Supplemental data

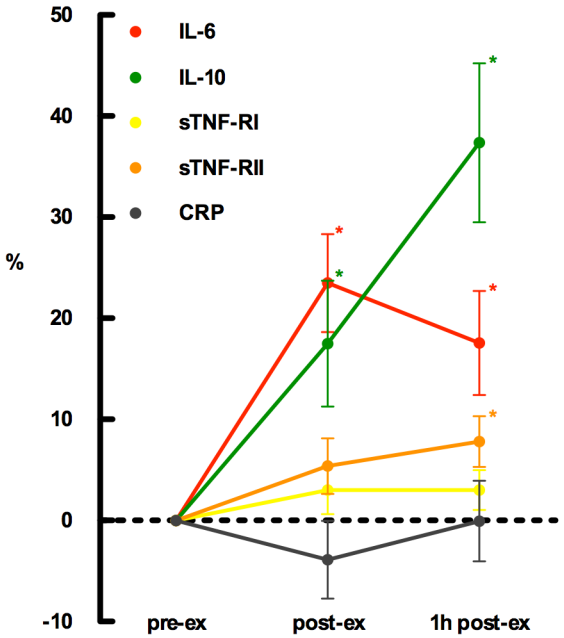

Figure 1. Effects of acute exercise: relative changes from pre-exercise in plasma markers of systemic inflammation. IL: interleukin; sTNF-R: soluble tumour necrosis factor receptor; CRP: C-reactive protein. Data are mean ± SEM (n=15). *$P < 0.05$ vs. pre-exercise for illustration purposes (statistical analysis performed on absolute values).

Supplemental methods

1. ENZYME-LINKED IMMUNOSORBENT ASSAYS

Interleukin-6 and interleukin-10 assays

ELISAs for detection of IL-6 and IL-10 levels in plasma samples were developed and optimised using the capture and detection antibodies included in BD OptEIA ELISA sets (IL-6: 555220 and IL-10: 555157, BD Biosciences, Oxford, UK), National Institute for Biological Standards and Control (NIBSC) standards (IL-6: 89/548 and IL-10: 93/722, NIBSC, Potters Bar, Hertfordshire, UK) and an ELISA Amplification System (19589-109, Invitrogen, Paisley, UK). 96-well ELISA plates (Nunc-Immuno 439454 MaxiSorp) were coated with the anti-human IL-6 or IL-10 capture antibody diluted at the lot-specific recommended dilution in 0.05 M sodium carbonate buffer at 100 µl/well, sealed and incubated overnight at 4ºC. The next day, plates were washed 3 times (at 300 µl/well) with tris-buffered saline (TBS) with 0.05% Tween 20 (TBS-T) and blocked for 1 h at room temperature with 5% BSA (Probumin, Millipore, Illinois, USA) in TBS at 200 µl/well. Plates were subsequently washed 3 times with TBS-T and duplicates of samples (neat), standards (serially diluted in TBS with 10% FCS from 200 pg/ml to 0.78 pg/ml) and blank (TBS with 10% FCS) were added at 100 µl/well. Plates were again sealed and incubated overnight at 4ºC. The following day, plates were washed 6 times with TBS-T and the biotinylated anti-human IL-6 or IL-10 detection antibody diluted at the lot-specific recommended dilution in TBS-T with 1% BSA was added at 100 µl/well. Plates were incubated for 2 h at room temperature and subsequently washed 7 times with 30 seconds soaks with TBS-T. Streptavidin (SAv)-alkaline phosphatase conjugate (554065, BD Biosciences, Oxford, UK) diluted 1:2000 in TBS with 1% BSA was then added at 100 µl/well and plates were incubated for a further 1 h at room temperature. After a final wash (7 times with 30 seconds soaks with TBS-T), the substrate and amplifier solutions (prepared according to the manufacturer's instructions) were added (at 50 µl/well each) in two subsequent steps (incubated for approximately 25 min at 25ºC each). The reaction was

stopped with 0.3 M sulphuric acid (at 50 µl/well) and the plates were immediately read at 490 nm. Samples' concentrations were determined by relation to a standard curve generated by plotting the standards' absorbances against the log of the standards' concentrations using a four-parameter logistic equation (Graphpad Prism version 5, Graphpad Software, La Jolla, CA, USA). All samples from the same patient were assayed on the same plate. Duplicates with a CV above 10% were repeated. The inter- and intra-assay CV were 4.4% and 3.4% for IL-6 and 5.1% and 3.8% for IL-10, respectively.

