Supplemental Material

Innate and adaptive immune cell subsets as risk factors for coronary heart disease in two population-based cohorts

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Supplemental Table I. Pilot study comparing cell phenotypes from fresh and cryopreserved samples

Study	**Sample Type**	**n**	**CD4** (%)	**Memory** (%)	**Naive** (%)	**Th1** (%)	**Th2** (%)
Pilot: All	Cryopreserved	20	39.7	56.7	28.6	NA	NA
CHS (pilot)	Cryopreserved	12	37.4	59.7	29.8	NA	NA
MESA (pilot)	Cryopreserved	8	43.1	52.4	26.8	12.3	3.06
MESA-Inflammation	Fresh	912	39.9	53.9	28.3	16.0	0.80

Data are presented as mean values. Peripheral blood mononuclear cell (PBMC) samples were cryopreserved in 1998-1999 in the Cardiovascular Health Study (CHS) and 2000-2002 in the Multi-Ethnic Study of Atherosclerosis (MESA). The MESA-Inflammation study evaluated cells isolated from fresh blood samples in 2005-2007. Cells were evaluated by flow cytometry as described in the Methods. Th1 and Th2 cells were not evaluated in CHS samples in the pilot study. $CD4^+$ cells were expressed as a percentage of lymphocytes. $CD4^+$ memory, $CD4^+$ naive, Th1, and Th2 cells were expressed as a percentage of $CD4^+$ cells.

Supplemental Table II. Antibodies used for immune cell phenotyping assays

Phenotype Assay	**Target Antigen**	**Clone ID** (Catalog #)	**Fluorophore**	**Isotype Control** Antibody [Clone ID] (Catalog #)
γδ T and Natural Killer cells	CD3	BW264/56 (130-094-363)	VioBlue	Mouse IgG2a [S43.10] (130-094-671)
	γδ TCR	11F2 (130-096-869)	PE	Mouse IgG1 [IS5-21F5] (130-092-212)
	CD56	REA196 (130-100-676)	PE-Vio770	REA Control [REA293] (130-104-616)
	CD16	VEP13 (130-100-430)	PerCP-Vio700	Mouse IgM [IS5-20C4] (130-098-595)
$CD4^+$ and $CD8^+$ subsets (cell stimulation assays)	CD4	VIT4 (130-092-374)	APC	Mouse IgG2a [S43.10] (130-091-836)
	CD8	BW135/80 (130-104-519)	VioBright FITC	Mouse IgG2a [S43.10] 130-104-512
	IFN-γ	45-15 (130-096-752)	PE-Vio770	Mouse IgG1 [IS5-21F5] (130-096-654)
	IL-4	7A3-3 (130-091-647)	PE	Mouse IgG1 [IS5-21F5] (130-092-212)
	IL-17A	CZ8-23G1 (130-096-656)	APC-Vio770	Mouse IgG1 [IS5-21F5] (130-096-653)
$CD4^+$ subsets	CD4	M-T466 (130-103-793)	PerCP-Vio700	Mouse IgG1 [IS5-21F5] (130-097-561)
	CD45RA	T6D11 (130-096-604)	APC-Vio770	Mouse IgG2b [IS6-11E5.11] (130-096-822)
	CD45RO	UCHL1 (130-099-044)	VioBlue	Mouse IgG2a [S43.10] (130-094-671)
	CD28	15E8 (130-092-921)	PE	Mouse IgG1 [IS5-21F5] (130-092-212)
	CD38	IB6 (130-099-151)	PE-Vio770	Mouse IgG2a [S43.10] (130-096-638)
	CD57	TB03 (130-092-141)	APC	Mouse IgM [IS5-20C4]

