Macrophages Directly Contribute to the Exaggerated Inflammatory Response in Cystic Fibrosis Transmembrane Conductance Regulator$^{-/-}$ Mice

Emanuela M. Bruscia[1], Ping-Xia Zhang[2], Elisa Ferreira[1], Christina Caputo[1], John W. Emerson[3], David Tuck[2], Diane S. Krause[2], and Marie E. Egan[1,4]

Departments of [1]Pediarics, [2]Laboratory Medicine, and [4]Cellular and Molecular Physiology, Yale University School of Medicine; and [3]Department of Statistics, Yale University, New Haven, Connecticut

Online Data Supplement

Online Supplementary Materials and Methods

In vivo LPS administration

WT, HET and CF mice received 3 doses of LPS (Sigma L8643) over 3 days. LPS (12.5 mg) was dissolved in 5.0 ml of warm (70-80^{o}C) PBS. After vigorous mixing, the solution was cooled to 37^{o}C. LPS was administered to the mice with a nebulizer (Pulmo-Aide Compressor, Natallergy California) connected to a container with vent holes. Five mls of solution were nebulized over 15 min. WT, HET and CF mice were treated simultaneously in each experiment.

Lung Histology

To collect the lungs for histopathology, a midline incision from sternum to diaphragm was performed. To remove blood from the pulmonary circulation, PBS was perfused via the right ventricle using a 20g needle. Lungs were inflated with 1ml fixative (10% neutral buffered formalin), removed from the chest of the mouse and placed in fixative. Paraffin embedded tissues were stained with H&E for morphological analysis.

Bronchoalveolar lavage (BAL) fluid collection and analysis.

BAL fluid, collected using standard methods, was stored in single–use aliquots at -80 ^{o}C until analysis. The total number of cells recovered in the BAL was determined using an automated cell counter (Hemavet Multispecies Hematology System, DREW Scientific, Inc.). For differential cell counting, cells were cytospun onto slides (20,000 cells/slide, 2 slides for BAL) and stained with Wright Giemsa. At least 200 cells per slide were classified. Values were expressed as the percentage of each different cell type. The total number of neutrophils and macrophages for each sample was then calculated.

For lung digestion, lungs were cut into pieces and incubated in a digestion solution composed of 150 U/ml of Collagenase IV (Worthington Biochemical Co, code #NPRO) and 50 U/ml DNAse I (Sigma, Cat# D4527) in culture media. Samples were incubated at 37^{o} C for 1 hour, after which they were mechanically passed though a 40 μM nylon cell strainer (BD Falcon 352340, Bedford MA). The cells were then centrifuged for 10min at 400 g (RT) and washed 2 times with PBS. The cells were stained for blood cell markers using directly conjugated antibodies against CD45, CD3, CD4, CD8, Mac-1, F4-80 and B220 and appropriated isotype controls (all purchased from BD-Bioscience). Data were collected with a FACS Calibur instrument and analyzed by FlowJo software.

Cytokine cDNA and intracellular protein analysis

For the LINCO-plex, data were collected in a Luminex instrument and analyzed with BeadView Software (Upstate NY).

Total RNA was isolated from 1x10^{6} cells using QiagenRNAMini Kits^{TM} or QiagenRNA Blood Mini Kits^{TM} (Valencia, CA). After RNase-free DNaseI treatment (Roche Molecular Biochemical, Germany), 2 μg total RNA was reverse transcribed using Superscript ^{TM} II RNaseH^{-} Reverse Transcriptase (Nitrogen, Grand Island, NY) with either 100 ηg random hexamers or 2 pmol specific primers (CFTR and GAPDH). Real time PCR analysis was performed with a BioRadiCycler using TaqMan technology. The primers were purchased from Applied Biosystems (IL-6, Mm00446190_m1 and KC, Mm00433859_m1) and used following the manufacturer's specifications. The relative amount of specific mRNA expression in macrophages was determined by standard curve method. Copy number was normalized to 18S levels (18S, Hs99999901_s1).

