The following is the abstract of the article discussed in the subsequent letter.

Rhind, Shawn G., John W. Castellani, Ingrid K. M. Brenner, Roy J. Shephard, Jiri Zamecnik, Scott J. Montain, Andrew J. Young, and Pang N. Shek. Intracellular monocyte and serum cytokine expression is modulated by exhausting exercise and cold exposure. Am J Physiol Regulatory Integrative Comp Physiol 281: R66–R75, 2001.—This study tested the hypothesis that exercise elicits monocyte cytokine expression and that prolonged cold exposure modulates such responses. Nine men (age, 24.6 ± 3.8 yr; \( \dot{V}\)O₂ peak, 56.8 ± 5.6 ml·kg⁻¹·min⁻¹) completed 7 days of exhausting exercise (aerobic, anaerobic, resistive) and underwent three cold, wet exposures (CW). CW trials comprised ≤6 h (six 1-h rest-work cycles) exposure to cold (5°C, 20 km/h wind) and wet (5 cm/h rain) conditions. Blood samples for the determination of intracellular and serum cytokine levels and circulating hormone concentrations were drawn at rest (0700), after exercise (~1130), and after CW (~2000). Whole blood was incubated with (stimulated) or without (spontaneous) lipopolysaccharide (LPS; 1 μg/ml) and stained for CD14 monocyte surface antigens. Cell suspensions were stained for intracellular cytokine expression and analyzed by flow cytometry. The proportion of CD14⁺ monocytes exhibiting spontaneous and stimulated intracellular expression of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF-α) increased after exercise, but these cells produced less IL-1β and TNF-α after CW when CW was preceded by exhausting exercise. Serum cytokine concentrations followed a parallel trend. These findings suggest that blood monocytes contribute to exercise-induced cytokinemia and that cold exposure can differentially modulate cytokine production, upregulating expression of IL-6 and IL-1 receptor antagonist but down-regulating IL-1β and TNF-α. The cold-induced changes in cytokine expression appear to be linked to enhanced catecholamine secretion associated with cold exposure.

The Cellular Origin of Plasma Cytokine Expression After Acute Exercise

To the Editor: In a very important study, Rhind et al. (4) recently reported that 1) intracellular monocyte cytokine production contributes to the exercise-induced cytokinemia, 2) the increase in cytokine production in monocytes corresponds with the increase in plasma cytokine concentration, and 3) the effects are likely mediated by alterations in the sympathoadrenal response. In reporting these results, Rhind et al. (4) have not compared their results with anomalous previously published work. In contrast with the data by Rhind et al. (4), convincing evidence demonstrates that monocytes do not contribute to the exercise-induced increase in plasma levels of interleukin (IL)-6 and tumor necrosis factor (TNF-α). In a recent study (5), published before the submission by Rhind et al. (4), human subjects exercised for 120 min on a cycle ergometer at 70% of peak oxygen uptake (\( \dot{V}\)O₂ peak) on two occasions with or without the ingestion of carbohydrate. Starkie et al. (5) observed that neither the number nor the percentage of monocytes producing IL-6 or TNF-α increased as a result of an acute exercise bout. Importantly, the amount of spontaneously produced cytokine in each monocyte was either unchanged or decreased after exercise (5). This demonstrated that the circulating monocytes could not have been the source of the exercise-induced cytokinemia. Moreover, carbohydrate ingestion, which blunted the epinephrine response, did not affect monocyte intracellular cytokine production. It was concluded, therefore, that circulating monocytes are not the source of the exercise-induced increase in plasma IL-6 and that attenuating the sympathoadrenal response has no effect on monocyte cytokine production. In this study (5), the increase in plasma IL-6 was subtle, and in a subsequent study (7), blood was sampled from highly performed marathon runners before and after a marathon run. Despite an increase in plasma IL-6 from 2 pg/ml at rest to 120 pg/ml after the marathon, the number of monocytes spontaneously producing IL-6 and the mean fluorescence intensity of the cells producing IL-6 actually decreased as a result of exercise. It was again concluded that monocytes do not contribute to the exercise-induced increase in IL-6 or, for that matter, TNF-α. Of note, the absence of any increase in monocyte intracellular cytokine production was observed in the face of large increases in epinephrine and norepinephrine, again suggesting that the catecholamines do not increase cytokine production by leukocytes. To confirm that adrenergic stimulation does not mediate leukocyte cytokine production, it was recently demonstrated that combined β- and α-adrenergic blockade does not affect the exercise-induced suppression of lymphocyte cytokine production (8). These previous studies (5, 7, 8) are not anomalous with existing literature. Indeed, Rhind et al. (4) concede that previous studies (one from their own laboratory) have not observed increases in cytokine gene expression in monocytes after prolonged exercise (2, 11). In addition, it has been demonstrated that IL-6 gene expression is not only observed in resting skeletal muscle but that gene expression increases severalfold in response to acute exercise (3, 6). In addition, in a recent study, which utilized the arteriovenous balance technique, Steensberg et al. (9) demonstrated that the exercise-induced increase in IL-6 could be accounted for solely by the release from contracting skeletal muscle during prolonged single-legged kicking. Taken together, the existing literature demonstrates that monocyte intracellular cytokine production does not increase in response to exercise.
to acute exercise and that contracting skeletal muscle is a more likely candidate.