				(130-093-176)
	CD27	M-T271 (130-104-845)	VioBright FITC	Mouse IgG1 [IS5-21F5] (130-104-513)
CD4$^+$ chemokine receptor assays	CD4	M-T466 (130-103-793)	PerCP-Vio700	Mouse IgG1 [IS5-21F5] (130-097-561)
	CD194 (CCR4)	REA279 (130-103-814)	PE-Vio770	Mouse IgG1 [IS5-21F5] (130-096-654)
	CD196 (CCR6)	REA190 (130-100-373)	APC	Mouse IgM [IS5-20C4] (130-093-176)
	CD183 (CXCR3)	REA232 (130-106-009)	VioBright FITC	Mouse IgG1 [IS5-21F5] (130-104-513)
	CD195 (CCR5)	REA245 (130-106-223)	PE	Mouse IgG1 [IS5-21F5] (130-092-212)
CD8$^+$ subsets	CD8	BW135/80 (130-097-911)	PerCP-Vio700	Mouse IgG2a [S43.10] (130-097-563)
	CD45RA	T6D11 (130-096-604)	APC-Vio770	Mouse IgG2b [IS6-11E5.11] (130-096-822)
	CD45RO	UCHL1 (130-099-044)	VioBlue	Mouse IgG2a [S43.10] (130-094-671)
	CD28	15E8 (130-092-921)	PE	Mouse IgG1 [IS5-21F5] (130-092-212)
	CD38	IB6 (130-099-151)	PE-Vio770	Mouse IgG2a [S43.10] 130-096-638
	CD57	TB03 (130-092-141)	APC	Mouse IgM [IS5-20C4] (130-093-176)
	CD27	M-T271 (130-104-845)	VioBright FITC	Mouse IgG1 [IS5-21F5] (130-104-513)
B Cell subsets	CD19	LT19 (130-096-643)	APC-Vio770	Mouse IgG1 [IS5-21F5] (130-096-653)
	CD5	UCHT2 (130-096-577)	APC	Mouse IgG1 [IS5-21F5] (130-092-214)
	CD27	M-T271 (130-104-845)	VioBright FITC	Mouse IgG1 [IS5-21F5] (130-104-513)
Monocyte subsets	CD14	TÜK4	PE-Vio770	Mouse IgG2a

		(130-096-628)		[S43.10] (130-096-638)
	CD16	VEP13 (130-100-430)	PerCP-Vio700	Mouse IgM [IS5-20C4] (130-098-595)
CD4⁺ T Regulatory cells	CD4	VIT4 (130-092-374)	APC	Mouse IgG2a [S43.10] (130-091-836)
	CD25	4E3 (130-104-274)	VioBright-FITC	Mouse IgG2b [IS6-11E5.11] (130-104-575)
	CD127	MB15-18C9 (130-099-719)	PE-Vio770	Mouse IgG2a [S43.10] (130-096-638)
	CD6	M-T411 (130-105-129)	APC-Vio770	Mouse IgG1 [IS5-21F5] (130-096-653)
All antibodies from Miltenyi Biotec (San Diego, CA). All assays used antibody dilutions as recommended by Miltenyi.				