Intracellular cytokine quantification was performed by flow cytometry. Briefly, cells were treated with LPS, as described above. Four hours before harvesting, the cells were incubated with Brefeldin A (BD Golgi-Plug, BD Bioscience) following the product protocol specification. Cells were harvested using a PBS-EDTA solution, stained with APC-labeled F4-80 (EBioscience, cat #11-4801) for 2 h and fixed in 2% PFA over night. Then cells were permeabilized with 0.1% saponin, 2% BSA in PBS and the intracellular staining of IL-6 was performed for 2 h on ice with anti-IL-6 antibodies (EBioscience, cat #11-7061). Intracellular IL-6 in CF macrophages was confirmed by Western Blot analysis. The Abs used for western blots were IL-6 Ab (MAB406, R&D Systems, Inc.), and GAPDH as control. Digital images were taken using, Gel Logic 2200 Imaging System (Kodak) and the densitometry using Image J software (http://rsb.info.nih.gov/ij/index.html).

Table E1: Mice used for *in vivo* LPS treatment

ID	SEX	AGE months	WEIGHT grams	TIME POINT hours	ID	SEX	AGE months	WEIGHT grams	TIME POINT hours	CF ID	SEX	AGE months	WEIGHT grams	TIME POINT hours
WT	M	3.0	29.0	0	HET	F	3.0	21.0	0	CF	M	2.0	20.0	0
WT	F	3.0	22.5	0	HET	F	4.0	23.0	0	CF	M	4.0	24.0	0
WT	F	3.0	22.0	0	HET	M	3.0	27.5	0	CF	F	3.5	19.0	0
WT	F	3.0	22.5	0	HET	F	3.0	22.0	0	CF	F	3.0	20.0	0
WT	M	2.5	28.0	0	HET	F	5.0	25.0	0	CF	F	3.0	18.5	0
WT	M	3.0	24.5	3	HET	F	4.0	19.9	3	CF	F	6.0	24.8	3
WT	M	3.0	25.0	3	HET	F	4.0	22.6	3	CF	M	9.0	31.7	3
WT	F	3.0	22.5	3	HET	M	5.0	33.3	3	CF	M	2.0	26.7	3
WT	F	2.5	18.0	3	HET	F	3.0	20.6	3	CF	M	4.0	22.8	3
WT	F	2.5	20.0	3	HET	F	3.0	17.7	3	CF	F	4.0	20.3	3
WT	M	2.5	26.0	3	HET	M	3.0	25.9	3	CF	F	4.0	18.9	3
					HET	F	2.0	19.9	3	CF	F	3.0	18.0	3
					HET	F	2.0	19.0	3	CF	F	3.5	18.0	3
					HET	M	2.0	24.0	3	CF	M	2.0	23.0	3
WT	M	2.0	26.5	6	HET	F	5.0	26.2	6	CF	F	4.0	24.7	6
WT	F	3.0	22.5	6	HET	M	4.0	32.7	6	CF	F	4.0	27.2	6
WT	F	2.0	21.5	6	HET	M	5.0	34.3	6	CF	F	3.0	22.3	6
WT	F	2.5	19.0	6	HET	F	3.0	19.4	6	CF	F	4.0	20.0	6
WT	F	2.5	20.0	6	HET	F	3.0	20.5	6	CF	M	4.0	21.7	6
WT	M	2.5	24.0	6	HET	M	3.0	27.9	6	CF	M	4.0	22.1	6
					HET	F	2.5	19.0	6	CF	F	3.5	19.0	6
					HET	F	2.5	20.0	6	CF	F	3.0	19.0	6
					HET	M	2.0	25.0	6	CF	M	3.5	23.0	6
WT	F	2.0	22.0	24	HET	M	5.0	33.0	24	CF	M	4.0	30.0	24
WT	F	2.0	23.3	24	HET	M	4.0	29.4	24	CF	F	4.0	24.4	24
WT	F	3.0	22.0	24	HET	F	5.0	26.8	24	CF	F	5.0	26.6	24
WT	F	2.5	20.0	24	HET	F	3.0	19.7	24	CF	F	4.0	19.8	24
WT	M	2.5	27.0	24	HET	F	3.0	21.3	24	CF	F	4.0	19.6	24
WT	M	2.5	29.0	24	HET	M	3.0	27.0	24	CF	M	4.0	21.0	24
					HET	F	3.0	18.0	24	CF	F	3.5	16.0	24
					HET	F	3.0	21.0	24	CF	F	3.5	20.0	24
					HET	M	3.0	25.0	24	CF	M	3.5	22.0	24

