Multiplex Quantification Identifies Novel Exercise-regulated Myokines/Cytokines in Plasma and in Glycolytic and Oxidative Skeletal Muscle*⑤

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Exercise is known to confer major health benefits, but the underlying mechanisms are not well understood. The systemic effects of exercise on multi-organ systems are thought to be partly because of myokines/cytokines secreted by skeletal muscle. The extent to which exercise alters cytokine expression and secretion in different muscle fiber types has not been systematically examined. Here, we assessed changes in 66 mouse cytokines in serum, and in glycolytic (plantaris) and oxidative (soleus) muscles, in response to sprint, endurance, or chronic wheel running. Both acute and short-term exercise significantly altered a large fraction of cytokines in both serum and muscle, twenty-three of which are considered novel exercise-regulated myokines. Most of the secreted cytokine receptors profiled were also altered by physical activity, suggesting an exercise-regulated mechanism that modulates the generation of soluble receptors found in circulation. A greater overlap in cytokine profile was seen between endurance and chronic wheel running. Between fiber types, both acute and chronic exercise induced significantly more cytokine changes in oxidative compared with glycolytic muscle. Further, changes in a subset of circulating cytokines were not matched by their changes in muscle, but instead reflected altered expression in liver and adipose tissues. Last, exercise-induced changes in cytokine mRNA and protein were only minimally correlated in soleus and plantaris. In sum, our results indicate that exercise regulates many cytokines whose pleiotropic actions may be linked to positive health outcomes. These data provide a framework to further understand potential crosstalk between skeletal muscle and other organ compartments. *Molecular & Cellular Proteomics 17: 1546–1563, 2018. DOI: 10.1074/mcp.RA118.000794.*

Physical inactivity is a major risk factor contributing to cardio-metabolic disorders in modern society (1, 2). Conversely, regular exercise confers systemic health benefits (3–6). The underlying mechanisms responsible for the positive physiological effects of exercise, however, are not well understood (7, 8). The systemic effects of exercise on multi-organ systems has long thought to be, at least in part, because of myokines and cytokines secreted by skeletal muscle in response to exercise (9–11). These exercise-induced myokines are believed to mediate crosstalk between skeletal muscle and other organ compartments. Depending on the method used and biological contexts, proteomics-based analyses of muscle "secretome" have identified hundreds of proteins secreted by muscle cells (12–19). The functions for most of these muscle-derived secretory proteins are poorly defined, and it is unclear whether their expression and secretion are regulated by muscle contraction *in vivo*. Importantly, biologically potent molecules, such as cytokines, that normally exist at low levels (typically in the ng/ml and pg/ml range) are generally not detected by current proteomics methods.

The cytokine interleukin (IL)[1]-6 is one of the first myokines shown to be actively secreted by contracting skeletal muscle in human (20, 21). Since its discovery, other cytokines such as IL-7 (22), IL-15 (23), leukemia inhibitory factor (LIF) (24), and vascular endothelial growth factor (VEGF) (25) have also been shown to be functionally relevant exercise-induced myokines, either acting in an autocrine/paracrine manner within muscle or acting via an endocrine fashion to affect biological processes in non-muscle tissues. Recent *in vitro* studies using electrical pulse stimulation of primary human myotubes and antibody-based detection approaches also identified significant numbers of contraction-induced myokines/cytokines (26, 27). Although these *in vitro* studies allow the unequivocal

identification of *bona fide* muscle cell-derived myokines, limitations to these cell models have also been noted. Although differentiated primary myotubes in culture disk serve as a reasonable proxy for mature muscle cells, they do not capture the three-dimensional muscle fibers in the context of other potentially important accessory cell types (*e.g.* vascular endothelial cells, satellite cells, immune cells, and sympathetic nerve cells), nor do the cultured myotubes fully resemble the metabolically distinct glycolytic and oxidative fiber types. Further, electrical pulse stimulation of myotubes does not fully recapitulate both the stimulatory and inhibitory pathways induced by exercising skeletal muscle (28). As such, *in vivo* studies in humans or animal models will complement and extend the *in vitro* studies, enabling a more complete understanding of skeletal muscle's response to exercise.

Cytokines, chemokines, and secreted cytokine receptors constitute a large family of secretory proteins with pleiotropic immune and non-immune functions (29–33). As such, changes in local and systemic levels of these molecules in response to exercise could potentially account, at least in part, for the positive effects of physical activities. The present studies aimed to address three related questions. First, although a handful of cytokines has thus far been shown to be secreted by exercising muscle *in vivo*, the extent to which different types of physical activity (sprint, endurance, voluntary wheel running) regulates cytokine expression and secretion in glycolytic *versus* oxidative muscle fibers has not been systematically examined. Most human exercise studies examined transcriptional and protein changes in vastus lateralis, a quadriceps muscle with mixed fiber types. The metabolism of *in vitro* differentiated primary human myotubes also tended to be glycolytic rather than oxidative. Thus, the use of mouse exercise models allows us to address questions that are difficult to study in humans and primary cell culture system. Second, it is generally assumed that changes in serum cytokine in response to exercise are directly related to changes in their expression and secretion in skeletal muscle. It remains unknown to what extent the changes in plasma cytokine levels is because of secondary adaptive changes in their expression and secretion in non-muscle tissues in response to exercise. This could potentially occur if the primary exercise-induced cytokines in turn stimulate the expression and secretion of secondary cytokines in other non-muscle tissues.

Thirdly, several transcriptomics studies have been carried out to look at changes in human muscle gene expression in response to exercise (34–37). Whether changes in mRNA level translate into changes in protein level in human skeletal muscle are often not examined, in large part limited by the availability of protein-specific antibodies and the cost of validating many candidate proteins.

With the use of the conventional ELISA method, it is not feasible to simultaneously measure the levels of large numbers of cytokines in a mouse because of limited blood volume that could be sampled. Technological advancement in recent years has made it feasible now to simultaneously quantify many cytokines in serum and tissues using only small volume of blood or quantity of tissue. Using a bead-based multiplex profiling method, we seek to measure cytokine levels in mice in response to an acute sprint or endurance run, or a short-term access to a running wheel for 1 week (representing chronic exercise). Here, we examined a total of 66 known cytokines (including secreted cytokine receptors and acute phase proteins) that represent a well-studied set of molecules with diverse functions and activities. These cytokines act on diverse cell types to modulate a variety of biological processes that include inflammation, chemotaxis, apoptosis, metabolism, angiogenesis, and cell proliferation (38). Given the pleiotropic systemic effects of exercise, we hypothesized that acute and chronic exercise would dynamically alter cytokine levels in circulation and in different muscle fiber types (*e.g.* oxidative and glycolytic). Indeed, our results show that exercise modulates the expression of a large number of cytokines in different muscle fiber types depending on the type of physical activity (sprint, endurance, or chronic wheel running). Many of these cytokines are considered novel exercise-regulated myokines. Concordance between mRNA and protein level was observed for only a small subset of cytokines. Further, we showed that a subset of circulating cytokines is likely induced and secreted in non-muscle tissues in response to exercise, highlighting the primary skeletal muscle response to exercise and the secondary adaptive response in non-muscle tissues.

EXPERIMENTAL PROCEDURES

Animals—Eight-week-old C57BL/6J male mice were purchased from The Jackson Labs. All mice were housed in polycarbonate cages under a 12:12-h light-dark photocycle (6 AM - 6 PM) and had access to food and water *ad libitum* throughout the study period. Mice were allowed to acclimate for 1 week before experimentation. All animal experiments were approved by the Animal Care and Use Committee of The Johns Hopkins University School of Medicine.

Mice were randomly divided into 4 groups of 10 mice for the following control or exercise paradigms: sedentary, sprint exercise, endurance exercise, and voluntary exercise. At 9 weeks of age, blood was collected from the tail vein for pre-exercise measurements from all mice, including the sedentary group. Although serum samples from the sedentary group were not used for analysis, blood was still collected from this group to normalize treatment and handling among all groups. Samples were collected at the same time of day for all

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[1] The abbreviations used are: IL, Interleukin; CCL, Chemokine (C-C motif) ligand; CXCL, Chemokine (C-X-C motif) ligand; CX3CL, Chemokine (C-X3-C motif) ligand; EPO, Erythropoietin; G-CSF, Granulocyte colony-stimulating factor; GM-CSF, Granulocyte macrophage colony-stimulating factor; IFN-γ, Interferon-γ; LIF, Leukemia inhibitory factor; M-CSF, Macrophage colony-stimulating factor; MCP, Monocyte chemoattractant protein; MIP, Macrophage inflammatory protein; TIMP, Tissue inhibitor of metalloproteinase; TNF-α, Tumor necrosis factor alpha; TNF-R, Tumor necrosis factor receptor; sRAGE, Soluble receptor for advanced glycation end products; VEGF, Vascular endothelial growth factor.

Body weight, total run time and distance, and pre- and postexercise blood glucose and lactate levels. All values shown as average $\pm$ S.E. n = 10 mice per group

	Body weight (g)	Pre-exercise Glucose (mg/dL)	Pre-exercise Lactate (mM)	Total run Time (min)	Total run distance (m)	Post-exercise Glucose (mg/dL)	Post-exercise Lactate (mM)
Sprint	25.7 $\pm$ 0.6	186 $\pm$ 6	2.0 $\pm$ 0.3	11 $\pm$ 1	156 $\pm$ 20	199 $\pm$ 9	11.0 $\pm$ 0.6
Endurance run	26.6 $\pm$ 0.6	176 $\pm$ 5	3.0 $\pm$ 0.4	72 $\pm$ 5	912 $\pm$ 85	210 $\pm$ 6	5.5 $\pm$ 0.3
Voluntary wheel running	25.7 $\pm$ 0.5	187 $\pm$ 6	–				
Sedentary	25.3 $\pm$ 0.4	193 $\pm$ 6	2.3 $\pm$ 0.2				

mice (10:30 AM). Serum was separated using Microvette® CB 300 LH (The SARSTEDT Group, Numbrecht, Germany), and separated serum was stored at −80 °C until analysis.

For sprint and endurance exercise conditions, mice were acclimatized to the treadmill (Exer 3/6 treadmill; Columbus Instruments, Columbus, OH) for 3 days before the exercise test as follows: day 1: mice were placed on the stationary treadmill for 10 min (with the shock grid on); day 2: mice were run on the treadmill at a speed of 5 m/min for 10 min at a 10° incline; day 3: mice were run on the treadmill at 10 m/min for 10 min at a 10° incline. A shock grid at the back of the treadmill encouraged mice to continue running by administering a mild shock each time they stepped off the belt. The acute exercise tests were performed on the fourth day when the mice were 10 weeks of age.

Sprint exercise: Baseline blood glucose and lactate levels were measured with glucose and lactate meters (Nova Biomedical, Waltham, MA) from a drop of blood obtained through the tail vein. Mice were run on the treadmill set to a fixed incline of 15° until exhaustion using the following speed protocol: 5 m/min for 1 min, 10 m/min for 2 min, then 2 min intervals of the following speeds (m/min): 10, 14, 18, 22, 26, 28, 30, 32, 34, 36, 38, 40. Mice were run in multiple groups beginning at 9:30 AM. A mouse was considered exhausted when it remained on the shock grid for 5 continuous seconds and, after being blown by one puff of air at 5 s, still didn't move back to treadmill within 2 s of the air puff. Immediately after finishing the run, blood glucose and lactate levels were measured (Table I). Within 5 min of finishing the run, mice were anesthetized with isoflurane, euthanized with cervical dislocation, and the following tissues were collected: blood from the retro-orbital sinus via heparin-coated capillary tubes (Fisher 22-362-566, Waltham, MA), soleus, plantaris, and gastrocnemius muscles, visceral (epididymal) and subcutaneous (inguinal) white adipose tissue, and liver. Separated serum and tissues were flash frozen in liquid nitrogen, and all samples were stored at −80 °C until analysis.