Supplemental Table III. Markers used for immune cell phenotyping

Cellular Markers	Phenotype	Data expressed as
Monocytes		
$CD14^{++}CD16^{-}$ (†)	Classical Monocytes	% of $CD14^{+}$ monocytes
$CD14^{+}CD16^{+}$ (†)	Intermediate Monocytes	% of CD14+ monocytes
$CD14^{+}CD16^{++}$ (†)	Non-Classical Monocytes	% of CD14+ monocytes
Innate Lymphocytes		
$CD3^{-}CD16^{+}CD56^{+}$	Natural Killer cells	% of lymphocytes
$CD3^{+}\gamma\delta TCR^{+}$	γδ T cells	% of $CD3^{+}$ cells
$CD4^{+}$ T helper (Th) Cells		
$CD4^{+}$	Pan $CD4^{+}$ T cells	% of lymphocytes
$CD4^{+}IFN\text{-}\gamma^{+}$ (†)	Th1	% of $CD4^{+}$ cells
$CD4^{+}IL\text{-}4^{+}$ (†)	Th2	% of $CD4^{+}$ cells
$CD4^{+}IL\text{-}17A^{+}$ (†)	Th17	% of $CD4^{+}$ cells
$CD4^{+}CXCR3^{+}CCR4^{-}CCR6^{-}$ (‡)	Th1	% of $CD4^{+}$ cells
$CD4^{+}CXCR3^{-}CCR4^{+}CCR6^{-}$ (‡)	Th2	% of $CD4^{+}$ cells
$CD4^{+}CXCR3^{-}CCR4^{+}CCR6^{+}$ (‡)	Th17	% of $CD4^{+}$ cells
$CD4^{+}CD25^{+}CD127^{-}$	T regulatory (Treg) cells	% of $CD4^{+}$ cells
$CD4^{+}CD45RA^{+}$	Naive $CD4^{+}$	% of $CD4^{+}$ cells
$CD4^{+}CD45RO^{+}$	Memory $CD4^{+}$	% of $CD4^{+}$ cells
$CD4^{+}CD25^{+}$	Activated or Treg $CD4^{+}$	% of $CD4^{+}$ cells
$CD4^{+}CD38^{+}$	Activated or mature $CD4^{+}$	% of $CD4^{+}$ cells
$CD4^{+}CD28^{-}$	Differentiated / Senescent	% of $CD4^{+}$ cells
$CD4^{+}CD57^{+}$	Differentiated / Senescent	% of $CD4^{+}$ cells
$CD4^{+}CD28^{-}CD57^{+}$	Differentiated / Senescent	% of $CD4^{+}$ cells
$CD4^{+}CD28^{-}CD57^{+}CD45RA^{+}$	TEMRA	% of $CD4^{+}$ cells
$CD8^{+}$ Cytotoxic T cells (Tc)		
$CD8^{+}$	Pan $CD8^{+}$ T cells	% of lymphocytes
$CD8^{+}IFN\text{-}\gamma^{+}$ (†)	Tc1	% of $CD8^{+}$ cells
$CD8^{+}IL\text{-}4^{+}$ (†)	Tc2	% of $CD8^{+}$ cells
$CD8^{+}IL\text{-}17A^{+}$ (†)	Tc17	% of $CD8^{+}$ cells
$CD8^{+}CD45RA^{+}$	Naive $CD8^{+}$	% of $CD8^{+}$ cells
$CD8^{+}CD45RO^{+}$	Memory $CD8^{+}$	% of $CD8^{+}$ cells
$CD8^{+}CD38^{+}$	Activated or mature $CD8^{+}$	% of $CD8^{+}$ cells
$CD8^{+}CD28^{-}$	Differentiated / Senescent	% of $CD8^{+}$ cells
$CD8^{+}CD57^{+}$	Differentiated / Senescent	% of $CD8^{+}$ cells
$CD8^{+}CD28^{-}CD57^{+}$	Differentiated / Senescent	% of $CD8^{+}$ cells
$CD8^{+}CD28^{-}CD57^{+}CD45RA^{+}$	TEMRA	% of $CD8^{+}$ cells
B cells		

$CD19^+$	Pan B cells	% of lymphocytes
$CD19^+CD5^+$	Transitional B cells	% of $CD19^+$ B cells
$CD19^+CD5^+CD27^-$	Transitional B cells	% of $CD19^+$ B cells
$CD19^+CD27^+$	Memory B cells	% of $CD19^+$ B cells
$CD19^+CD5^-CD27^+$	Memory B cells	% of $CD19^+$ B cells
(†): Measured in MESA only. (‡): Measured in CHS only.		