For each group mice are age, sex and weight matched. The average weight per group± standard deviation are as follows: at the 0 hour (control/untreated) time point WT= 24.4 ± 3.0 g , HET= 23.7± 2.6g, CF= 20.3±2.2 g ; at the 3 hour time point WT= 22.7± 3.1 g , HET= 22.5± 4.7g, CF= 22.7±4.6 g; at the 6 hour time point WT= 22.2± 2.7 g , HET= 25± 5.8g, CF= 22.1±2.7 g; at the 24 hour time point WT= 23.9± 3.4 g , HET= 24.6± 4.9g, CF= 22.1±4.2 g. The average age per group± standard deviation are as follows: at the 0 hour (control/untreated) time point WT= 2.9± 0.2 months , HET= 3.6± 0.9 months, CF= 3.1± 0.8 months; at the 3 hour time point WT= 2.8± 0.3 months , HET= 3.1± 1.1 months, CF= 4.2± 2.2 months; at the 6 hour time point WT= 2.4± 0.4 months , HET= 3.3± 1.1 months, CF= 3.5± 0.9 months; at the 24 hour time point WT= 2.4± 0.4 months, HET= 3.5± 0.9 months, CF= 3.9± 0.5 months.

Table E2: Statistical analysis for total and differential BAL cells

Time	Type of test	Comparison	Total BAL Cells	Neutrophils	Macrophages
No LPS	t-test	WTvsHET	**0.028**	n/a	0.072
	Wilcoxon rank-sum test		**0.046**	n/a	**0.034**
	t-test	HETvsCF	0.064	n/a	0.244
	Wilcoxon rank-sum test		**0.027**	n/a	0.140
	t-test	WTvsCF	**0.006**	n/a	**0.037**
	Wilcoxon rank-sum test		**0.011**	n/a	**0.021**
3h LPS	t-test	WTvsHET	0.246	0.245	0.241
	Wilcoxon rank-sum test		0.362	0.319	0.219
	t-test	HETvsCF	0.513	0.449	0.675
	Wilcoxon rank-sum test		0.830	0.851	0.606
	t-test	WTvsCF	0.345	0.360	0.352
	Wilcoxon rank-sum test		0.477	0.477	0.360
6h LPS	t-test	WTvsHET	**0.043**	**0.046**	**0.001**
	Wilcoxon rank-sum test		**0.044**	**0.044**	**0.005**
	t-test	HETvsCF	0.228	0.183	0.317
	Wilcoxon rank-sum test		0.145	0.081	0.154
	t-test	WTvsCF	**0.014**	**0.014**	**0.001**
	Wilcoxon rank-sum test		**0.026**	**0.018**	**0.003**
24h LPS	t-test	WTvsHET	0.197	0.285	0.586
	Wilcoxon rank-sum test		0.279	0.438	0.500
	t-test	HETvsCF	0.086	0.098	0.289
	Wilcoxon rank-sum test		0.097	0.060	0.135
	t-test	WTvsCF	**0.014**	**0.022**	0.197
	Wilcoxon rank-sum test		**0.015**	**0.033**	0.254

p-values <0.05 are in bold
n/a= statistical test not applicable due to the lack of Neutrophils in the BAL of untreated mice

Supplementary data Figure Legends

Figure E1: Phenotypic and morphological characterization of BM-derived and alveolar macrophages.

BM-derived macrophages (**A**) were differentiated from BM progenitors cells by stimulation with M-CSF. After 10 days, almost all of the cells expressed macrophage markers. For each experiment performed, we have checked the percentage of MAC-1-positive and F4/80 positive population after the differentiation protocol, which were always ≥ 90%.