Endurance exercise: Baseline blood glucose and lactate levels were measured and then mice were run on the treadmill at a fixed incline of 10° until exhaustion using the following speed protocol: 10 m/min for 30 min, then an increase of 2 m/min every 15 min thereafter. Mice were run in multiple groups beginning at 9:30 AM. Mice were considered exhausted using the same criteria as detailed above. After measuring post-run glucose and lactate values (Table I), mice were euthanized within 5 min of finishing the run and tissues collected in the same manner as described above.

Voluntary exercise: At 10 weeks of age, mice were separated into individual cages and a running wheel (Columbus Instrument) was placed in each cage such that mice had free access to it. This represents a form of chronic exercise. After 1 week, mice were sacrificed, and tissues were collected as described above. Running wheels were left in the cages until the time of sacrifice (10:30 AM).

Sedentary control: At 10 weeks of age, mice that are normally housed in the cage (with no running wheel) were sacrificed and tissues were collected as described above. Mice were sacrificed at 10:00 AM.

Throughout the study, all mice were treated in the exact same manner, except for the exercise itself, to facilitate accurate analysis by minimizing any potential confounding factors.

Muscle Lysate Preparation—One plantaris and one soleus muscle from each mouse was used. Muscles were lysed in 500 μl PBS supplemented with protease inhibitor mixture (Sigma, St. Louis, MO). First, muscles were homogenized with a 1 ml glass dounce homogenizer (Wheaton, Millville, NJ), using the loose pestle followed by the tight pestle. Subsequently, the suspension was subjected to sonication to complete the tissue lysis (Branson Sonifier 150; 5–10 s at the lowest setting). Homogenates were centrifuged at 12,000 $\times$ g for 20 min at 4 °C and the supernatant was transferred to a new tube. Total protein content in the supernatant was quantified with a Bradford protein assay (Sigma), and the cleared lysate was stored at −80 °C until analysis.

Multiplex Assays for Measuring Cytokine Protein Level—Multiplex assays for 66 mouse cytokines (covering most of the well-studied cytokines) were performed using a Luminex bead-based multiplex system according to manufacturer's protocol (EMD Millipore, Billerica, MA). This method is sensitive and reproducible (39, 40) and has been validated in multiple studies (27, 41). The following 4 assay kits (covering 66 analytes according to their dynamic range) from Millipore were used to measure the concentrations of cytokines in mouse serum, soleus homogenate, and plantaris homogenate: MCYTO-MAG-70K, MECY2MAG-73K, MSCRMAG-42K, and MTH17MAG-47K. IFN-γ protein levels in soleus and plantaris were measured with the kit MCYTOMAG-70K. However, IFN-γ levels in serum were measured using a more sensitive assay kit MECY2MAG-73K. Many cytokine receptors are known to be proteolytic cleaved and the soluble ligand-binding ectodomain circulates in plasma (42). Thus, secreted sCD30, sIL-1RI, sIL-1RII, sIL-2Ra, sIL-4R, sIL-6R, sTNFRI, sTNFRII, sVEGFR1, sVEGFR2, sVEGFR3, sgp130, and sRAGE were also measured as part of the 66 cytokines profiled. The experiments were set up according to the manufacturer's protocol and the assays were performed as we have previously described (43). In brief, standards were provided for each mouse cytokine, from which standard curves were generated. Serum was diluted according to the manufacturer's protocol and cleared muscle lysates were assayed undiluted. For soleus lysates, an average of 18.05 $\pm$ 6.79 pg protein was added per well, and for plantaris lysates, an average of 38.84 $\pm$ 10.43 pg protein was added per well. All samples (n = 10 mice per group) and standards were analyzed using a Luminex 200 instrument (Luminex, Austin, TX) and XPonent 3.1 Software (Millipore, Billerica, MA). Concentrations were determined for each of the 66 mouse cytokines relative to an appropriate 6-point regression standard curve in which the mean fluorescence for each cytokine standard was transformed into known concentrations (pg/ml or ng/ml). For muscle lysates, cytokine concentrations were normalized to total protein before further analysis. The multiplex assays were carried out on three separate occasions, each time with different samples: (1) pre- and post-exercise serum from sprint and endurance mice, (2) pre- and post-exercise serum from voluntary running mice, and (3) all muscle lysates from all 4

exercise regimes. All samples that were used for direct comparison or normalization were run on the same plate.

QuantiGene Plex Assay (QGP) for Measuring Cytokine mRNA Level—QuantiGene Plex assays enable simultaneous quantification of up to 80 genes of interest in a single well of a 96-well plate. This method is hybridization-based and incorporates branched DNA (bDNA) technology (44, 45). Because it relies on signal amplification rather than target amplification for direct measurement of RNA transcripts, no RNA isolation and reverse transcription is needed. QuantiGene assay has comparable or better linear range and relative accuracy when compared with standard real-time PCR method (46). All Gene-specific QuantiGene probes were validated and provided by Thermo Fisher Scientific. Soleus and plantaris muscle fibers harvested from mice were weighed, and placed on dry ice until ready to process. Five to fifteen milligrams of tissue was used in sample preparation. Tissues were lysed in 300 μl of homogenizing solution (QGP Assay Kit, Thermo Fisher Scientific, Waltham, MA) per 10 mg tissue, using an MP24 fastprep (MP Biomedicals, Santa Ana, CA) with MP lysing matrix A tubes (Cat #6910–100, MP Biomedicals). Tissue homogenates were heated at 65 °C for 1 h, with vortexing every 10 min. Heated samples were centrifuged at room temperature for 15 min at 16,000 $\times$ *g* to sediment the matrix, and the supernatant was transferred to clean microcentrifuge tubes. The supernatant was clarified by repeating the above centrifugation step, and the resultant supernatant transferred to another clean microcentrifuge tube. Supernatants were added directly to the QGP hybridization plate following the addition of master mix prepared according to manufacturer's instructions. The hybridization plate was incubated overnight at 57 °C with shaking at 600 rpm. The next day, the plate was processed according to manufacturer's instructions, and read on a Luminex 200 instrument (Luminex, Austin, TX). Data was analyzed and fold change calculated according to manufacturer's instructions, using *Hprt* and *Actb* as reference genes. Statistical analyses were performed using a one-way ANOVA with Dunnett's post-test with the sedentary group as the control.

Quantitative Real-time PCR Analysis—Total RNA was isolated from liver, visceral (epididymal) and subcutaneous (inguinal) white adipose tissues using Trizol® (Life Technologies, Carlsbad, CA). Random primers and GoScript reverse transcriptase (Promega) were used to generate cDNA. Quantitative real-time PCR analyses were performed on CFX Connect Real-Time system (Bio-Rad, Hercules, CA) using iTaq Universal SYBR Green Supermix (Bio-Rad). The data were normalized to 36B4 (also known as acidic ribosomal phosphoprotein P0 (RPLP0)) to generate a ΔCt value, and then a ΔΔCt was obtained by normalizing the data to the mean ΔCt of the control group (47). 36B4 gene was selected for normalization because it had the least variation across tissue samples. All primers used for real-time PCR analyses are listed in supplemental Table S1.

Confirmatory Experiment with a Second Cohort of Mice—A second independent cohort of endurance and sedentary mice was used to confirm some of the findings obtained from the multiplex assays. As before, 8-week-old C57BL/6J mice were purchased from Jackson Labs for the experiments with *n* = 10 per group. However, data for the endurance group reflects *n* = 6 because 2 mice refused to run despite being adequately acclimated to the treadmill and 2 mice were running when the treadmill experienced technical issues so had to be taken out of the study. The experiments were conducted exactly as described previously except for timing of the pre-exercise blood draws. Given that a large volume of serum was needed to satisfy the volume requirements for multiple ELISAs, blood was drawn on two separate occasions before the exercise experiment: once 3 weeks before the endurance run and once 2 weeks before the run. Serum from both blood draws was combined before it was assayed. Mice were allowed to recover for two full weeks after the last pre-exercise blood draw as

opposed to the 1 week of recovery with the first cohort. Thus, the mice in this second cohort were ~2 weeks older and a few grams heavier than their counterparts in the first cohort on the day of the experiment. Otherwise, all data including time to exhaustion, blood glucose, and blood lactate levels matched very well between the two cohorts.

Soleus and plantaris lysates were prepared as described except both muscles were used, and the volume of buffer was reduced to 350 μl. These changes were implemented to increase the signal in the ELISAs.

The following cytokines were measured via ELISA in the pre- and post-exercise serum from the endurance group and soleus and plantaris lysates from all endurance and sedentary mice: MCP-5, KC, MIP-2, MIP-3, IFNγ, and sTNFRI. ELISAs for all targets were purchased from R&D Systems except for sTNFRI, which was purchased from Thermo Scientific. Assays were carried out according to the manufacturer's protocol. Muscle lysates were assayed undiluted, and cytokine concentrations in these samples were first normalized to total protein before further analysis.

Data analysis—For a given analyte, not all samples were successfully detected in the multiplex analysis. Data for a given analyte was analyzed when analyte levels were detected in at least 3 out of the 10 samples. To enable paired *t* test analysis (where N $\geq$ 3), serum analyte levels must be detected in both pre- and post-exercise samples from the same mouse. It should be noted that the majority (~90%) of statistically significant data were obtained from sample size *n* = 9–10. For statistical analysis, a paired *t* test was used to analyze pre-exercise *versus* post-exercise serum data. The nonparametric Wilcoxon matched pairs test was used to analyze data in which the differences (post minus pre) were not Gaussian. Lysate data were analyzed by 1-way-ANOVA. The means from each exercise condition were compared with that from the sedentary condition using Dunnett's post-test. $p < 0.05$ was considered significant. All data is reported as mean $\pm$ S.E. Data was analyzed using Graph Pad Prism 7 software. For each data set, false discovery rate analysis was also performed with GraphPad Prism 7 using the recommended two-stage step-up method of Benjamini, Krieger and Yekutieli. The false discovery rate (Q) was set to 5%.

RESULTS

Three exercise paradigms—sprint, endurance exercise, and voluntary wheel running—representing acute and chronic forms of physical activity, were used in the present study to assess changes in circulating serum cytokines, as well as their protein levels in glycolytic (plantaris) and oxidative (soleus) muscles. A bead-based multiplex profiling method was used to quantify 66 cytokines in both serum and muscle lysates. We could reliably detect and measure up to 52 cytokines from among the 66 cytokines profiled (Tables II and III). This is not surprising given that some cytokines are expressed at very low levels and are only up-regulated in the context of immune activation in response to infection. Remarkably, a large fraction of the cytokines detected were significantly altered by acute and chronic exercise (Tables II and III). When adjusted for false discovery, most of the significant changes remain (supplemental Table S2 and S3). Using a second independent cohort of mice subjected to an endurance run, we confirmed the validity of the multiplex assay using the standard, conventional ELISA method. Of the six chosen targets analyzed, three (CCL12, CXCL1, and