Supplemental Table IV. Characteristics of immune cell traits in MESA and CHS

	MESA		CHS	
	n	Mean (SD) (%)	*n*	Mean (SD) (%)
Classical monocyte ($CD14^{++}CD16^{-}$)	922	74.5 (10.1)	NA	-
Intermediate monocyte ($CD14^{+}CD16^{+}$)	922	18.1 (7.1)	NA	-
Non-Classical monocyte ($CD14^{+}CD16^{++}$)	922	7.4 (7.4)	NA	-
Natural killer	1087	5.0 (5.7)	806	5.5 (5.1)
δγ T	1087	6.6 (6.1)	789	5.0 (5.0)
$CD4^{+}$	1051	50.0 (11.1)	863	53.7 (13.2)
Th1*	770	15.3 (9.0)	516	20.1 (9.0)
Th2*	770	2.9 (1.8)	516	6.7 (4.4)
Th17*	770	2.2 (1.5)	516	4.3 (3.6)
Treg ($CD4^{+}CD25^{+}CD127^{-}$)	1035	5.0 (2.2)	791	6.1 (5.1)
Naive ($CD4^{+}CD45RA^{+}$)	1051	26.1 (12.0)	863	26.0 (11.8)
Memory ($CD4^{+}CD45RO^{+}$)	1051	51.8 (13.4)	863	42.1 (13.0)
$CD4^{+}CD25^{+}$	1036	32.5 (11.4)	791	27.1 (13.2)
$CD4^{+}CD38^{+}$	1051	26.2 (12.2)	863	30.3 (15.6)
$CD4^{+}CD28^{-}$	1051	13.9 (9.9)	863	21.7 (14.7)
$CD4^{+}CD57^{+}$	1051	22.4 (12.9)	862	25.5 (14.4)
$CD4^{+}CD28^{-}CD57^{+}$	1051	9.8 (8.4)	863	13.5 (9.9)
$CD4^{+}CD28^{-}CD57^{+}CD45RA^{+}$	1051	5.6 (5.3)	862	7.8 (6.4)
$CD8^{+}$	1062	23.7 (9.3)	881	16.8 (9.6)
Tc1 ($CD8^{+}IFN\text{-}\gamma^{+}$)	770	41.3 (17.9)	NA	-
Tc2 ($CD8^{+}IL\text{-}4^{+}$)	770	7.1 (5.0)	NA	-
Tc17 ($CD8^{+}IL\text{-}17A^{+}$)	770	5.5 (5.8)	NA	-
$CD8^{+}CD45RA^{+}$	1062	52.3 (14.7)	882	38.7 (15.0)
$CD8^{+}CD45RO^{+}$	1062	21.8 (10.7)	875	25.9 (12.8)
$CD8^{+}CD38^{+}$	1062	23.6 (12.2)	882	35.5 (21.1)
$CD8^{+}CD28^{-}$	1062	55.6 (15.8)	882	45.7 (17.5)
$CD8^{+}CD57^{+}$	1062	59.4 (15.3)	720	44.9 (17.2)
$CD8^{+}CD28^{-}CD57^{+}$	1062	44.5 (15.7)	882	33.0 (15.7)
$CD8^{+}CD28^{-}CD57^{+}CD45RA^{+}$	1062	32.7 (14.2)	720	6.0 (7.9)
$CD19^{+}$ B cells	1089	11.3 (7.4)	806	16.8 (13.1)
$CD19^{+}CD5^{+}$	1089	54.8 (22.4)	807	52.7 (25.4)
$CD19^{+}CD27^{+}$	1089	42.7 (16.4)	807	38.0 (19.1)
$CD19^{+}CD5^{+}CD27^{-}$	1089	30.8 (18.3)	806	29.6 (24.3)
$CD19^{+}CD5^{-}CD27^{+}$	1089	18.7 (12.7)	806	15.0 (12.1)