Alveolar macrophages (**B**) were harvested from unstimulated mice by BAL and cultured overnight prior stimulation. Cells were 100% macrophages.

Figure E2: Lung H&E of CFTR+/- (HET) mice treat with 3 doses of LPS and sacrificed at 6h, 24h and 72h after last dose of LPS. As observed for WT and CF (Figure 1B), the lungs histology of HET mice reflected the PMN infiltration during LPS treatment. Although the lung histiology is not quantitative, HET lungs have a pattern of PMN infiltration between WT and CF, which is supported by the quantitative BAL differential cell count (Figure 1A)

Figure E3: Saline nebulization do not alter the histology or cytokine secretion in mice

(A) H&E of untreated (control, CTR) WT, HET and CF lung and WT lung of mice treated with saline and sacrificed 6h later. (B) Total BAL cell counting in untreated and saline nebulized mice (please note: y axis scale is $x10^5$).

Figure E4: Comparison of BAL cytokine concentrations in LPS treated WT, HET and CF mice. Average BAL cytokine concentrations at baseline (0h) and after (3h, 6h, 24h) LPS treatment in WT (black), HET (green) and CF (red) mice. Each time point represents the mean BAL cytokine concentration from 9 mice (HET and CF) and 6 mice (WT). The cytokines

represented here did not show significant differences between the genotypes; cytokines with notable differences were presented in Figure 2 (see text).

Figure E1

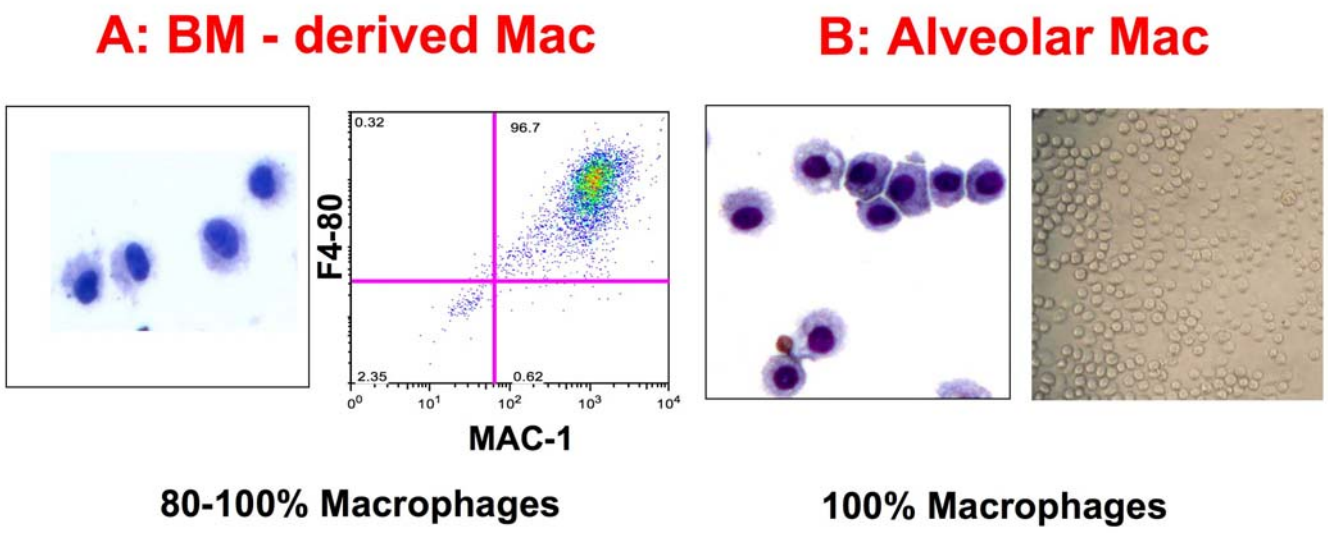

A: BM - derived Mac
B: Alveolar Mac
0.32
96.7
2.35
0.62
F4-80
MAC-1
80-100% Macrophages
100% Macrophages