TABLE II

*Summary of cytokine protein levels in serum in response to sprint, endurance run, or voluntary wheel running for 1 week. All values shown as average ± S.E. Those cytokines that are below the detection limit of the assay are indicated by "–". n = 10 mice per group. *p < 0.05; **p < 0.01; ***p < 0.001*

| | Serum cytokine | | | | | |
| | Sprint | | Endurance | | 1-week wheel running | |
Analyte	Pre-exercise (pg/ml)	Postexercise (pg/ml)	Pre-exercise (pg/ml)	Postexercise (pg/ml)	Pre-exercise (pg/ml)	Post-exercise (pg/ml)
G-CSF	290 ± 26	341 ± 73	343 ± 69	250 ± 33	3244 ± 457	**787 ± 149***
Eotaxin-1/CCL11	1265 ± 171	1526 ± 233	1417 ± 261	1654 ± 216	1344 ± 378	1475 ± 342
GM-CSF	77 ± 32	52 ± 10	–	30 ± 9	48 ± 11	69 ± 30
IFN-β1	–	–	–	–	–	–
IFN-γ	491 ± 50	**337 ± 49**	472 ± 37	**285 ± 36***	367 ± 54	**113 ± 10**
IL-1α	111 ± 15	125 ± 12	125 ± 25	122 ± 22	148 ± 27	247 ± 84
IL-1β	37 ± 15	20 ± 7	21 ± 5	37 ± 14	–	23 ± 6
IL-2	–	–	–	–	8 ± 1	6 ± 3
IL-3	–	–	–	–	–	–
IL-4	–	–	–	–	–	–
IL-5	13 ± 2	9 ± 1		15 ± 3	11 ± 1	11 ± 2
IL-6	15 ± 4	30 ± 11	11 ± 2	9 ± 0	56 ± 10	**15 ± 3***
IL-7	13 ± 2				5 ± 1	6 ± 1
IL-9	377 ± 131	443 ± 308	669 ± 549	501 ± 155	229 ± 45	272 ± 40
IL-10	13 ± 3	10 ± 2	–	–	4 ± 1	9 ± 4
IL-11	304 ± 49	274 ± 21	416 ± 40	476 ± 115	–	–
IL-12 (p40)	15 ± 3	17 ± 2	10 ± 1	14 ± 3	15 ± 5	15 ± 4
IL-12 (p70)		20 ± 5		26 ± 9	12 ± 5	17 ± 6
IL-13	36 ± 4	42 ± 1	32 ± 0	40 ± 4	11 ± 2	10 ± 2
IL-15	33 ± 17	42 ± 17	64 ± 51	78 ± 26	63 ± 27	100 ± 36
IL-16	3525 ± 311	**2590 ± 431***	4062 ± 338	**2321 ± 186***	4761 ± 747	**1509 ± 147**
IL-17A	–	–	–	–	–	–
IL-17A/F	1922 ± 77	**1567 ± 73***	1919 ± 126	1828 ± 295	286 ± 39	203 ± 15
IL-17F	–	–	–		10 ± 5	14 ± 8
IL-20	1431 ± 187	**2183 ± 343***	1568 ± 220	2427 ± 446	434 ± 91	359 ± 76
IL-21	–	–	–	–	–	–
IL-22	7 ± 3	53 ± 49	9 ± 2	10 ± 2	15 ± 5	9 ± 2
IL-23	388 ± 66	884 ± 212	410 ± 53	762 ± 106	735 ± 91	352 ± 61
IL-27	1212 ± 186	1311 ± 137	920 ± 120	1110 ± 72	1307 ± 307	1201 ± 102
IL-28B	–	17104 ± 9808	–	–	–	119 ± 22
IL-33	–	2062 ± 2016	–	98 ± 31	–	–
IP-10/CXCL10	159 ± 9	154 ± 19	172 ± 13	**109 ± 19***	246 ± 19	229 ± 7
KC/CXCL1	125 ± 25	**309 ± 78***	143 ± 27	**284 ± 30**	619 ± 110	**392 ± 52***
LIF	–	–	–	–	–	–
LIX/CXCL5	5100 ± 1955	4250 ± 1027	3935 ± 1499	4771 ± 1302	717 ± 224	2998 ± 1013
MCP-1/CCL2	102 ± 25	102 ± 13	61 ± 8	77 ± 7	27 ± 6	49 ± 9
M-CSF	21 ± 7	35 ± 10		23 ± 9	14 ± 3	14 ± 4
MIG/CXCL9	171 ± 13	208 ± 17	188 ± 22	172 ± 17	136 ± 14	151 ± 20
MIP-1α/CCL3	74 ± 11	90 ± 7	68 ± 5	85 ± 16	40 ± 11	51 ± 14
MIP-1β/CCL4	41 ± 6	38 ± 4	35 ± 0	36 ± 13	46 ± 5	37 ± 4
MIP-2/CXCL2	199 ± 32	215 ± 21	124 ± 13	**169 ± 15***	206 ± 56	270 ± 28
RANTES/CCL5	24 ± 4	26 ± 3	17 ± 2	19 ± 2	16 ± 2	16 ± 3
TNF-α	9 ± 0	11 ± 2	9 ± 1	14 ± 3	15 ± 4	15 ± 4
VEGF	–	–	–	–	–	–
Exodus-2/CCL21	10433 ± 392	10817 ± 635	11022 ± 379	11095 ± 433	20853 ± 1796	20777 ± 1125
Fractalkine/CX3CL1	2431 ± 92	2607 ± 116	2427 ± 57	**2770 ± 119***	609 ± 47	730 ± 47
MDC/CCL22	394 ± 46	492 ± 53	496 ± 48	455 ± 40	161 ± 34	136 ± 20
MCP-5/CCL12	416 ± 36	392 ± 33	368 ± 37	**293 ± 31***	252 ± 36	**160 ± 23**
MIP-3α/CCL20	269 ± 11	237 ± 9	272 ± 11	**215 ± 10***	102 ± 6	**74 ± 4**
MIP-3β/CCL19	687 ± 52	676 ± 71	745 ± 70	776 ± 122	143 ± 29	77 ± 12
TARC/CCL17	327 ± 13	**227 ± 12***	359 ± 61	**176 ± 9**	73 ± 10	56 ± 6
TIMP-1	4641 ± 198	4515 ± 678	4842 ± 222	**2905 ± 52***	5184 ± 372	**3313 ± 179***
sCD30	–	–	–	–	–	67 ± 0
sgp130	658 ± 69	**430 ± 53***	527 ± 32	459 ± 43	616 ± 56	649 ± 54
sIL-1RI	1760 ± 298	1679 ± 198	2189 ± 263	1705 ± 210	2914 ± 362	1966 ± 378
sIL-1RII	6343 ± 149	6348 ± 117	6445 ± 153	6685 ± 134	11780 ± 446	11412 ± 281
sIL-2Ra	366 ± 25	376 ± 20	367 ± 14	392 ± 32	467 ± 21	448 ± 26
sIL-4R	2434 ± 477	1851 ± 257	2481 ± 311	**1601 ± 153**	2859 ± 204	2302 ± 163
sIL-6R	11627 ± 508	10710 ± 437	11691 ± 226	11713 ± 487	19337 ± 639	17804 ± 754
sRAGE	252 ± 63	332 ± 140	347 ± 154	–	–	–

TABLE II—*continued*

	Serum cytokine					
	Sprint		Endurance		1-week wheel running	
Analyte	Pre-exercise (pg/ml)	Postexercise (pg/ml)	Pre-exercise (pg/ml)	Postexercise (pg/ml)	Pre-exercise (pg/ml)	Post-exercise (pg/ml)
sTNFRI	2786 ± 205	2823 ± 196	3032 ± 252	**2101 ± 139***	8200 ± 597	**6461 ± 372***
sTNFRII	5833 ± 559	5028 ± 292	5851 ± 663	**5156 ± 589****	9591 ± 572	**7926 ± 288***
sVEGFR1	997 ± 349	618 ± 100	507 ± 70	507 ± 74	368 ± 79	707 ± 240
sVEGFR2	33031 ± 1833	28423 ± 2901	29170 ± 1504	36032 ± 1903	–	–
sVEGFR3	26831 ± 1438	28009 ± 711	29247 ± 448	29785 ± 888	61281 ± 2402	62438 ± 2043
EPO	1257 ± 98	**1393 ± 82***	1030 ± 85	1143 ± 73	123 ± 10	100 ± 16
# of cytokine detected	48		45		49	
# of cytokine changed	8		13		10	
% of cytokine changed	17%		29%		20%	

CXCL2) were significantly different between pre- and post-exercise serum, and the direction of change matched that of the multiplex data (supplemental Fig. S1). Similarly, three targets were significantly different between sedentary and exercised soleus, and the direction of change matched that of the multiplex data (CCL12, IFN-γ, and sTNFRI). In agreement with the multiplex data, none of the targets (except sTNFRI) were significantly different between sedentary and exercised plantaris as measured by ELISA.

In serum, we observed significant changes in eight (17%), thirteen (29%), and ten (20%) circulating cytokines in response to a single bout of sprint, endurance run, and 1-week voluntary wheel running, respectively (Table II). A much greater overlap was seen between a single bout of endurance run and voluntary wheel running (Fig. 1). Except for CXCL1, the direction of change in serum levels was consistent among all the overlapping cytokines in three of the exercise paradigms. Although there was overlap, a single bout of sprint and endurance run also altered different sets of cytokines (Fig. 1). Of the serum cytokines altered by acute or chronic exercise, 4 were up-regulated and 14 were downregulated.

In oxidative soleus muscle, an even greater proportion of the detected cytokine was altered by acute and chronic exercise relative to the sedentary control group (Table III). The protein levels of eleven (24%), twenty-one (47%), and twenty-six (62%) cytokines were significantly changed in response to a single bout of sprint, endurance run, and 1-week voluntary wheel running, respectively. Again, we observed a much greater overlap between a single bout of endurance run and 1-week voluntary wheel running (Fig. 2A). However, the direction of change (up or downregulated) among the overlapping cytokines was different depending on the types of physical activity (Table III and Fig. 2A). Despite significant overlap, different types of physical activity clearly altered different sets of cytokines in soleus muscle (Fig. 2A). Of the 34 cytokines whose levels changed in soleus muscle by acute or chronic exercise, 11 were up-regulated, 11 were downregulated, and 12 were either up or downregulated depending on the types of physical activity.

A very different cytokine profile was seen for the glycolytic (plantaris) when compared with the oxidative (soleus) muscle. Relative to the sedentary control group, the protein levels of four (8%), three (6%), and twenty-two (50%) cytokines were significantly changed in plantaris muscle in response to a single bout of sprint, endurance run, and 1-week voluntary wheel running, respectively (Table III). In striking contrast to the soleus muscle, only 3–4 cytokines were altered by a single bout of sprint or endurance run in the plantaris muscle (Fig. 2B). Most of the cytokine changes in plantaris were seen in mice subjected to 1-week of voluntary wheel running. Minimal (1–2 cytokines) overlap was observed in plantaris for the three different types of exercise. Of the 25 cytokines altered in plantaris muscle by acute or chronic exercise, 4 were up-regulated, 20 were downregulated, and 1 was either up or downregulated depending on the types of physical activity.

Next, we asked how many of the systemic changes in circulating serum cytokines paralleled the local protein changes in soleus and plantaris. This would help address which among the systemic cytokine changes in blood is directly because of the expression and secretion from skeletal muscle. Surprisingly, for a single bout of sprint, no overlap in cytokine profiles was seen between serum, soleus, and plantaris muscles (Fig. 3A). Different sets of cytokines were apparently altered in oxidative (soleus) and glycolytic (plantaris) muscles, with most of the changes seen in soleus muscle fibers. Combined, a total of 21 cytokines were altered by sprint, with 13 upregulated, 7 downregulated, and 1 with opposite changes between serum and soleus muscle.

For a single bout of endurance run, most of the cytokine changes were seen in serum and soleus muscle (Fig. 3B). However, no overlap (common to all three) was seen between serum, soleus, and plantaris. Although seven cytokine changes showed overlap between serum and soleus muscle, the direction of change (up or downregulated) was different for three of the cytokines (Fig. 3B). Combined, a total of 28 cytokines were altered by a single bout of endurance run, with 13 upregulated, 12 downregulated, and 3 with opposite changes between serum and soleus muscle.