Soluble tumour necrosis factor receptors I and II assays

ELISAs for detection of sTNF-RI and sTNF-RII in plasma samples were developed and optimised using the capture and detection antibodies and standards included in R&D DuoSet ELISA Development kits (sTNF-RI: DY225 and sTNF-RII: DY726, R&D Systems, Abingdon, UK). 96-well ELISA plates (Nunc-Immuno 439454 MaxiSorp) were coated with the anti-human sTNF-RI or sTNF-RII capture antibody (previously reconstituted according to the manufacturer's instructions) diluted 1:200 in 0.05 M sodium carbonate buffer at 65 µl/well, sealed and incubated overnight at 4ºC. The next day, plates were washed 4 times (at 200 µl/well) with PBS with 0.1% Tween 20 (PBS-T.1) and blocked for 1 h at room temperature with 1% BSA (Probumin, Millipore, Illinois, USA) in PBS at 100 µl/well. Plates were subsequently washed 4 times with PBS-T.1 and duplicates of samples (diluted 1:10 in PBS), standards (serially diluted in PBS with 1% BSA from 10 ng/ml to 10 pg/ml and 18 ng/ml to 18 pg/ml for sTNF-RI and sTNF-RII assays, respectively) and blank (PBS with 1% BSA) were added at 50 µl/well. Plates were again sealed and incubated overnight at 4ºC. The following day, plates were washed 4 times with PBS-T.1 and the biotinylated anti-human sTNF-RI or sTNF-RII detection antibody (previously reconstituted according to the manufacturer's instructions) diluted 1:200 in PBS was added at 50 µl/well. Plates were incubated for 2 h at room temperature and subsequently washed 4 times with PBS-T.1. HRP Avidin D (A-2004, Vector Laboratories, Peterborough, UK) diluted 1:2000 in PBS was then added at 50 µl/well

and plates were incubated for a further 1 h at room temperature. After a final wash (4 times with PBS-T.1), an OPD substrate solution (S2045, Dako, Glostrup, Denmark), prepared according to the manufacturer's instructions, was added at 50 µl/well. Approximately 5 min after or when suitable colour has developed, the reaction was stopped with 1 M sulphuric acid (at 75 µl/well) and the plates were immediately read at 490 nm. Samples' concentrations were determined by relation to a standard curve generated by plotting the standards' absorbances against the log of the standards' concentrations using a four-parameter logistic equation (Graphpad Prism version 5, Graphpad Software, La Jolla, CA, USA). All samples from the same patient were assayed on the same plate. Duplicates with a CV above 10% were repeated, as were samples that fell beyond the dynamic range of each assay standard curve (adjusting the dilution factor accordingly). The inter- and intra-assay CV were 3.6% and 2.6% for sTNF-RI and 4.5% and 2.9% for sTNF-RII, respectively.

C-reactive protein assay

An ELISA for detection of CRP levels in plasma samples was developed and optimised using a method adapted from Pawluczyk *et al.*[1] 96-well ELISA plates (Nunc-Immuno 439454 MaxiSorp) were coated with anti-human CRP rabbit polyclonal antibody (235752, Calbiochem, Merck Chemicals, Nottingham, UK) diluted to a final concentration of 5 µg/ml in 0.05 M sodium carbonate buffer at 65 µl/well, sealed and incubated overnight at 4ºC. The next day, plates were washed 4 times (at 200 µl/well) with PBS-T.1 and blocked for 1 h at room temperature with 1% BSA in PBS at 100 µl/well. Plates were subsequently washed 4 times with PBS-T.1 and duplicates of samples (diluted 1:100 in PBS), CRP standards (85/506, NIBSC, Potters Bar, Hertfordshire, UK, serially diluted in PBS with 1% BSA from 1 µg/ml to 1 ng/ml) and blank (PBS with 1% BSA) were added at 50 µl/well. Plates were again sealed and incubated overnight at 4ºC. The following day, plates were washed 4 times with PBS-T.1 and anti-human CRP mouse monoclonal antibody (ab8279, Abcam, Cambridge, UK) diluted 1:750 in PBS was added at 50 µl/well. Plates were incubated for 2 h at room