Figure E2

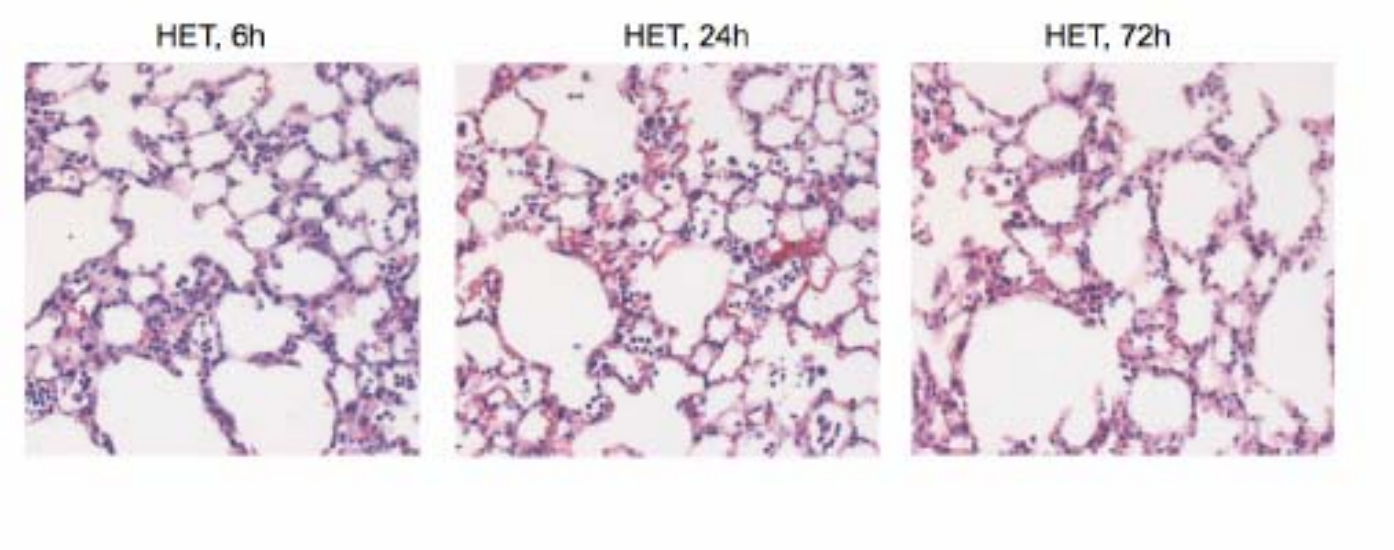


Figure E 3