TABLE III

Summary of cytokine protein levels in soleus and plantaris muscles in response to sprint, endurance run, or voluntary wheel running for 1 week. All values are normalized to the control sedentary group and shown as fold change $\pm$ S.E. Those cytokines that are below the detection limit of the assay are indicated by "–". n = 10 mice per group. *p < 0.05; **p < 0.01; ***p < 0.001

Analyte	Soleus (protein)			Plantaris (protein)		
	Sprint/Sedentary (Fold change)	Endurance/Sedentary (Fold change)	Voluntary wheel running/Sedentary (Fold change)	Sprint/Sedentary (Fold change)	Endurance/Sedentary (Fold change)	Voluntary wheel running/Sedentary (Fold change)
G-CSF	–	–	–	–	–	–
Eotaxin-1/CCL11	1.09 ± 0.08	0.82 ± 0.03	***0.57 ± 0.01**	1.10 ± 0.07	0.96 ± 0.10	**0.64 ± 0.05**
GM-CSF	1.57 ± 0.34	1.35 ± 0.21	–	0.36 ± 0.08	0.43 ± 0.13	0.17 ± 0.03
IFN-β1	–	–	–	–	–	–
IFN-γ	*1.47 ± 0.12*	*1.55 ± 0.22*	***0.10 ± 0.01**	0.67 ± 0.10	0.97 ± 0.21	**0.15 ± 0.02**
IL-1α	*1.23 ± 0.10*	***1.40 ± 0.08**	***0.20 ± 0.01**	0.83 ± 0.07	1.05 ± 0.18	**0.31 ± 0.02**
IL-1β	*1.57 ± 0.17*	***1.86 ± 0.18**	1.13 ± 0.03	1.17 ± 0.08	*1.36 ± 0.10*	0.78 ± 0.05
IL-2	1.21 ± 0.08	*1.28 ± 0.09*	***0.18 ± 0.01**	0.80 ± 0.08	1.02 ± 0.17	**0.32 ± 0.02**
IL-3	–	–	–	–	–	–
IL-4	1.07 ± 0.13	0.81 ± 0.13	–	0.76 ± 0.09	0.97 ± 0.18	**0.32 ± 0.02**
IL-5	2.16 ± 0.46	1.14 ± 0.20	0.36 ± 0.06	0.63 ± 0.21	0.49 ± 0.18	0.20 ± 0.10
IL-6	*1.29 ± 0.10*	**1.42 ± 0.12**	***0.17 ± 0.01**	0.81 ± 0.08	1.06 ± 0.18	***0.22 ± 0.01**
IL-7	–	–	–	0.74 ± 0.15	1.48 ± 0.54	–
IL-9	1.13 ± 0.06	***1.35 ± 0.07**	***0.22 ± 0.02**	0.86 ± 0.08	1.08 ± 0.14	***0.19 ± 0.02**
IL-10	*2.22 ± 0.51*	1.33 ± 0.17	1.08 ± 0.26	0.79 ± 0.14	0.94 ± 0.20	1.24 ± 0.16
IL-11	–	–	–	–	–	–
IL-12 (p40)	**1.73 ± 0.18**	***2.10 ± 0.15**	***0.16 ± 0.01**	0.75 ± 0.13	0.86 ± 0.13	***0.10 ± 0.01**
IL-12 (p70)	–	–	–	–	–	–
IL-13	–	–	–	0.48 ± 0.11	0.75 ± 0.19	0.21 ± 0.03
IL-15	1.65 ± 0.22	**2.06 ± 0.21**	*0.14 ± 0.02*	*0.56 ± 0.10*	0.76 ± 0.11	***0.05 ± 0.01**
IL-16	0.89 ± 0.08	0.71 ± 0.03	***1.88 ± 0.18**	1.14 ± 0.08	0.98 ± 0.09	0.90 ± 0.11
IL-17A	–	–	–	0.63 ± 0.10	0.90 ± 0.18	–
IL-17A/F	–	–	–	–	–	–
IL-17F	–	–	–	–	–	–
IL-20	1.23 ± 0.08	1.56 ± 0.13	*1.70 ± 0.19*	0.71 ± 0.13	1.62 ± 0.54	0.97 ± 0.23
IL-21	–	–	–	–	–	–
IL-22	*1.51 ± 0.18*	1.35 ± 0.17	**0.28 ± 0.01**	0.83 ± 0.11	0.71 ± 0.11	***0.23 ± 0.01**
IL-23	1.28 ± 0.13	1.22 ± 0.26	–	0.47 ± 0.06	0.53 ± 0.08	–
IL-27	–	–	–	0.77 ± 0.08	0.91 ± 0.17	–
IL-28B	–	–	–	0.65 ± 0.07	0.71 ± 0.11	–
IL-33	1.04 ± 0.11	*0.61 ± 0.07*	*0.64 ± 0.06*	1.27 ± 0.17	0.86 ± 0.12	0.62 ± 0.07
IP-10/CXCL10	*1.58 ± 0.14*	*1.61 ± 0.19*	1.01 ± 0.13	0.81 ± 0.05	0.96 ± 0.10	***0.25 ± 0.05**
KC/CXCL1	1.52 ± 0.11	**1.72 ± 0.26**	*0.45 ± 0.02*	0.61 ± 0.0769	1.01 ± 0.17	***0.12 ± 0.01**
LIF	–	–	–	–	–	–
LIX/CXCL5	–	–	–	0.49 ± 0.09	0.85 ± 0.21	–
MCP-1/CCL2	1.22 ± 0.16	1.46 ± 0.31	0.96 ± 0.18	1.21 ± 0.19	1.15 ± 0.14	0.91 ± 0.20
M-CSF	1.68 ± 0.32	0.90 ± 0.21	0.34 ± 0.03	0.75 ± 0.07	0.89 ± 0.16	***0.24 ± 0.01**
MIG/CXCL9	1.51 ± 0.09	1.56 ± 0.21	1.35 ± 0.27	1.06 ± 0.06	1.01 ± 0.08	***0.39 ± 0.07**
MIP-1α/CCL3	1.45 ± 0.16	1.54 ± 0.14	1.18 ± 0.06	1.22 ± 0.09	***1.51 ± 0.10**	**0.60 ± 0.07**
MIP-1β/CCL4	–	–	–	–	–	–
MIP-2/CXCL2	1.58 ± 0.29	***2.89 ± 0.39**	1.30 ± 0.18	0.45 ± 0.12	0.82 ± 0.26	0.18 ± 0.05
RANTES/CCL5	*1.46 ± 0.15*	*1.49 ± 0.18*	***0.21 ± 0.01**	0.76 ± 0.10	0.73 ± 0.13	***0.19 ± 0.02**
TNF-α	–	–	–	–	–	–
VEGF	**0.64 ± 0.09**	***0.63 ± 0.05**	***0.38 ± 0.03**	***0.62 ± 0.05**	**0.70 ± 0.05**	*0.76 ± 0.07*
Exodus-2/CCL21	0.70 ± 0.12	*0.54 ± 0.11*	0.69 ± 0.06	1.08 ± 0.07	0.80 ± 0.05	*0.66 ± 0.11*
Fractalkine/CX3CL1	–	–	**0.62 ± 0.05**	–	1.09 ± 0.34	–
MDC/CCL22	1.12 ± 0.10	1.16 ± 0.10	0.80 ± 0.06	0.87 ± 0.07	1.04 ± 0.17	0.63 ± 0.11
MCP-5/CCL12	0.61 ± 0.08	*0.42 ± 0.06*	*1.51 ± 0.18*	0.73 ± 0.08	0.73 ± 0.15	0.92 ± 0.24
MIP-3α/CCL20	1.11 ± 0.06	*1.20 ± 0.05*	***0.33 ± 0.01**	0.84 ± 0.06	0.90 ± 0.14	***0.29 ± 0.03**
MIP-3β/CCL19	1.20 ± 0.16	1.00 ± 0.11	**1.65 ± 0.14**	0.96 ± 0.09	1.15 ± 0.08	0.86 ± 0.11
TARC/CCL17	–	–	–	–	–	–
TIMP-1	0.76 ± 0.09	0.55 ± 0.06	***4.16 ± 0.67**	0.87 ± 0.11	0.78 ± 0.13	*3.01 ± 1.01*
sCD30	0.95 ± 0.05	0.82 ± 0.07		0.77 ± 0.03	0.88 ± 0.13	0.83 ± 0.26
sgp130	1.09 ± 0.11	1.34 ± 0.11	*0.60 ± 0.09*	1.39 ± 0.11	1.64 ± 0.25	2.02 ± 1.12
sIL-1RI	1.10 ± 0.09	1.14 ± 0.04	***0.57 ± 0.03**	0.86 ± 0.06	1.03 ± 0.07	**0.66 ± 0.06**
sIL-1RII	1.04 ± 0.10	0.78 ± 0.05	0.78 ± 0.06	*1.65 ± 0.33*	1.19 ± 0.08	0.82 ± 0.11
sIL-2Ra	–	–	–	–	–	–
sIL-4R	–	–	–	–	–	–
sIL-6R	1.08 ± 0.17	0.80 ± 0.10	0.94 ± 0.07	**2.10 ± 0.41**	1.48 ± 0.14	0.69 ± 0.07
sRAGE	1.19 ± 0.10	1.28 ± 0.14	***0.17 ± 0.01**	0.73 ± 0.06	0.82 ± 0.13	***0.16 ± 0.06**
sTNFRI	0.87 ± 0.06	**0.73 ± 0.06**	0.82 ± 0.05	1.10 ± 0.07	1.07 ± 0.05	0.82 ± 0.14
sTNFRII	0.89 ± 0.06	0.70 ± 0.05	***1.66 ± 0.13**	1.11 ± 0.07	1.08 ± 0.06	1.05 ± 0.14
sVEGFR1	1.05 ± 0.06	*0.79 ± 0.03*	***0.64 ± 0.03**	1.23 ± 0.12	1.32 ± 0.09	0.71 ± 0.07

TABLE III—*continued*

| Analyte	Soleus (protein)			Plantaris (protein)		
	Sprint/Sedentary (Fold change)	Endurance/Sedentary (Fold change)	Voluntary wheel running/Sedentary (Fold change)	Sprint/Sedentary (Fold change)	Endurance/Sedentary (Fold change)	Voluntary wheel running/Sedentary (Fold change)
sVEGFR2	*0.84 ± 0.04	***0.61 ± 0.03	*0.83 ± 0.04	0.91 ± 0.05	0.79 ± 0.06	0.88 ± 0.10
sVEGFR3	0.89 ± 0.11	0.90 ± 0.07	0.88 ± 0.04	1.52 ± 0.10	1.49 ± 0.15	1.46 ± 0.37
EPO	1.24 ± 0.10	*1.40 ± 0.09	–	0.63 ± 0.08	0.90 ± 0.17	–
# of cytokine detected	45	45	42	51	52	44
# of cytokine changed	11	21	26	4	3	22
% of cytokine changed	24%	47%	62%	8%	6%	50%
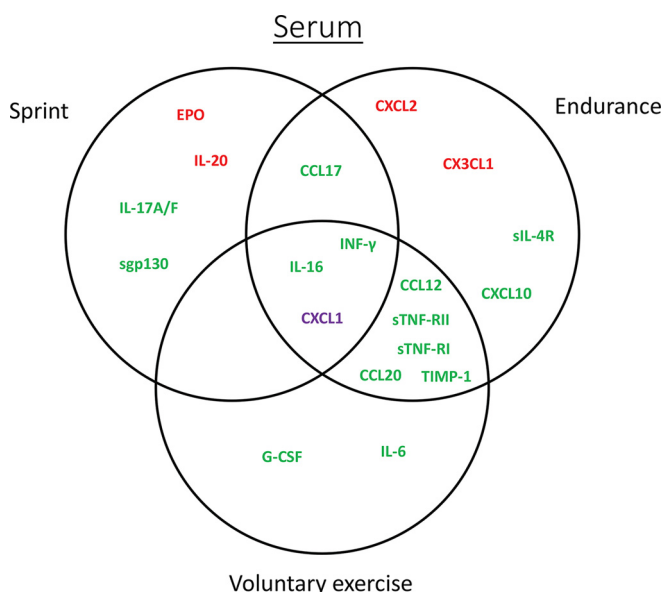
FIG. 1. **Vein diagram of cytokines altered by sprint, endurance run, or voluntary wheel running in serum.** Upregulated cytokines are highlighted in red. Downregulated cytokines are highlighted in green. Cytokines that are upregulated in one exercise paradigm but downregulated in another are highlighted in purple.