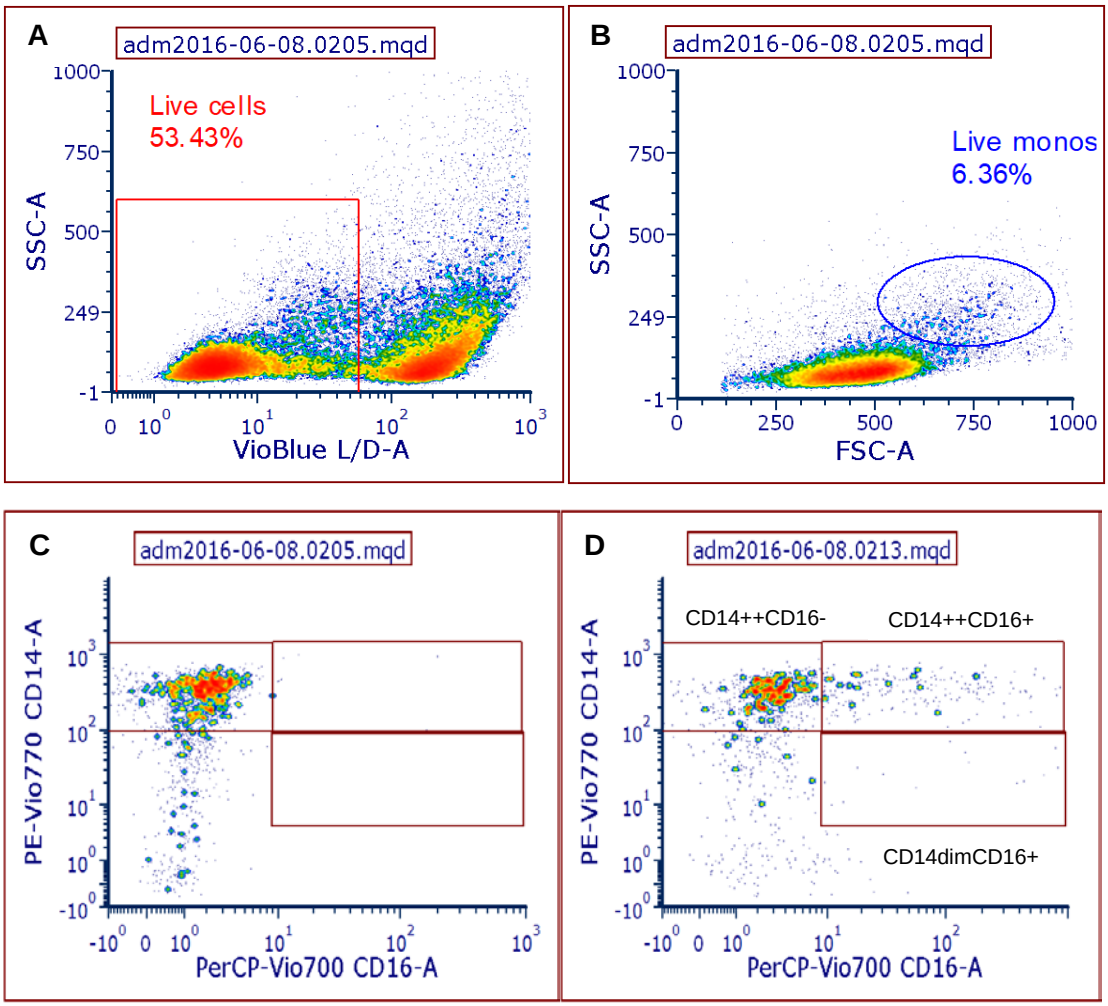
n represents the number of participants with data for each immune cell trait. * Th1, Th2, and Th17 cells were phenotyped using different methods in MESA and CHS. MESA used intracellular cytokine staining; CHS stained cell surface receptors. Monocyte and Tc1/2/17 cell assays were not performed in CHS.

