated to our knowledge. It must be considered that antisense or miRNAs could attenuate the binding of the target sequence to microarray probes and that standard RT-PCR can not differentiate whether a sense or an antisense sequence is amplified.

In addition to these pitfalls which apply to HSP70 or IL6 mRNA detec-

tion another problem is the regulation of translation by the polyA tail length of an mRNA or in principle, the polyA tail length difference between transcripts at all. Short oligonucleotide microarrays use T7 polymerase protocols to process and amplify the mRNA. The efficiency of this protocol may depend on the length of the polyA tail of the transcript. On the one hand only polyA positive RNA should be detectable with this method (64). On the other hand, 18s rRNA as a well known poly A negative RNA is used as a housekeeping gene by Affymetrix on various microarrays and expression values generally exceed those of most of the other transcripts. Therefore the influence of the polyA tail length on the expression level of a transcript determined by short oligonucleotide microarrays is not yet clear.

Conclusion

Different biophysical and humoral responses following exercise use common pathways to induce IEG expression profiles as described in Chapters 2 and 3 and illustrated in Figure 1. While the downstream cascades that follow IEG expression are highly complex interactive networks, IEG expression itself may only show limited variation in response patterns. Focusing studies on a level where we expect relatively low complexity is appropriate at the current stage of the technical procedures to study the transcriptome. An additional argument for focusing at the IEG level and therefore at the first line of transcriptional response is the increasing evidence for a much bigger size of the transcriptome and its fascinating interactivity. Future gene expression studies at the IEG level in exercise immunology may also contribute to unravel this interactivity and they will certainly help to bridge the gap between exercise and the induced phenotypic adaptation.

List of Abbreviations:

ARE	Adenosine- and Uracil-rich element
CArG box	CC(A/T)6GG box
cis-NAT	in cis natural antisense transcript
CPE	cytoplasmic polyadenylation element
eHSP	extracellular heat shock protein
hnRNA	heterogeneous nuclear RNA
HSP	heat shock protein
IEG	immediate early gene
miRNA	microRNA
ncRNA	non-coding RNA
PBMC	peripheral blood derived mononuclear cell
RNAi	RNA interference
TonE / ORE	tonicity / osmotic response element
UTR	untranslated region

List of genes mentioned:

AUF1 / HNRD	Heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA-binding protein 1, 37kD)
CD81	Cluster of differentiation 81
c-fos	FBJ murine osteosarcoma viral oncogene homolog
c-jun	Jun oncogene
EGR1	Early growth response gene 1
ERK	Elk-related tyrosine kinase
GSTM3	Glutathione S-transferase M3
HSPA1A	Heat shock 70kDa protein 1A
HSPA1B	Heat shock 70kDa protein 1B
HUR/ELAVL1	Hu antigen R; embryonic lethal, abnormal vision, drosophila, homolog-like 1
IL1-ra/IL1RN	Interleukin 1 receptor antagonist
IL2	Interleukin 2
IL6	Interleukin 6
IL8	Interleukin 8
JNK / MAPK8	c-Jun N-terminal kinase 1; mitogen-activated protein kinase 8
MAPKAP-K2	Mitogen-activated protein kinase-activated protein kinase 2
p38 / MAPK14	Mitogen-activated protein kinase 14; p38 mitogen activated protein kinase
PU1 / SPI1	spleen focus forming virus (SFFV) proviral; PU-box binding transcription factor 1
RDBP	RD RNA-binding protein
SAPK / MAPK9	stress-activated protein kinase
TMFα	Tumor necrosis factor α

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