temperature and subsequently washed 4 times with PBS-T.1. HRP conjugated anti-mouse immunoglobulins rabbit polyclonal antibody (P0260, Dako, Glostrup, Denmark) diluted 1:1000 in PBS was then added at 50 µl/well and plates were incubated for a further 1 h at room temperature. After a final wash (4 times with PBS-T.1), an OPD substrate solution (S2045, Dako, Glostrup, Denmark), prepared according to the manufacturer's instructions, was added at 50 µl/well. Approximately 5 min after or when suitable colour has developed, the reaction was stopped with 1 M sulphuric acid (at 75 µl/well) and the plates were immediately read at 490 nm. Samples' concentrations were determined by relation to a standard curve generated by plotting the standards' absorbances against the log of the standards' concentrations using a four-parameter logistic equation (Graphpad Prism version 5, Graphpad Software, La Jolla, CA, USA). All samples from the same patient were assayed on the same plate. Duplicates with a CV above 10% were repeated, as were samples that fell beyond the dynamic range of each assay standard curve (adjusting the dilution factor accordingly). The inter- and intra-assay CV were 3.2% and 2.9%, respectively.

2. FLOW CYTOMETRY DATA ANALYSIS

T-lymphocyte analysis

SSC vs. FSC plots of all cells acquired were used to gate on the lymphocyte population by morphology (Figure 2 a). Subsequent SSC vs. FL1 or FL3 plots of the lymphocyte population were used to gate, respectively, on the CD4 positive population ($CD4^{+}$, helper T-cells; Figure 2 b) or on the CD8 brightly positive population ($CD8^{++}$, cytotoxic T-cells; Figure 2 c). This gating strategy for cytotoxic T-cells was used because it is known that all lymphocytes that express CD8 at high fluorescence intensities also express CD3 and that this population forms the majority of the total cytotoxic T-cell blood pool, while on the other hand, lymphocytes that express CD8 at low fluorescence intensities also include natural killer cells.[2,3] Further FL2 vs. FL1 or FL3 plots of $CD4^{+}$ or $CD8^{++}$ lymphocytes, respectively, were used to determine the percentage the $CD4^{+}$ and $CD8^{++}$ lymphocytes expressing CD69 and the geometric mean of fluorescence intensity (GMFI) of CD69 in these cells (Figure 2 d, e, f and g). The unstimulated samples were used to define the threshold of positive staining and only SEB-stimulated data are reported.

Monocyte analysis

FL2 vs. FL1 plots of all cells acquired were used to gate on the $CD14^{+}CD86^{+}$ cells (Figure 3 a). Subsequent FL3 vs. FL1 plots of the $CD14^{+}CD86^{+}$ cells were used to gate on the $CD14^{+}CD86^{+}$HLA-DR^{+} cells (Figure 3 b). Backgating analysis was then performed to ensure the gated cells fell on the monocyte region by morphology on SSC vs. FSC plots (Figure 3 c). Further FL2 or FL3 histogram plots of $CD14^{+}CD86^{+}$HLA-DR^{+} monocytes were used to determine, respectively, the GMFI of CD86 and HLA-DR in these cells (Figure 3 d and f). Negative unstained controls were used to define the threshold of positive staining. This analysis was carried for both unstimulated and SEB-stimulated samples and SEB-stimulated data are reported as a ratio to the unstimulated condition.