Of the three different types of exercise, the greatest number of cytokines changes were seen in the 1-week voluntary wheel running group relative to the sedentary control group (Fig. 3C). Large numbers of cytokine changes showed overlap between serum and soleus or plantaris, as well as as between oxidative (soleus) and glycolytic (plantaris) fibers. Interestingly, most of the cytokine changes induced by voluntary wheel running were in the "downregulated" direction (Fig. 3C; highlighted in green); in contrast, a large fraction of the cytokine changes seen in sprint and endurance run were in the "up-regulated" direction (Fig. 3A and 3B; highlighted in red). Combined, a total of 34 cytokines were altered by 1-week voluntary wheel running, with 2 up-regulated, 28 downregulated, 4 with opposite changes between different tissues.

We next examined changes in cytokine mRNA levels in oxidative (soleus) and glycolytic (plantaris) muscles in response to the three different types of exercise. We used the QuantiGene Plex assays to simultaneously measure the mRNA levels of 63 cytokines (Table IV). Except for CXCL9 transcript, we were able to detect the mRNA of 62 cytokines.

Relative to the sedentary control group, the mRNA levels of six (10%), eight (13%), and fourteen (22%) cytokines were significantly changed in soleus in response to sprint, endurance run, and voluntary wheel running, respectively. In plantaris muscle, however, the mRNA levels of five (8%), thirteen (21%), and five (8%) cytokines were significantly changed in response to sprint, endurance, and voluntary wheel running, respectively. Surprisingly, most of the mRNA changes in soleus and plantaris show relatively little overlap between the three types of physical activity (Fig. 4A and 4B). In soleus muscle, 10 cytokine mRNA were up-regulated, 7 downregulated, and 3 changed in the opposing directions depending on the types of exercise. In plantaris muscle, 5 cytokine mRNA were up-regulated, 12 downregulated, and 1 changed in the opposite direction between endurance and chronic wheel running.

Next, we addressed the issue of concordance between changes in cytokine mRNA and protein expression in soleus and plantaris muscles. In oxidative (soleus) muscle, parallel reductions in transcript and protein were observed for IL-15, VEGF, sIL1RI, sVEGFR1, sVEGFR2, and sgp130, whereas parallel increases were only observed for CCL12 and TIMP-1. In glycolytic (plantaris) muscle, parallel reductions in transcript and protein were observed for VEGF and CCL21, whereas a parallel increase was only observed for TIMP-1. Essentially, most of the cytokine changes reported here showed discordance between transcript and protein levels (comparing Tables III and IV).

Given that the changes in a subset of serum cytokines were not matched by the corresponding changes in their protein levels in muscle, we addressed whether this could be because of secondary adaptive changes in their expression in nonmuscle tissues. Acute and chronic exercise can alter systemic metabolism in response to energetic demands, and we have previously shown that different metabolic states alters circulating cytokine levels (43). Thus, we determined the expression of cytokine transcripts in liver and two major fat depots (visceral and subcutaneous white adipose tissues), major metabolic organs known to produce and secrete a wide array of cytokines and chemokines in response to different physiological stimuli. We focused our analyses only on those cytokines whose altered serum levels were not accounted for by changes in protein levels in skeletal muscle in response to
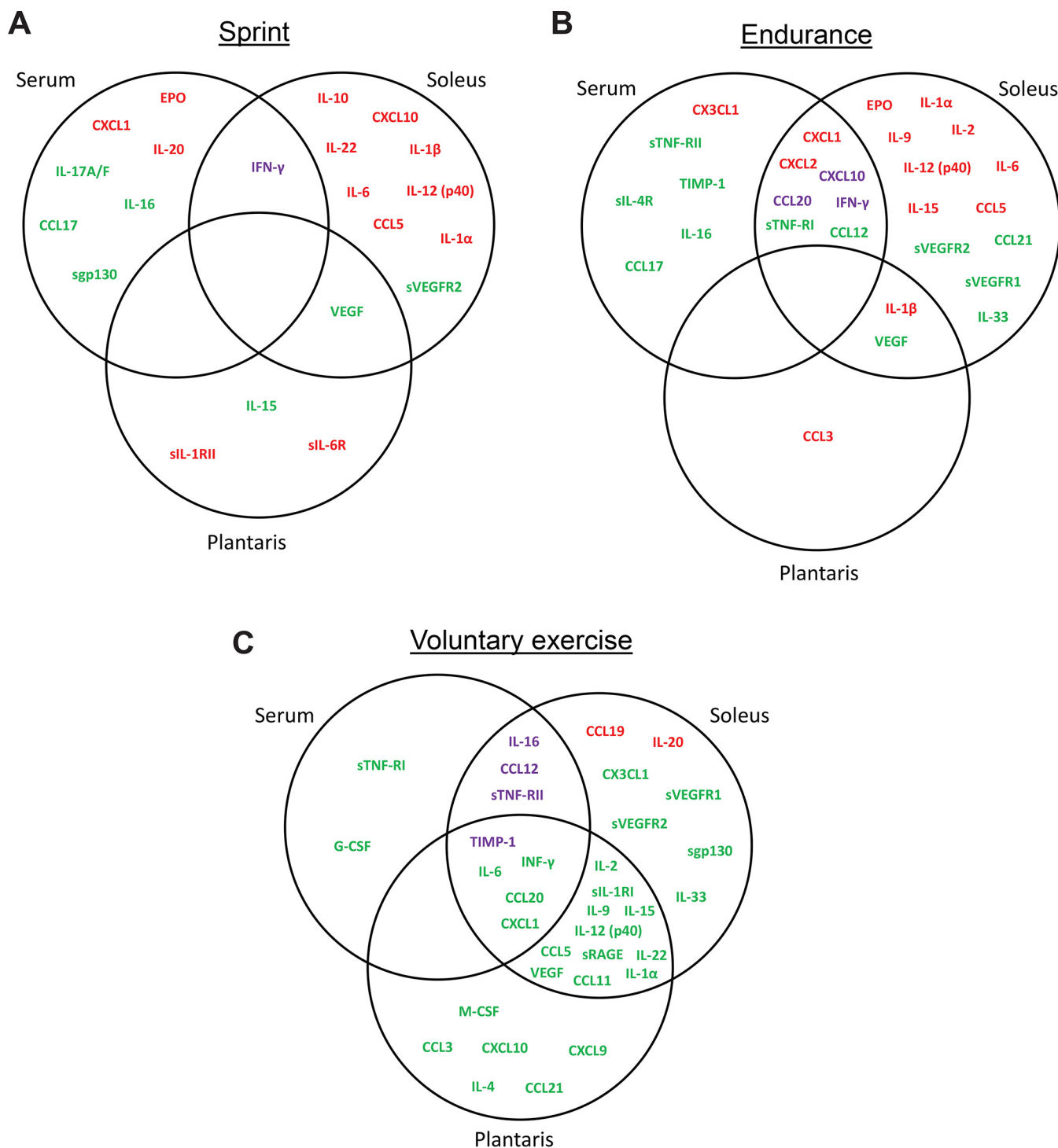
FIG. 2. **Vein diagram of cytokines altered by sprint, endurance run, or voluntary wheel running in (A) soleus and (B) plantaris muscles.** Upregulated cytokines are highlighted in red. Downregulated cytokines are highlighted in green. Cytokines that are upregulated in one exercise paradigm but downregulated in another are highlighted in purple.

exercise. As shown in Fig. 5, depending on the types of exercise, expression of cytokine transcripts could be either up- or down-regulated in the liver and/or adipose tissues. With a couple exceptions, most of these changes in mRNA paralleled the cytokine changes seen in circulation. For example, for a single bout of sprint, increased serum EPO and IL-20 levels were paralleled by increased mRNA expression in visceral white adipose tissue, whereas reduced serum IL-17F, sgp130, and INF-γ levels were paralleled by reduction in mRNA expression in either visceral or subcutaneous adipose tissue. For a single bout of endurance run, reduced serum sIL-4R, sTNF-RII, TIMP-1, IL-16, INF-γ, and CCL20 levels were paralleled by reduced mRNA expression in liver and/or adipose tissue. For voluntary wheel running, reduction in serum sTNF-RII, TIMP-1, and CCL12 levels were paralleled by reduced mRNA expression in subcutaneous adipose tissue. These results suggest that changes in a subset of circulating cytokines could, at least in part, be accounted for by the corresponding changes in transcript levels in liver and/or adipose tissues.

DISCUSSION

Here, we undertook an antibody-based profiling approach to determine the extent by which exercise modulates local cytokine levels in glycolytic and oxidative muscle fibers, as well as their systemic circulating levels, in response to three different types of physical activity—an acute sprint and endurance run, or chronic exercise in the form of voluntary wheel running. We chose to focus on cytokine family members as

potential myokines given their well-known pleiotropic functions that could potentially link exercise to myriad physiological effects that include immunity (48), inflammation (6), metabolism (43), food intake (32, 49), and autophagy (50).

All the significant changes in cytokine mRNA and protein levels are summarized in Table V. From our antibody-based profiling studies of exercise-regulated cytokines, several notable observations are highlighted. (1) Chronic exercise in the form of wheel running consistently induced more cytokine changes than an acute single bout of sprint or endurance run. This is perhaps not surprising as repeated bouts of exercise lead to skeletal muscle adaptation (51). (2) Acute and chronic exercise appear to alter distinct sets of cytokines in serum and skeletal muscle, with varying degrees of overlap depending on the type of physical activity. This presumably reflects differences in skeletal muscle adaptation to repeated voluntary wheel running *versus* a stress-like response to an acute bout of exercise. (3) There is more overlap in cytokine profile between a single bout of endurance exercise and chronic wheel running, particularly in serum and oxidative soleus muscle. This suggests that exercise intensity affects the nature of the cytokine response, as endurance and chronic wheel running represent low-intensity exercise whereas an exhaustive sprint reflects high-intensity exercise. (4) We observed more exercise-regulated cytokine changes in skeletal muscle than in serum. This could reflect a higher local concentration of cytokines and hence our greater ability to reliably detect their differences. The circulating plasma levels of most

FIG. 3. **Vein diagram of cytokines altered by (A) sprint, (B) endurance run, or (C) voluntary wheel running in serum and in soleus and plantaris muscles.** Up-regulated cytokines are highlighted in red. Downregulated cytokines are highlighted in green. Cytokines that are up-regulated in one tissue but downregulated in another are highlighted in purple.

cytokines are in the nanogram and picogram per milliliter range. Because of the dilution effect of plasma, only relatively large changes in cytokine secretion into circulation by skeletal muscle in response to exercise would be detected. Alternatively, a subset of exercise-regulated cytokines act locally

within skeletal muscle and are not secreted into circulation. (5) Interestingly, between muscle fiber types, we observed significantly more cytokine changes in oxidative soleus compared with glycolytic plantaris muscles irrespective of the types of exercise (sprint, endurance, wheel running). With

TABLE IV

Summary of cytokine mRNA levels in soleus and plantaris muscles in response to sprint, endurance run, or voluntary wheel running for 1 week. All values are normalized to the control sedentary group and shown as fold change ± S.E. Those cytokines whose mRNA that are not detected are indicated by "–". n = 10 mice per group. *p < 0.05; **p < 0.01; ***p < 0.001