Why then do the data of Rhind et al. (4) contradict the evidence that suggests that monocytes do not contribute to the increase in plasma cytokines after acute exercise? The methodology appears robust; in fact, it is remarkably similar to the methodology extensively reported in a previous study (5). However, there are subtle differences in the analyses of the data that require consideration. Rhind et al. (4) report their intracellular data as percentage of CD14+ cells producing each individual cytokine. Although reporting percentages provides useful information, it does not allow for sufficient analyses to make definitive conclusions. Since the authors do not provide intracellular data as percentage of CD14+ cells producing each individual cytokine, one cannot truly ascertain whether the cytokine production by monocytes actually contributes to the exercise-induced increase in plasma cytokine concentration. To fully interpret monocyte intracellular cytokine production, the percentage of monocytes producing each cytokine must be reported in conjunction with the number of monocytes producing cytokine and the fluorescence intensity. In the case of Rhind et al. (4), it is crucial because of mitogen binding. CD14+ may not be the ideal cell surface marker antibody, particularly when analyzing lipopolysaccharide (LPS)-stimulated blood, because LPS has been found to bind to CD14+ and increase its cell surface expression (1). It would appear, therefore, that CD33+ is a more appropriate cell surface staining antibody to utilize. Notwithstanding any methodological flaws associated with the data of Rhind et al. (4), it is plausible that the data represent an accurate indication of the immunological response to exercise. In fact, we believe that their data are complementary to those published previously. However, it is surprising to us that the authors would suggest that the findings “demonstrate that blood monocytes are a source of IL-1β, IL-1 receptor antagonist (IL-1ra), IL-6, and TNF-α production after acute, strenuous exercise” when they appear not to have examined the effect of acute, strenuous exercise on monocyte cytokine production. Despite the fact that the authors state that blood samples were obtained “at rest (0700), 30 min after fatiguing exercise (~1130), and 30 min after cold wet exposure (~2000) on days 1 and 8,” this appears not to be the case. Figures 1–3 of their study all indicate that exercise was not performed on day 1. Therefore, it is quite plausible that the combination of 8 days of exercise and cold, wet exposure would lead to cytokine production in immunocompetent cells. However, to suggest that the results indicate that monocytes are a source of plasma cytokine concentration after “acute” exercise appears misleading. Furthermore, the assertion that monocyte cytokine production is mediated by the sympathoadrenal response is based on a temporal relationship and not supported by studies where the catecholamines have been perturbed by an experimental intervention.

REFERENCES


10. Ullum H, Haar PM, Dianant M, Palme Halker-Kristensen J, and Pedersen BK. Exercise enhances plasma IL-6 but does not change IL-α, IL-1ß, IL-6 or TNF-α pre-mRNA in BMNC. J Appl Physiol 77: 93–97, 1994.