Supplemental Table V. Associations of innate and adaptive immune cell subsets included as secondary hypotheses with incident myocardial infarction

	Hazards Ratios (confidence intervals) of MI		
	MESA	**CHS**	**MESA-CHS Meta-analysis**
$CD14^{++}CD16^{-}$	1.12 (0.83, 1.51)	NA	NA
$CD14^{+}CD16^{+}$	0.88 (0.63, 1.23)	NA	NA
$CD14^{+}CD16^{++}$	0.97 (0.72, 1.31)	NA	NA
Natural killer	0.99 (0.76, 1.29)	1.02 (0.83, 1.26)	1.01 (0.86, 1.19)
γδ T	1.04 (0.81, 1.34)	1.04 (0.84, 1.28)	1.04 (0.88, 1.22)
$CD4^{+}$	0.92 (0.69, 1.24)	0.91 (0.74, 1.12)	0.91 (0.77, 1.08)
$CD4^{+}CD25^{+}$	0.91 (0.70, 1.20)	1.11 (0.91, 1.36)	1.07 (0.91, 1.25)
$CD4^{+}CD38^{+}$	1.01 (0.74, 1.38)	1.08 (0.88, 1.34)	1.06 (0.89, 1.26)
$CD4^{+}CD57^{+}$	1.16 (0.88, 1.53)	1.04 (0.85, 1.27)	1.08 (0.92, 1.27)
$CD4^{+}CD28^{-}CD57^{+}$	1.10 (0.83, 1.47)	0.97 (0.78, 1.20)	1.01 (0.85, 1.20)
$CD4^{+}CD28^{-}CD57^{+}CD45RA^{+}$	1.07 (0.82, 1.40)	0.97 (0.78, 1.21)	1.01 (0.85, 1.19)
$CD8^{+}$	1.13 (0.86, 1.48)	1.04 (0.83, 1.30)	1.08 (0.91, 1.28)
Tc1 ($CD8^{+}$IFN-γ^{+})	1.21 (0.89, 1.63)	NA	NA
Tc2 ($CD8^{+}$IL-4^{+})	1.05 (0.76, 1.44)	NA	NA
Tc17 ($CD8^{+}$IL-$17A^{+}$)	1.12 (0.84, 1.48)	NA	NA
$CD8^{+}CD45RA^{+}$	1.03 (0.78, 1.37)	1.08 (0.88, 1.32)	1.06 (0.90, 1.25)
$CD8^{+}CD45RO^{+}$	0.93 (0.72, 1.21)	0.95 (0.77, 1.17)	0.94 (0.80, 1.11)
$CD8^{+}CD38^{+}$	1.03 (0.78, 1.36)	0.99 (0.80, 1.24)	1.01 (0.85, 1.20)
$CD8^{+}CD28^{-}$	1.16 (0.87, 1.55)	1.09 (0.87, 1.35)	1.11 (0.93, 1.33)
$CD8^{+}CD57^{+}$	1.17 (0.90, 1.53)	1.04 (0.84, 1.29)	1.09 (0.92, 1.29)
$CD8^{+}CD28^{-}CD57^{+}$	1.18 (0.90, 1.55)	1.07 (0.86, 1.32)	1.11 (0.94, 1.31)
$CD8^{+}CD28^{-}CD57^{+}CD45RA^{+}$	1.14 (0.88, 1.49)	0.98 (0.78, 1.24)	1.05 (0.88, 1.25)
$CD19^{+}$	0.96 (0.69, 1.35)	0.95 (0.75, 1.20)	0.95 (0.79, 1.15)
$CD19^{+}CD5^{+}$	0.93 (0.72, 1.20)	1.02 (0.82, 1.26)	0.98 (0.83, 1.16)
$CD19^{+}CD27^{+}$	1.02 (0.75, 1.41)	1.06 (0.85, 1.32)	1.05 (0.88, 1.26)
$CD19^{+}CD5^{+}CD27^{-}$	0.93 (0.71, 1.22)	0.97 (0.78, 1.22)	0.95 (0.80, 1.14)
$CD19^{+}CD5^{-}CD27^{+}$	1.05 (0.78, 1.41)	1.01 (0.81, 1.25)	1.02 (0.86, 1.22)