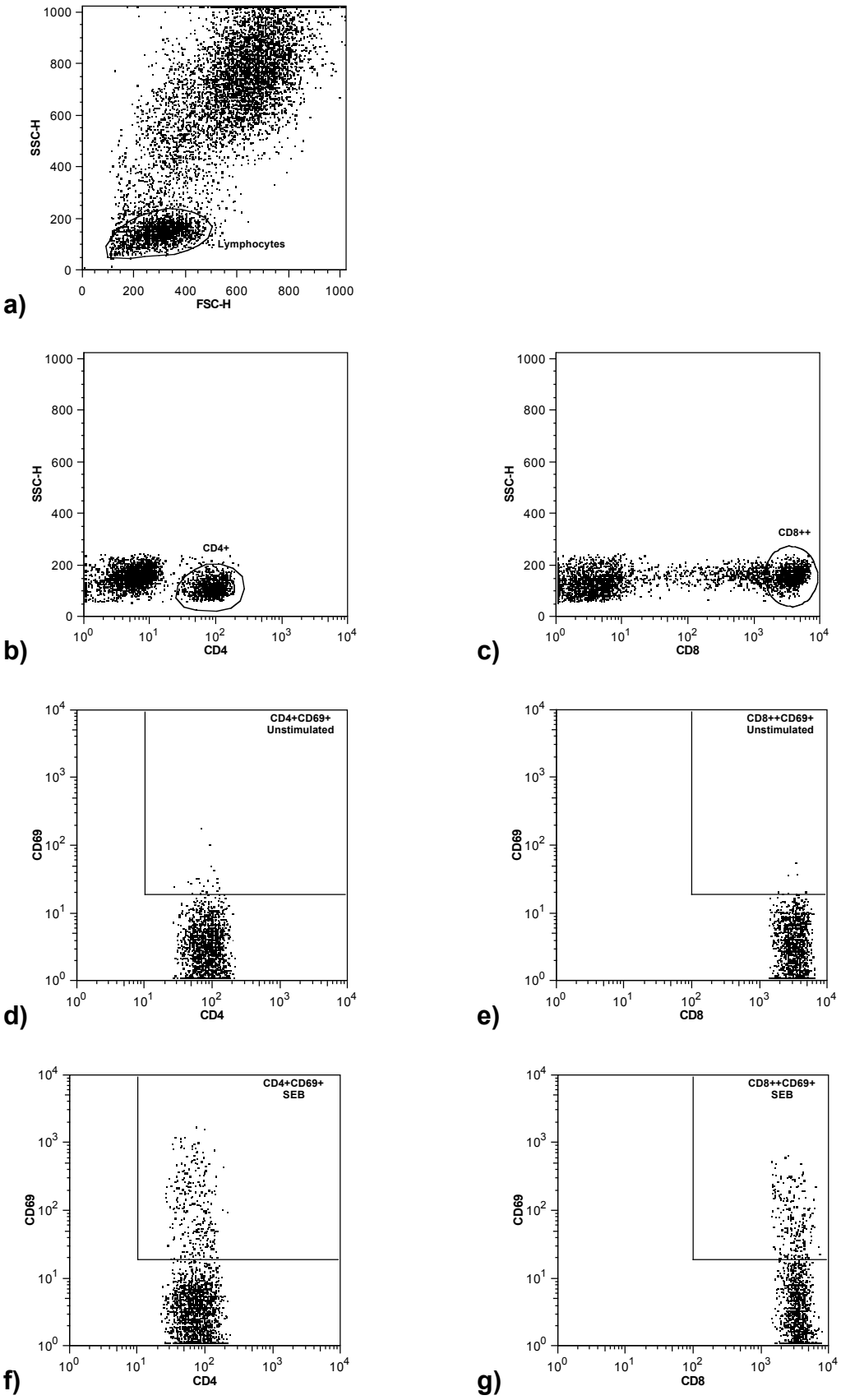

Figure 2. T-lymphocyte flow cytometry data analysis.