Accesion #	Gene	Soleus Muscle (mRNA)			Plantaris Muscle (mRNA)		
		Sprint/Sedentary Fold change	Endurance/sedentary Fold change	Voluntary wheel running/sedentary Fold change	Sprint/Sedentary Fold change	Endurance/sedentary Fold change	Voluntary wheel running/sedentary Fold change
NM_009971	G-CSF/Csf-3	0.71 ± 0.13	0.45 ± 0.14	0.87 ± 0.18	1.10 ± 0.29	–	0.91 ± 0.22
NM_009969	GM-CSF/Csf-2	1.40 ± 0.18	1.10 ± 0.16	0.92 ± 0.21	1.13 ± 0.12	0.72 ± 0.05	0.80 ± 0.08
NM_010510	IFN-β1	1.50 ± 0.23	1.27 ± 0.19	1.06 ± 0.26	1.09 ± 0.16	0.82 ± 0.07	0.87 ± 0.08
NM_008337	IFN-γ	1.33 ± 0.21	1.25 ± 0.23	0.92 ± 0.20	0.93 ± 0.10	0.73 ± 0.06	0.81 ± 0.10
NM_010554	IL-1α	1.42 ± 0.37	2.18 ± 0.54	0.98 ± 0.15	1.37 ± 0.18	0.58 ± 0.05	0.94 ± 0.17
NM_008361	IL-1β	1.28 ± 0.12	1.02 ± 0.17	1.37 ± 0.13	1.14 ± 0.15	0.73 ± 0.07	0.89 ± 0.11
NM_008366	IL-2	1.40 ± 0.45	2.33 ± 1.3	0.59 ± 0.11	1.24 ± 0.30	0.74 ± 0.13	1.24 ± 0.37
NM_010556	IL-3	1.60 ± 0.19	1.25 ± 0.19	0.98 ± 0.19	1.14 ± 0.13	0.97 ± 0.08	0.91 ± 0.21
NM_021283	IL-4	1.35 ± 0.20	1.05 ± 0.21	0.80 ± 0.19	**2.31 ± 0.42	1.37 ± 0.22	1.55 ± 0.24
NM_010558	IL-5	1.34 ± 0.21	1.20 ± 0.20	0.95 ± 0.23	1.00 ± 0.16	0.71 ± 0.07	0.77 ± 0.08
NM_031168	IL-6	1.53 ± 0.25	1.38 ± 0.20	1.28 ± 0.28	0.91 ± 0.10	0.73 ± 0.06	0.81 ± 0.11
NM_008371	IL-7	1.50 ± 0.23	1.29 ± 0.14	0.93 ± 0.20	1.05 ± 0.12	0.94 ± 0.13	0.85 ± 0.10
NM_008373	IL-9	1.22 ± 0.10	1.13 ± 0.13	0.79 ± 0.17	1.32 ± 0.15	0.71 ± 0.07	0.84 ± 0.13
NM_010548	IL-10	1.34 ± 0.19	1.35 ± 0.33	0.95 ± 0.09	1.08 ± 0.08	0.73 ± 0.06	0.82 ± 0.19
NM_008350	IL-11	1.22 ± 0.18	1.03 ± 0.12	0.95 ± 0.16	1.14 ± 0.11	0.89 ± 0.06	0.89 ± 0.08
NM_008352	IL-12 (p40)/ IL-12B	1.47 ± 0.16	1.19 ± 0.18	*1.54 ± 0.15	1.24 ± 0.17	0.82 ± 0.06	1.02 ± 0.17
NM_008351	IL-12 (p70)	1.17 ± 0.16	1.09 ± 0.20	0.96 ± 0.19	1.04 ± 0.10	*0.71 ± 0.06	0.75 ± 0.07
NM_008355	IL-13	1.39 ± 0.20	1.35 ± 0.38	0.87 ± 0.18	1.01 ± 0.13	0.79 ± 0.11	0.87 ± 0.23
NM_008357	IL-15	1.07 ± 0.06	0.90 ± 0.07	***0.59 ± 0.04	1.09 ± 0.07	1.04 ± 0.04	1.16 ± 0.05
NM_010551	IL-16	1.05 ± 0.06	**0.67 ± 0.04	0.86 ± 0.06	1.10 ± 0.08	0.83 ± 0.15	0.95 ± 0.07
NM_010552	IL-17A/CTLA8	1.47 ± 0.20	1.26 ± 0.18	1.06 ± 0.24	1.00 ± 0.14	0.87 ± 0.06	0.78 ± 0.09
NM_021380	IL-20	0.99 ± 0.11	*0.57 ± 0.13	**0.53 ± 0.10	0.97 ± 0.07	***0.55 ± 0.06	*0.75 ± 0.06
NM_021782	IL-21	1.29 ± 0.18	1.06 ± 0.13	0.96 ± 0.22	1.26 ± 0.19	0.81 ± 0.08	0.90 ± 0.12
NM_016971	IL-22	1.52 ± 0.27	1.20 ± 0.19	1.07 ± 0.30	1.12 ± 0.14	0.69 ± 0.07	0.78 ± 0.11
NM_031252	IL-23	1.52 ± 0.27	1.33 ± 0.19	1.03 ± 0.25	0.96 ± 0.12	0.80 ± 0.07	0.82 ± 0.08
NM_145636	IL-27	1.25 ± 0.10	1.07 ± 0.14	0.90 ± 0.11	1.30 ± 0.07	**0.90 ± 0.08	1.08 ± 0.17
NM_177396	IL-28B/IFNl3	1.29 ± 0.16	0.94 ± 0.11	0.78 ± 0.17	1.08 ± 0.12	0.67 ± 0.05	0.76 ± 0.12
NM_029594	IL-31	1.45 ± 0.26	1.15 ± 0.15	1.02 ± 0.23	0.96 ± 0.14	0.71 ± 0.05	0.73 ± 0.07
NM_133775	IL-33	1.16 ± 0.08	*1.31 ± 0.11	0.94 ± 0.06	1.27 ± 0.10	1.29 ± 0.10	0.91 ± 0.08
NM_021274	IP-10/CXCL10	1.10 ± 0.17	1.53 ± 0.22	*2.57 ± 0.72	0.72 ± 0.08	0.64 ± 0.15	0.76 ± 0.11
NM_008176	KC/CXCL1	***1.74 ± 0.11	1.36 ± 0.15	0.94 ± 0.10	***2.65 ± 0.45	1.45 ± 0.18	0.99 ± 0.12
NM_008501	LIF	1.26 ± 0.18	0.95 ± 0.10	1.00 ± 0.16	0.95 ± 0.07	**0.64 ± 0.04	0.81 ± 0.06
NM_009141	LIX/CXCL5	1.68 ± 0.35	1.46 ± 0.21	1.63 ± 0.36	1.35 ± 0.16	1.02 ± 0.06	1.09 ± 0.13
NM_011333	MCP-1/CCL2	1.24 ± 0.11	1.21 ± 0.09	*2.59 ± 0.81	1.21 ± 0.27	1.12 ± 0.18	1.37 ± 0.23
NM_007778	M-CSF/Csf-1	0.97 ± 0.05	**0.67 ± 0.03	0.81 ± 0.09	0.96 ± 0.04	***0.58 ± 0.03	0.94 ± 0.06
NM_008599	MIG/CXCL9	–	–	–	–	–	–
NM_011337	MIP-1α/CCL3	1.45 ± 0.20	1.06 ± 0.17	1.02 ± 0.21	0.95 ± 0.12	0.74 ± 0.07	0.78 ± 0.08
NM_013652	MIP-1β/CCL4	1.25 ± 0.13	1.00 ± 0.17	1.02 ± 0.13	1.12 ± 0.15	*0.62 ± 0.10	0.90 ± 0.06
NM_009140	MIP-2/CXCL2	1.56 ± 0.25	1.31 ± 0.21	1.16 ± 0.30	1.11 ± 0.16	0.90 ± 0.09	0.93 ± 0.10
NM_013653	RANTES/CCL5	0.91 ± 0.07	0.71 ± 0.18	***2.22 ± 0.30	0.88 ± 0.14	0.55 ± 0.09	1.18 ± 0.21
NM_013693	TNF-α	1.16 ± 0.21	0.91 ± 0.14	1.09 ± 0.18	1.11 ± 0.16	0.81 ± 0.04	**1.63 ± 0.17
NM_001287056	VEGF-A	0.97 ± 0.11	0.85 ± 0.03	***0.41 ± 0.03	*0.85 ± 0.06	*0.87 ± 0.02	***0.76 ± 0.02
NM_002989	Exodus-2/CCL21	1.09 ± 0.16	1.20 ± 0.07	0.78 ± 0.10	1.03 ± 0.06	0.79 ± 0.10	*0.73 ± 0.07
NM_009142	Fractalkine/CX3CL1	1.03 ± 0.12	0.83 ± 0.08	1.25 ± 0.11	0.93 ± 0.07	**0.70 ± 0.03	1.01 ± 0.08
NM_009137	MDC/CCL22	1.34 ± 0.20	1.25 ± 0.31	1.41 ± 0.27	1.17 ± 0.15	0.91 ± 0.08	1.31 ± 0.22

TABLE IV—*continued*

Accesion #	Gene	Soleus Muscle (mRNA)			Plantaris Muscle (mRNA)		
		Sprint/Sedentary Fold change	Endurance/sedentary Fold change	Voluntary wheel running/sedentary Fold change	Sprint/Sedentary Fold change	Endurance/sedentary Fold change	Voluntary wheel running/sedentary Fold change
NM_011331	MCP-5/CCL12	1.32 ± 0.21	1.00 ± 0.11	*1.64 ± 0.19	1.05 ± 0.12	0.74 ± 0.05	0.92 ± 0.09
NM_016960	MIP-3α/CCL20	1.36 ± 0.20	1.07 ± 0.12	1.01 ± 0.21	1.05 ± 0.12	0.76 ± 0.08	0.87 ± 0.09
NM_011888	MIP-3β/CCL19	1.23 ± 0.09	1.00 ± 0.15	0.77 ± 0.11	1.10 ± 0.10	0.79 ± 0.04	0.77 ± 0.07
NM_011332	TARC/CCL17	1.38 ± 0.16	1.05 ± 0.13	1.17 ± 0.22	1.16 ± 0.14	0.60 ± 0.06	0.83 ± 0.09
NM_011593	TIMP-1	0.84 ± 0.12	0.75 ± 0.05	***8.37 ± 2.16	0.88 ± 0.14	*0.46 ± 0.05	***2.3 ± 0.23
NM_009401	CD30/ Tnfrsf8	1.36 ± 0.26	1.39 ± 0.25	1.14 ± 0.28	1.04 ± 0.13	0.67 ± 0.04	0.91 ± 0.10
NM_010560	gp130/ IL-6ST	**1.13 ± 0.03	*0.88 ± 0.03	***0.64 ± 0.03	1.05 ± 0.03	**0.87 ± 0.02	0.91 ± 0.03
NM_008362	IL-1RI	1.08 ± 0.06	1.12 ± 0.03	***0.71 ± 0.02	1.03 ± 0.05	0.95 ± 0.05	0.95 ± 0.06
NM_010555	IL-1RII	1.40 ± 0.14	1.27 ± 0.11	0.92 ± 0.13	1.05 ± 0.05	0.90 ± 0.07	0.88 ± 0.08
NM_008367	IL-2Ra	*1.53 ± 0.14	1.22 ± 0.13	0.68 ± 0.13	*1.30 ± 0.11	1.01 ± 0.05	0.78 ± 0.07
NM_001008700	IL-4R	*1.39 ± 0.14	**1.48 ± 0.08	1.18 ± 0.12	1.08 ± 0.07	1.14 ± 0.05	0.94 ± 0.05
NM_010559	IL-6R	*1.30 ± 0.10	*1.31 ± 0.07	**0.62 ± 0.06	1.16 ± 0.11	**1.37 ± 0.05	0.87 ± 0.05
NM_007425	RAGE/Ager	1.12 ± 0.11	1.13 ± 0.12	1.15 ± 0.13	1.25 ± 0.10	0.91 ± 0.07	0.96 ± 0.10
NM_011609	TNF-RI/Tnfrsf1a	1.16 ± 0.07	0.93 ± 0.03	0.88 ± 0.04	1.06 ± 0.04	**0.86 ± 0.03	1.04 ± 0.02
NM_011610	TNF-RII/Tnfrsf1b	1.27 ± 0.12	1.21 ± 0.08	1.26 ± 0.09	1.16 ± 0.05	0.95 ± 0.04	1.01 ± 0.04
NM_010228	VEGFR1/Flt1	*1.27 ± 0.09	1.10 ± 0.04	***0.52 ± 0.06	1.14 ± 0.08	0.99 ± 0.03	0.84 ± 0.03
NM_010612	VEGFR2/Kdr	0.87 ± 0.06	***0.55 ± 0.03	***0.50 ± 0.04	**0.77 ± 0.05	***0.43 ± 0.02	0.95 ± 0.04
NM_008029	VEGFR3/Flt4	1.10 ± 0.10	1.13 ± 0.06	0.81 ± 0.07	1.03 ± 0.05	0.96 ± 0.05	0.98 ± 0.04
	# of cytokine changed	6	8	14	5	13	5
	% of cytokine changed	10%	13%	22%	8%	21%	8%