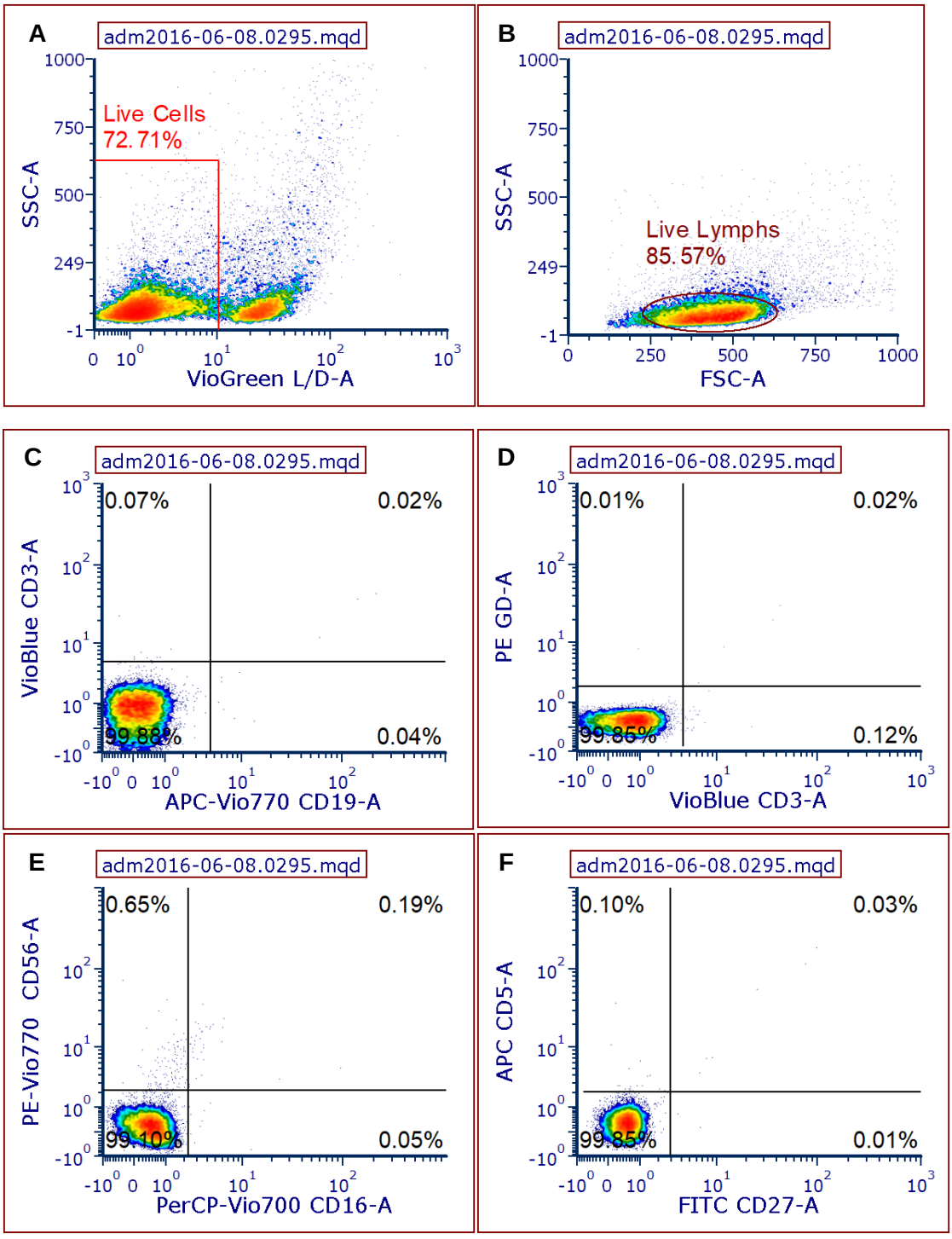
Cell phenotypes were analyzed per 1-SD higher values using Cox models with sampling weights and robust (sandwich) standard error estimates. CIs reflect the Bonferroni-adjusted significance level of $p<0.0015$. Models were adjusted for age, sex, race/ethnicity, education, clinical site, systolic blood pressure, use of antihypertensive medication, low-density lipoprotein cholesterol, use of statins, smoking status, and diabetes. NA indicates phenotypes that were not evaluated in CHS.