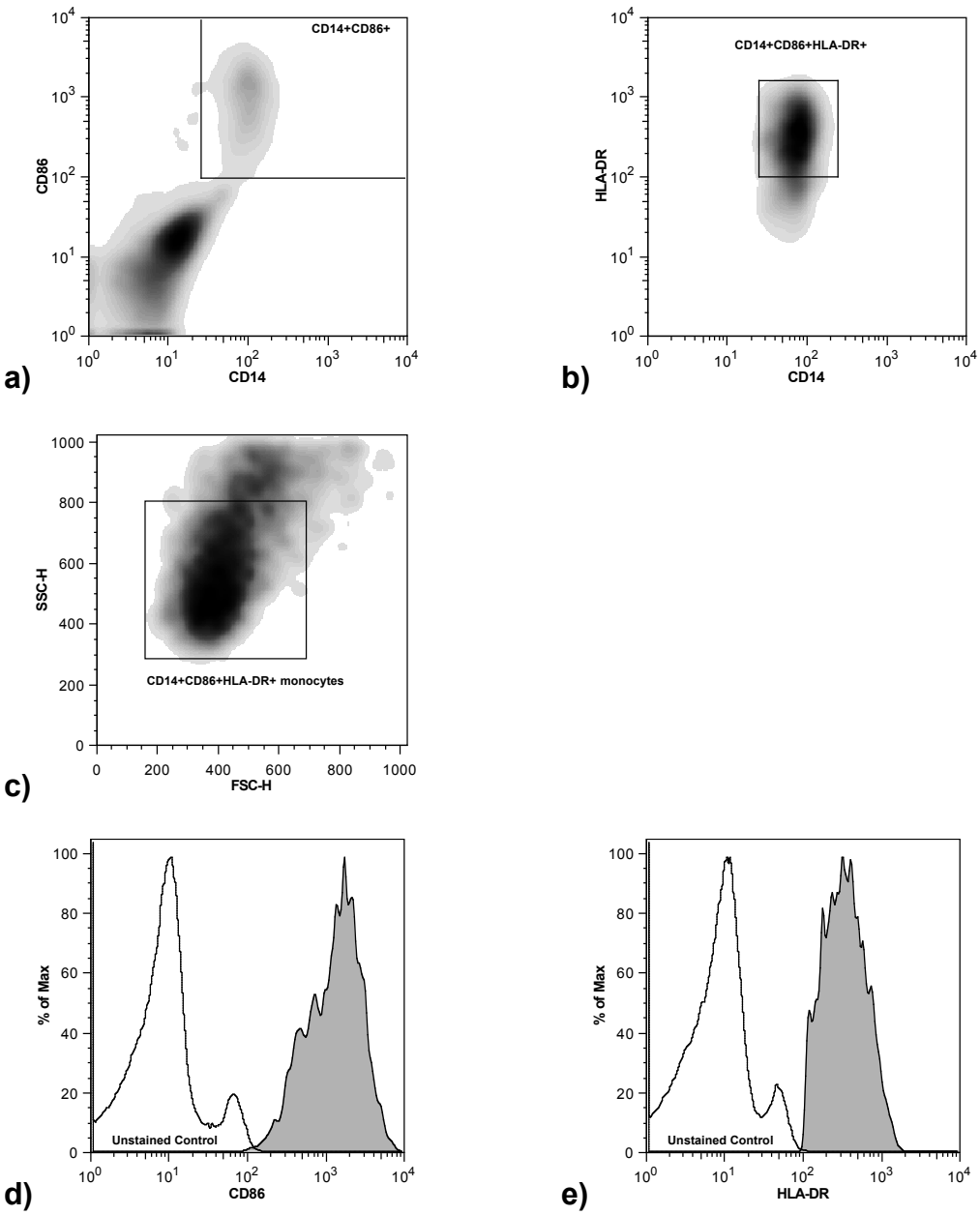

Figure 3. Monocyte flow cytometry data analysis.

References

1. Pawluczyk IZA, Yang B, Patel SR, Saleem MA, Topham PS: Low-level C-reactive protein levels exert cytoprotective actions on human podocytes. *Nephrol Dial Transplant* 26: 2465–2475, 2011

2. Simpson RJ, Florida-James GD, Cosgrove C, Whyte GP, Macrae S, Pircher H, Guy K: High-intensity exercise elicits the mobilization of senescent T lymphocytes into the peripheral blood compartment in human subjects. *J Appl Physiol* 103: 396–401, 2007

3. Campbell JP, Guy K, Cosgrove C, Florida-James GD, Simpson RJ: Total lymphocyte CD8 expression is not a reliable marker of cytotoxic T-cell populations in human peripheral blood following an acute bout of high-intensity exercise. *Brain Behav Immun* 22: 375–380, 2008