some exceptions (52), in most human exercise studies, gene and protein expression analyses were performed on quadriceps muscle biopsies consisting of mixed fiber types. As such, cytokine response to exercise in different muscle fiber types in human is largely unknown. Our study thus highlighted the utility of animal models in addressing questions difficult to answer in human-based exercise studies. (6) In oxidative soleus muscle, we observed significant overlaps in cytokine profiles in response to sprint, endurance run, and chronic wheel running. In striking contrast, only minimal overlaps in cytokine profiles were observed in glycolytic plantaris muscle. Thus, not only more cytokines are changed in soleus compared with plantaris, those changes seen in soleus also show greater overlaps between different types of physical activity. These types of unexpected insights relevant to skeletal muscle physiology illustrate the advantage of assessing gene and protein expression in different muscle fiber types. (7) In a single bout of exhaustive sprint and endurance run, we observed significantly more cytokines being up-regulated than downregulated in serum and in muscle. This may reflect normal physiological stress responses associated with acute exercise. In marked contrast, chronic voluntary wheel running predominantly resulted in cytokine down-regulation in serum and muscle. These differences likely reflect skeletal muscle adaptation to chronic exercise and may be linked to general improvements in inflammatory profiles associated with regular exercise.

IL-6 is the first and most widely studied exercise-regulated cytokine (53). As expected based on previous studies in humans, we observed a significant increase in IL-6 protein level in mouse skeletal muscle in response to a single bout of sprint or endurance exercise (Table III). Interestingly, only oxidative (soleus), but not glycolytic (plantaris), muscle fibers show increased IL-6 protein level in response to sprint or endurance run. One week of voluntary wheel running, however, resulted in reduced IL-6 in both soleus and plantaris, as well as in serum. This reduction presumably reflects skeletal muscle adaptation to repeated bouts of exercise. In healthy humans subjected to 10 weeks of endurance training, contraction-induced IL-6 expression was reduced (54). Intriguingly, neither sprint nor endurance run acutely raised the circulating level of IL-6 in serum (Table II). This could be because of exercise intensity, or insufficient quantities of IL-6 being secreted into systemic circulation to acutely raise its serum level. Although most human studies reported increase plasma IL-6 levels after high intensity exercise, some studies did not observe a similar increase in plasma IL-6 after a lower intensity exercise (37). It has been noted that circulating levels of cytokines can vary widely between individuals subjected to different types of exercise (28).

Intriguingly, most of the secreted cytokine receptors (10 out of 13) profiled are altered by exercise. These include sgp130, sRAGE, sIL-1RI, sIL-1RII, sIL-4R, sIL-6R, sTNF-RI, sTNF-RII, sVEGFR1, and sVEGFR2. The soluble form of cytokine recep-
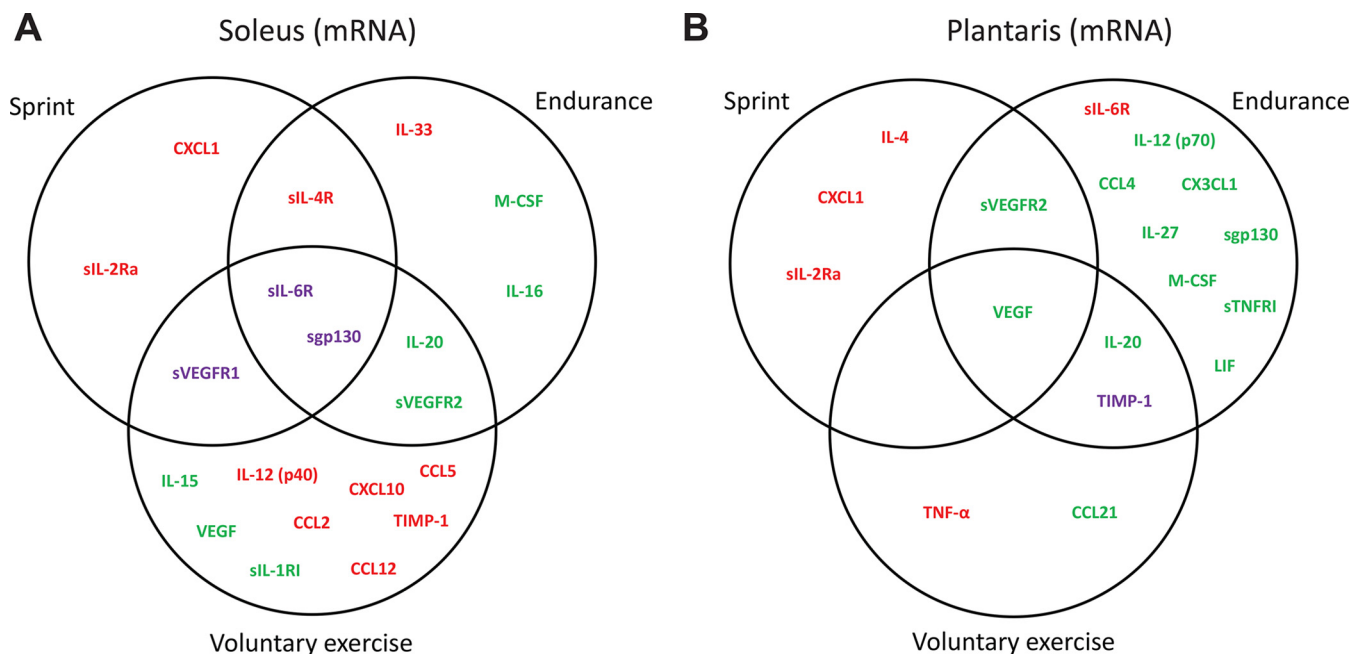
FIG. 4. **Vein diagram of cytokine mRNA altered by sprint, endurance run, or voluntary wheel running in (A) soleus and (B) plantaris muscles.** Up-regulated cytokines are highlighted in red. Downregulated cytokines are highlighted in green. Cytokines that are up-regulated in one exercise paradigm but downregulated in another are highlighted in purple.

tors in plasma can be generated by multiple mechanisms that include ectodomain shedding by zinc metalloproteinases (*e.g.* TACE/ADAM17 and ADAM19), as well as by alternative mRNA splicing (42). Protease-mediated release of receptor ectodomain has been demonstrated for TNF-RI, TNF-RII, IL-1RII, IL-4R, IL-6R, whereas the soluble form of gp130, RAGE, VEGFR1, and VEGFR2 are generated by alternative mRNA splicing (42, 55). Although previous studies have looked at soluble IL-6R levels in response to exercise in humans (56, 57), our current study represents the first attempt to quantify changes in multiple secreted cytokine receptors in response to exercise. Our results suggest that soluble cytokine receptors could be generated by potentially novel exercise-regulated proteolytic and alternative mRNA splicing mechanisms. In principle, these soluble receptors could influence cytokine signaling by functioning as receptor agonists or antagonists.

Interestingly, there is a subset of 17 circulating cytokines whose changes in serum were not matched by their corresponding changes in muscle. Presumably, these cytokines (EPO, IL-20, IL-17A/F, sgp130, sIL-4R, CCL17, G-CSF, CX3CL1, CXCL1, IL-16, TIMP-1, sTNF-RII, sTNF-RI, IFN-γ, CXCL10, CCL20, and CCL12) are produced and secreted by non-muscle tissues. These changes could be attributed to several potential mechanisms that are dependent or independent of contracting skeletal muscle. Primary cytokines, and other potential myokines, that are secreted into circulation by contracting skeletal muscle could convey a signal to other non-muscle tissues to alter the expression and secretion of secondary cytokines. Liver and adipose tissues are

also known to produce and secrete a variety of different cytokines. Given the energetics demands of contracting skeletal muscle, indirect metabolic changes in liver and adipose tissue induced by exercise could potentially also alter the expression and secretion of secondary cytokines. In support, we indeed observed parallel changes in cytokine expression in liver and/or adipose tissue in response to exercise that could, at least in part, account for their changes in circulation but not in skeletal muscle. Thus, our data highlighted the primary cytokine response of skeletal muscle and the secondary cytokine response in non-muscle tissues resulting from potential exercise-mediated tissue crosstalk.

When skeletal muscles isolated from the same mice were subjected to quantitative mRNA analysis, we found only minimal concordance between cytokine mRNA and protein levels, irrespective of exercise regimen. These observations suggest the importance of posttranscriptional regulation of cytokine expression and secretion in contracting skeletal muscle *in vivo*. Some of these posttranscriptional mechanisms could involve exercise-regulated stabilization of cytokine mRNA transcripts and/or microRNA (miRNA) mediated regulation of mRNA translation (28). Given that posttranscriptional control of cytokine/myokine expression in response to exercise is largely unexplored, our data provided a basis and impetus to further explore these underappreciated mechanisms in the context of physical activity.

Of the 66 cytokines profiled, a surprisingly large fraction is altered by acute and/or short-term exercise. Most of these altered cytokines are shown for the first time to be regulated by exercise *in vivo*. In recent studies, Scheler *et al.* profiled 23
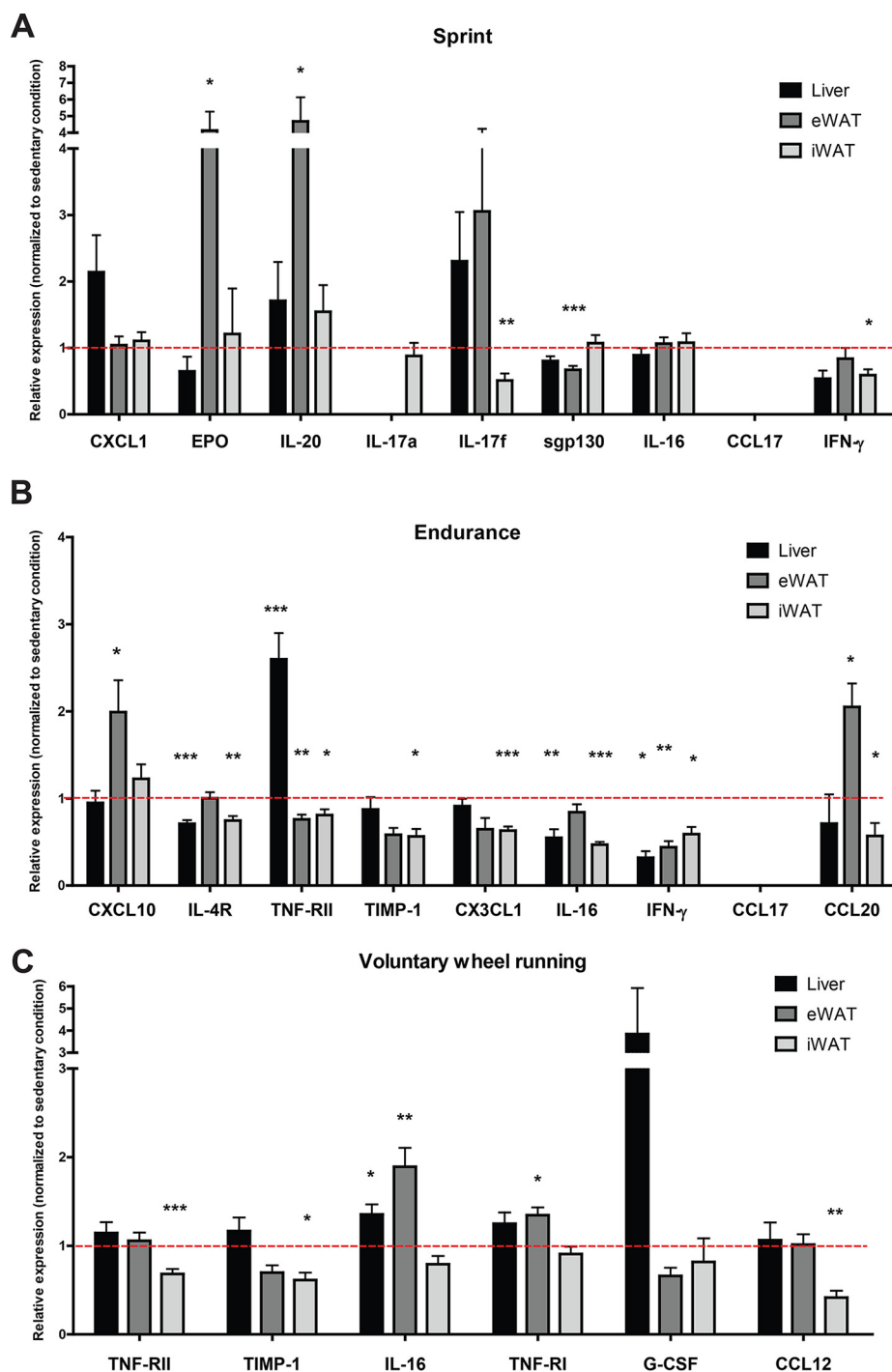
FIG. 5. **Quantitative real-time PCR analysis of select cytokine transcripts in liver and adipose tissues in response to acute and chronic exercise.** Relative mRNA expression of different cytokines in liver, visceral (epididymal) and subcutaneous (inguinal) fat depots. We only focus our analysis on those cytokines whose altered circulating levels were not matched by the corresponding changes in protein levels in skeletal muscle. All mRNA expression values were normalized to 36B4. These values were then normalized to the sedentary condition and the data are shown as average $\pm$ S.E. All p values were calculated using an unpaired t test with Welch's correction (comparing the exercise condition to the sedentary condition). *< 0.05; ** $p < 0.01$; *** $p < 0.001$.