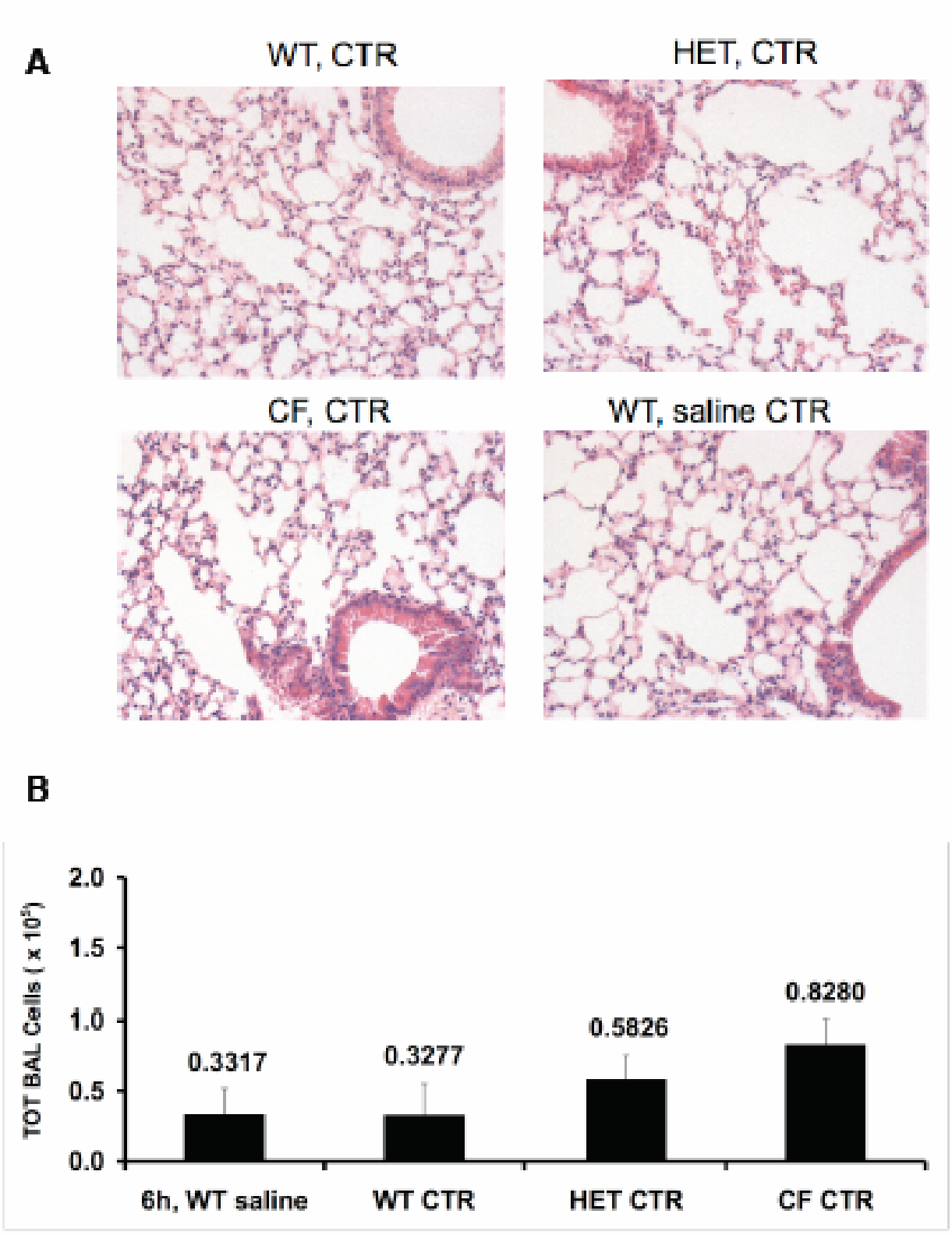
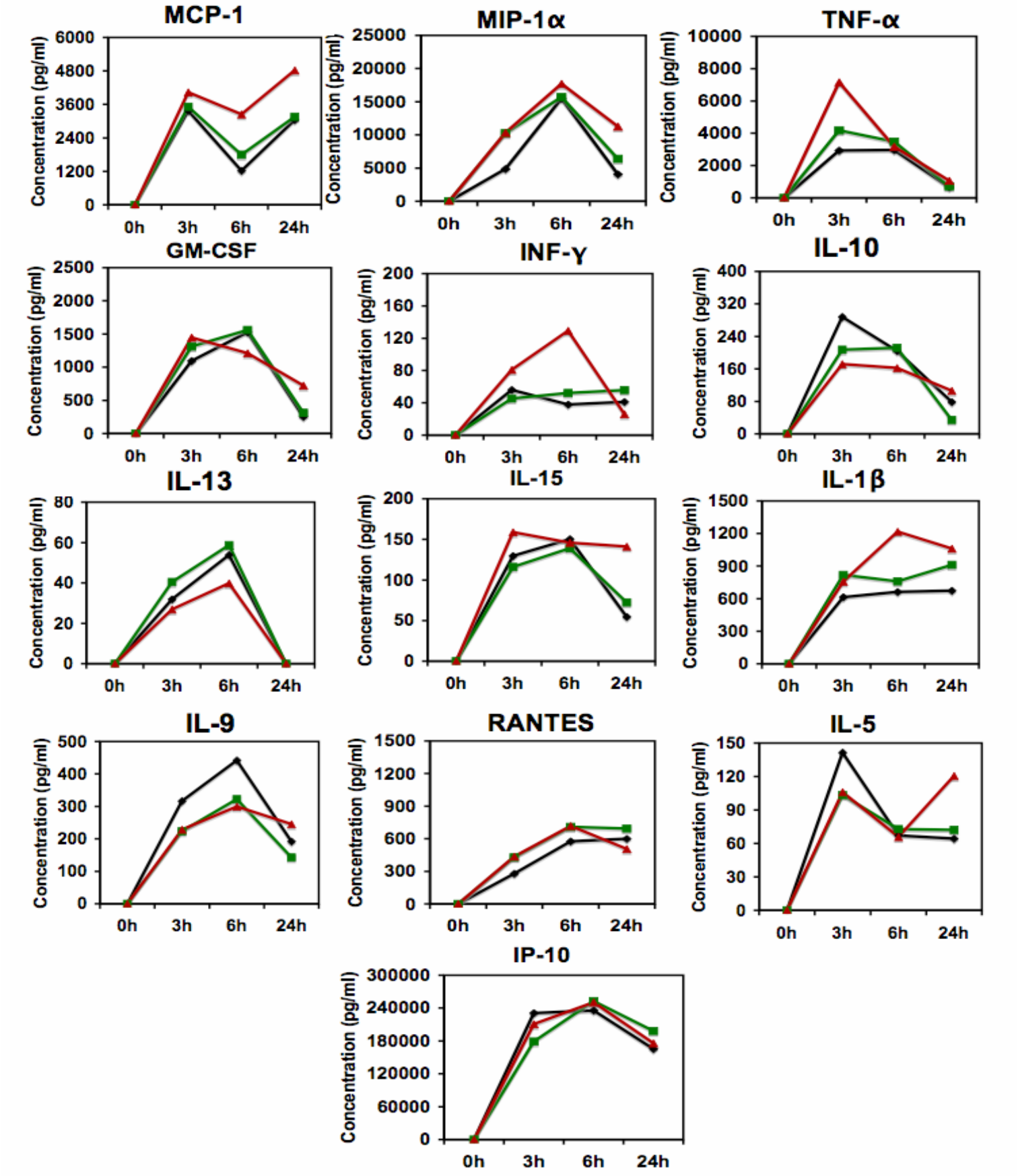