Mark A. Febbraio
Copenhagen Muscle Research Centre
Rigshospitalet, Section 7652
DK-2200 Copenhagen N, Denmark
E-mail: m.febbairo@physiology.unimelb.edu.au

Rebecca L. Starkie
Department of Physiology
The University of Melbourne
Parkville, Victoria 3010, Australia

REPLY

To the Editor: We appreciate the opportunity to respond to the letter by Febbraio and Starkie with respect to our publication in the American Journal of Physiology: Regulatory, Integrative and Comparative Physiology (9) concerning the unresolved issue of the cellular origin of cytokines during exercise. Febbraio and Starkie contend that blood “monocytes are not the source” of the exercise-induced cytokinemia, suggesting instead that “contracting skeletal muscle is a more

REFERENCES
likely candidate” for peripheral cytokine release. We agree with their assertion that methodological differences between our studies could account for the disparate findings concerning intracellular (IC) monocyotic cytokine production; however, in light of evidence presented in this rebuttal, we submit that the data of Febbraio and colleagues do not prove categorically that monocytes cannot be a source of cytokines with acute exercise.

Although we concur that the choice of cell surface markers is crucial to the validity of results obtained when assessing IC cytokine expression, we dispute the view that the pan-myeloid marker CD33 is “more appropriate” than CD14 as a cell surface marker for blood monocytes. In fact, increased sophistication in multiparameter cell phenotype analysis by flow cytometry demonstrates that monocytes are an extremely heterogeneous cell population (comprising at least 5 subsets) with respect to their expression of an array of surface markers and their functional characteristics, including the pattern of cytokines they produce (1). Recognizing this heterogeneity, we consider the use of any single “lineage-specific” surface marker to be less than ideal for the identification of blood monocytes.

Consequently, we routinely employ a multicolor staining protocol, which has been adopted for the consensus definition of human blood monocytes (18). This approach relies on coexpression of CD14, CD16, CD33, and HLA-DR surface antigens and provides a clear distinction of both phenotypically and functionally discrete monocyte subpopulations (1). This includes the identification of two main monocyte subsets, on the basis of their cytokine production profiles: a CD14bright, CD16dim/−CD33bright subset, which expresses low levels of cytokines, and a CD14dimCD16brightCD33dim subset, which expresses high levels of inflammatory cytokines (Fig. 1, A and B, respectively). The latter subset has been likened to a “circulating macrophage” (12, 19) and is known to be rapidly and selectively mobilized from the marginal pool (via β-adrenergic mechanisms) with exercise (3, 7, 16). Also, using this staining combination allows for the exclusion of CD33bright, CD14−CD16−, and HDL-DR+ peripheral blood dendritic cells; this is notable because dendritic cells exhibit a unique pattern of cytokine production (1, 2) and also increase appreciably with exercise (4, 17).

Although we were unable to exploit this multicolor staining approach in our collaborative field study with the U.S. Army (9), we have used it extensively to identify a panel of IC cytokines in critically ill patients (10, 11) and subsequently to characterize IC cytokine expression with acute exercise (Fig. 2). While IC cytokine expression may not change significantly with acute exercise when gating on total CD14 monocytes, a more detailed subset examination demonstrates significant increases in the expression (percentage and MFI) of IC cytokines by CD14dimCD16bright monocytes. For example, in the case of IL-6, a postexercise doubling of MFI by CD14dimCD16bright cells is offset by concomitant reductions in the CD14brightCD16dim− subset. At the same time, CD14dimCD16bright cell numbers increase dramatically (3- to 4-fold) with exercise (7, 16); hence it follows that net peripheral cytokine production by this subset would be enhanced after exercise.

Given this, we feel that measures of total monocyotic cytokine expression, in the absence of specific subset analysis, are inadequate and may lead to the erroneous conclusion that monocyotic cytokine production is unaffected by exercise. Likewise, we believe that the use of CD33 (in conjunction with side scatter) as the sole marker for the putative identification of blood monocytes is inappropriate and misleading because it does not adequately allow for the distinction of these important functional subsets and may in fact lead to con-
Fig. 2. Analysis of blood monocyte subsets by flow cytometry. A and B: dot plots show representative data illustrating the sequential gating method developed to identify CD14\textsuperscript{bright}CD16\textsuperscript{dim} and CD14\textsuperscript{dim}CD16\textsuperscript{bright} subpopulations in lysed whole blood after 4-color staining. C–E display the pattern of differential intracellular (IC) IL-1\beta, TNF-\alpha, and IL-6 expression at rest and after cycle ergometer exercise (EX; 75\% Vo\textsubscript{2peak}) to exhaustion in 6 healthy donors. Evaluation of monocytic cytokine production was based on both the percentage (±SE) of positive cells and mean fluorescence intensity (MFI; channel ± SE) from 5,000 events within each monocyte subset. Differences in MFI for all cytokines were measured on a single parameter FL2 histogram using a linear scale (arbitrary units scaled from 0–10,000) after subtracting MFI values obtained from corresponding isotype-matched negative controls. *Significant differences in IC cytokine expression by ANOVA, \( P < 0.01 \). FITC, fluorescein isothiocyanate; APC, allophycocyanin; PerCP, peridinin chlorophyll protein.