different cytokines in cultured human myotubes using similar bead-based multiplex approach and showed that 11 cytokines are regulated by electrical pulse stimulation (27); these include IL-8, CXCL1, IL-6, LIF, IL-4, IL-13, IL17A, IL-1β, G-

CSF, TNF, and CCL2. Raschke *et al.* used an antibody array to profile 179 peptides (covering different classes of secretory proteins in addition to cytokines), and identified 23 novel exercise regulated cytokines in electrical pulse stimulated

TABLE V

Summary of all the statistically significant changes in cytokine mRNA and protein levels in response to sprint (S), endurance run (E), and voluntary wheel running (VWR)

Analyte	Serum Protein			Soleus Protein			Soleus mRNA			Plantaris Protein			Plantaris mRNA		
	S	E	VWR	S	E	VWR	S	E	VWR	S	E	VWR	S	E	VWR
G-CSF			↓												
Eotaxin-1/CCL11						↓									
GM-CSF															
IFN-β1															
IFN-γ	↓	↓	↓	↑	↑	↓						↓			
IL-1α				↑	↑	↓						↓			
IL-1β				↑	↑						↑				
IL-2					↑	↓						↓			
IL-3															
IL-4												↓	↑		
IL-5															
IL-6			↓	↑	↑	↓						↓			
IL-7															
IL-9					↑	↓						↓			
IL-10				↑											
IL-11															
IL-12 (p40)				↑	↑	↓			↑			↓			
IL-12 (p70)														↓	
IL-13															
IL-15					↑	↓			↓	↓		↓			
IL-16	↓	↓	↓			↑		↓							
IL-17A															
IL-17A/F	↓														
IL-17F															
IL-20	↑					↑		↓	↓					↓	↓
IL-21															
IL-22				↑		↓						↓			
IL-23															
IL-27														↓	
IL-28B															
IL-33					↓	↓	↑					↑			
IP-10/CXCL10		↓		↑	↑	↓			↑			↓			
KC/CXCL1	↑	↑	↓		↑	↓	↑					↓	↑		
LIF														↓	
LIX/CXCL5															
MCP-1/CCL2									↑						
M-CSF								↓				↓		↓	
MIG/CXCL9												↓			
MIP-1α/CCL3											↑			↓	
MIP-1β/CCL4														↓	
MIP-2/CXCL2		↑			↑										
RANTES/CCL5				↑	↑	↓			↑			↓			
TNF-α															↑
VEGF				↓	↓	↓			↓	↓	↓	↓	↓	↓	↓
Exodus-2/CCL21					↓							↓			
Fractalkine/CX3CL1		↑				↓								↓	
MDC/CCL22															
MCP-5/CCL12		↓	↓		↓	↑					↑				
MIP-3α/CCL20		↓	↓		↑	↓						↓			
MIP-3β/CCL19						↑									
TARC/CCL17	↓	↓													
TIMP-1		↓	↓			↑			↑			↑		↓	↑
sCD30															
sgp130	↓					↓	↑	↓	↓						
sIL-1RI						↓			↓			↓			
sIL-1RII										↑					
sIL-2Ra							↑						↑		
sIL-4R		↓					↑	↑							
sIL-6R							↑	↑	↓	↑				↑	
sRAGE						↓						↓			
sTNFRI		↓	↓		↓									↓	
sTNFRII		↓	↓			↑									
sVEGFR1					↓	↓	↑		↓						
sVEGFR2				↓	↓	↓		↓	↓				↓	↓	
sVEGFR3															
EPO	↑				↑										

human myotubes (26); these include IL-1α, IL-3, IL-10, IL-16, IL-22, IL-28A, IL-29, IL-31, IFN-γ, TPO, RAGE, CCL1, CCL4, CCL15, CCL17, CCL18, CCL21, CCL23, CCL24, CCL26, CXCL6, CXCL9, and CXCL13. Of the known exercise-regulated cytokines reported in cultured human myotubes (26, 27), we were able to confirm most them *in vivo*, in particular those cytokines that were included in our multiplex assay. This suggests that many of the cytokine responses we observed are most likely because of changes in the expression and secretion of cytokines from skeletal myotubes. Our results validate the use of rodent exercise models to study cytokine/myokine response in humans. Excluding these recently described exercise-regulated cytokines in cultured human myotubes, our *in vivo* mouse studies—involving three different exercise regimens and two different muscle fiber types—identified an additional 23 novel exercise-regulated cytokines/myokines not previously reported. These include IL-9, IL-12 (p40), IL-20, IL-33, M-CSF, CXCL10, CXCL2, CCL5, CX3CL1, CCL3, CCL12, CCL19, CCL20, EPO, TIMP-1, sgp130, sIL-1RI, sIL-1RII, sIL-6R, sTNF-RI, sTNF-RII, sVEGFR1, and sVEGFR2. Of these novel exercise-regulated myokines, a subset (IL-20, CXCL2, CX3CL1, CXCL10, CCL12, CCL20, sTNF-RI, sTNF-RII, TIMP) can be considered "exercise factors" based on the definition that they are produced by skeletal muscle in response to exercise and are secreted into the circulation (11).

Most of the exercise-regulated cytokines we uncovered play myriad roles in the immune and non-immune systems. Many cytokine functions are physiological context-dependent, and their effects are cell-type specific (38); their excessive production or suppression are often causally linked to disease. In the context of exercise, the levels of a surprisingly large number of cytokines are changed either locally within skeletal muscle or systemically. It appears that both the pro- and anti-inflammatory cytokines are altered by acute and chronic exercise in rodents (Table V) and in humans (58). It has been suggested that the beneficial local and systemic effects of chronic exercise are because of exercise-induced hormesis, whereby a low level of stress up-regulates existing cellular and molecular pathways that improve whole-body physiology (*e.g.* metabolism and immunity) (58, 59). Thus, it may be a specific combination of cytokines (with diverse and opposing functions) whose direction, magnitude, and duration of change underlie exercise-induced hormesis. In this context, it is worth noting that chronic exercise with running wheel induced more cytokine changes than either an acute low-intensity (endurance) or high-intensity (sprint) exercise. Although this is beyond the scope of the present study, future investigations using genetic gain- or loss-of-function mouse models of select cytokines are clearly needed to further pinpoint the biological effects of muscle-derived cytokines/myokines in muscle and non-muscle tissues.

Several limitations and caveats are noted in the present studies. First, we did not analyze all the known cytokines discovered to date. This is limited by cost and the availability of cytokine-specific multiplex reagents. Second, we did not use an *in vitro* exercise model (*e.g.* EPS-stimulated myotubes) to directly show that the cytokine changes we see in soleus and plantaris muscle fibers are indeed derived from skeletal myotubes and not from other potential cell types such as satellite cells, endothelial cells, fibroblastic cells, and resident immune cells. It is known that eccentric or resistance exercise, or prolonged running (*e.g.* marathon), in humans result in muscle damage, leading to the influx of inflammatory leukocytes that can be the source of cytokines up-regulated in muscle [extensively reviewed in (60)]. Although we cannot rule out this possibility, the type and duration of exercise the mice were subjected to in our study generally do not cause significant muscle damage. Indeed, when obese mice were subjected to endurance interval training, significantly less inflammation and macrophage infiltration were observed in skeletal muscle (61). Because our *in vivo* study confirmed most of the exercise-regulated cytokines in cultured human myotubes (26, 27), we presume the novel exercise-regulated cytokines found in the present study are likely to be derived from myotubes. Third, we only measured transcript, and not protein, levels of a select set of cytokines in liver and adipose tissues. Thus, whether changes in mRNA translate into changes in protein levels remain to be determined. Fourth, we did not directly demonstrate the release of cytokines from exercised muscle into circulation. Although this may be technically challenging to perform in mice, *in situ* cytokine flux analysis will provide further insights into how different muscle fiber types modulate the production and secretion of cytokines in response to different exercise. Fifth, although mice run only during the dark cycle (62), one potential caveat of the voluntary exercise group was that the mice had access to the running wheel until the morning before they were sacrificed. However, when we examined the cytokine profiles, the number and type of cytokine changes were significantly different between acute (sprint and endurance run) and chronic (wheel running) exercise groups despite some overlaps (Fig. 1 and 2), suggesting that we could discriminate between the two exercise effects. Sixth, the pre-exercise values of some cytokines in the voluntary wheel running group were significantly higher than the pre-exercise values for the sprint and endurance groups. The reason for this is unknown. Although all mice were handled identically, the serum samples from the wheel running group were not analyzed at the same time as the sprint and endurance groups. However, samples that were directly compared with one another or used for normalization were always analyzed on the same plate; specifically, serum samples from the pre- and post-sprint and endurance groups were run together while the samples from the pre- and post-wheel running were run together. Our analyses focused on the changes in cytokine levels in each pre- and post-exercise condition. We do not directly compare serum data between exercise regimens. Thus, the differences in absolute values

between exercise conditions should not affect the interpretation of our data. Despite these limitations, our current studies reveal important insights concerning exercise regulation of local and systemic cytokine levels.

In summary, our current cytokine profiling studies in mice, along with other published studies on exercise-regulated myokines in humans and animal models, indicate that physical activity can significantly alter the skeletal muscle secretome. Many myokines/cytokines have pleiotropic functions, and some may have opposite effects depending on biological and physiological contexts. Given this complexity, we speculate that a right combination, magnitude, and direction of myokine changes induced by regular exercise, rather than one or two dominant secretory proteins, are likely responsible for the multi-organ effects and health benefits of exercise. The inherent systems-level response to physical activity may pose a major challenge to produce an "exercise pill" that can mimic and recapitulate all the local and systemic physiological and adaptive processes induced and suppressed by regular exercise.

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