A
WT, CTR
HET, CTR
CF, CTR
WT, saline CTR
B
TOT BAL Cells (x 10³)
2.0
1.5
1.0
0.5
0.0
0.3317
0.3277
0.5826
0.8280
6h, WT saline
WT CTR
HET CTR
CF CTR

Figure E4:


MCP-1
Concentration (pg/ml)
6000
4800
3600
2400
1200
0
0h
3h
6h
24h
MIP-1α
Concentration (pg/ml)
25000
20000
15000
10000
5000
0
0h
3h
6h
24h
TNF-α
Concentration (pg/ml)
10000
8000
6000
4000
2000
0
0h
3h
6h
24h
GM-CSF
Concentration (pg/ml)
2500
2000
1500
1000
500
0
0h
3h
6h
24h
INF-γ
Concentration (pg/ml)
200
160
120
80
40
0
0h
3h
6h
24h
IL-10
Concentration (pg/ml)
400
320
240
160
80
0
0h
3h
6h
24h
IL-13
Concentration (pg/ml)
80
60
40
20
0
0h
3h
6h
24h
IL-15
Concentration (pg/ml)
200
150
100
50
0
0h
3h
6h
24h
IL-1β
Concentration (pg/ml)
1500
1200
900
600
300
0
0h
3h
6h
24h
IL-9
Concentration (pg/ml)
500
400
300
200
100
0
0h
3h
6h
24h
RANTES
Concentration (pg/ml)
1500
1200
900
600
300
0
0h
3h
6h
24h
IL-5
Concentration (pg/ml)
150
120
90
60
30
0
0h
3h
6h
24h
IP-10
Concentration (pg/ml)
300000
240000
180000
120000
60000
0
0h
3h
6h
24h