Founding results due to the inclusion of peripheral blood dendritic cells.

Furthermore, we dismiss the idea that LPS stimulation can cause an artifactual increase in CD14 surface marker expression. This notion is simply not supported by the literature or by our own findings. On the contrary, there is overwhelming evidence that long-term LPS stimulation (≥24 h) leads to CD14 receptor shedding and downregulation of CD14 surface density. We have extensively evaluated the kinetics of CD14 receptor expression using multiple incubation times, and in our hands, LPS stimulation does not significantly reduce either the percentage of CD14\textsuperscript{+} cells or their MFI, over relatively short (3–4 h) incubation periods. Indeed, the bulk of the published literature on monocytic IC cytokine staining supports the use of anti-CD14 as the single marker of choice (1, 6, 13).

Another important methodological issue that deserves mention is related to the addition of the cytokine secretion inhibitor brefeldin A (BFA) to unstimulated whole blood for the analysis of spontaneous IC cytokine production. We have learned from experience with clinical specimens that when measuring spontaneous monocytic cytokine expression, it is critical to prespike heparinized vacutainers with BFA before blood draw. This ensures a more accurate snapshot of in vivo cytokine expression at the time of sampling by preventing the loss of cytokines from the producing cells during specimen handling. In fact, we have observed as much as a 10% increase in the proportion of cytokine-positive monocytes using this approach, compared with post-sampling addition of BFA. Obviously, this is very important when trying to accurately quantify the relatively small changes in cytokine production with exercise and could be especially significant in field trials, where longer sample processing times are common, such as the design involving marathon runners described by Starkie et al. (15).

Recent studies present a compelling argument that active skeletal muscle is a significant source of IL-6 (and possibly other cytokines) with exercise (5, 8); however, these studies do not preclude the contribution of peripheral immune cells to circulating cytokemia. In fact, the issue of which cell types within muscle are responsible for enhanced cytokine production is unresolved. Myoblasts are known to synthesize various cytokines, but it remains plausible that resident or infiltrating cells (including cells of the monocytic lineage) contribute to intramuscular cytokine production. Moreover, recent data from Starkie et al. (14) identify a discordance between changes in intramuscular IL-6 mRNA levels and plasma IL-6 concentration after exercise, leading them to conclude that muscle cannot “solely account for exercise-induced increases in plasma IL-6” and that “IL-6 production by tissues other than skeletal muscle” must exist. Nevertheless, the authors discount the possibility that blood monocytes might contribute to IL-6 production on the basis of their previous findings (15), which used CD33 alone to quantify monocytic IL-6. We would strongly suggest that this conclusion warrants reevaluation and further postulate that circulating CD14\textsuperscript{+}CD16\textsuperscript{+} monocytes could well contribute to the shortfall in IL-6 production.

Collectively, the data presented here clearly demonstrate that specific subsets of blood monocytes (i.e., CD14\textsuperscript{+}CD16\textsuperscript{+} cells) can be a source of cytokine production in association with acute exercise and that it is no longer sufficient to define monocytes on the basis of a single marker. A more appropriate approach for the identification of blood monocytes and their distinct functional attributes (i.e., cytokine production) re-
quires the use of multiple cell surface markers. We propose that future exercise immunology studies should follow a more standardized method for the identification of monocytes to better characterize the functional role of these important exercise-responsive cells. In short, reports of the demise of the monocyte with exercise seem greatly exaggerated.

REFERENCES


Shawn G. Rhind
Biomedical Sciences Section
Defence & Civil Institute of Environmental Medicine
Toronto, Ontario M3M 3B9, Canada
E-mail: shawn.rhind@dciem.dnd.ca

Pang N. Shek
Biomedical Sciences Section
Defence & Civil Institute of Environmental Medicine
Toronto, Ontario M3M 3B9, Canada