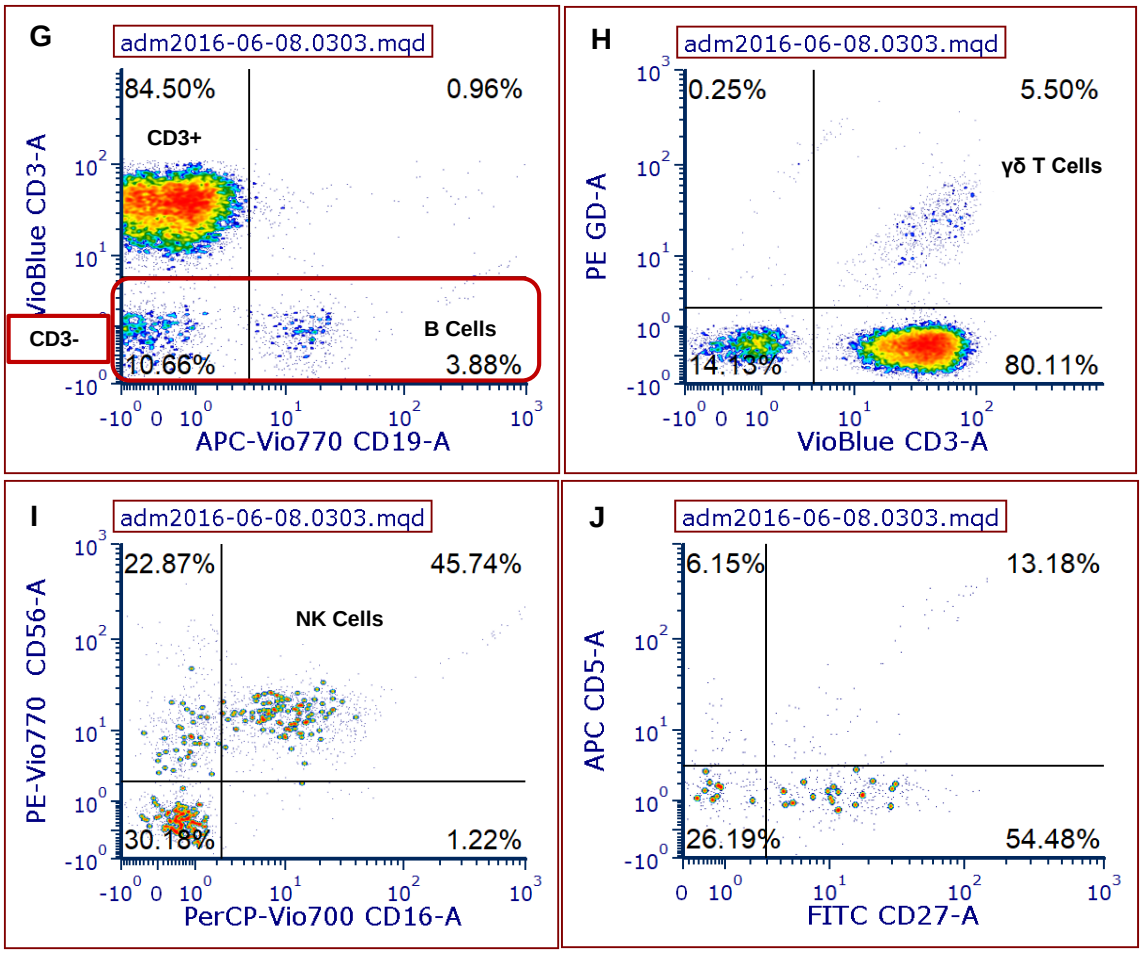
Supplemental Figure I. Monocyte flow cytometry gating scheme



A.) PBMCs gated on live cells; B.) Gate on monocytes within the live cell gate; C.) CD14+ vs. CD16 isotype within the monocyte gate; D.) CD14+ vs. CD16+ within the monocyte gate.

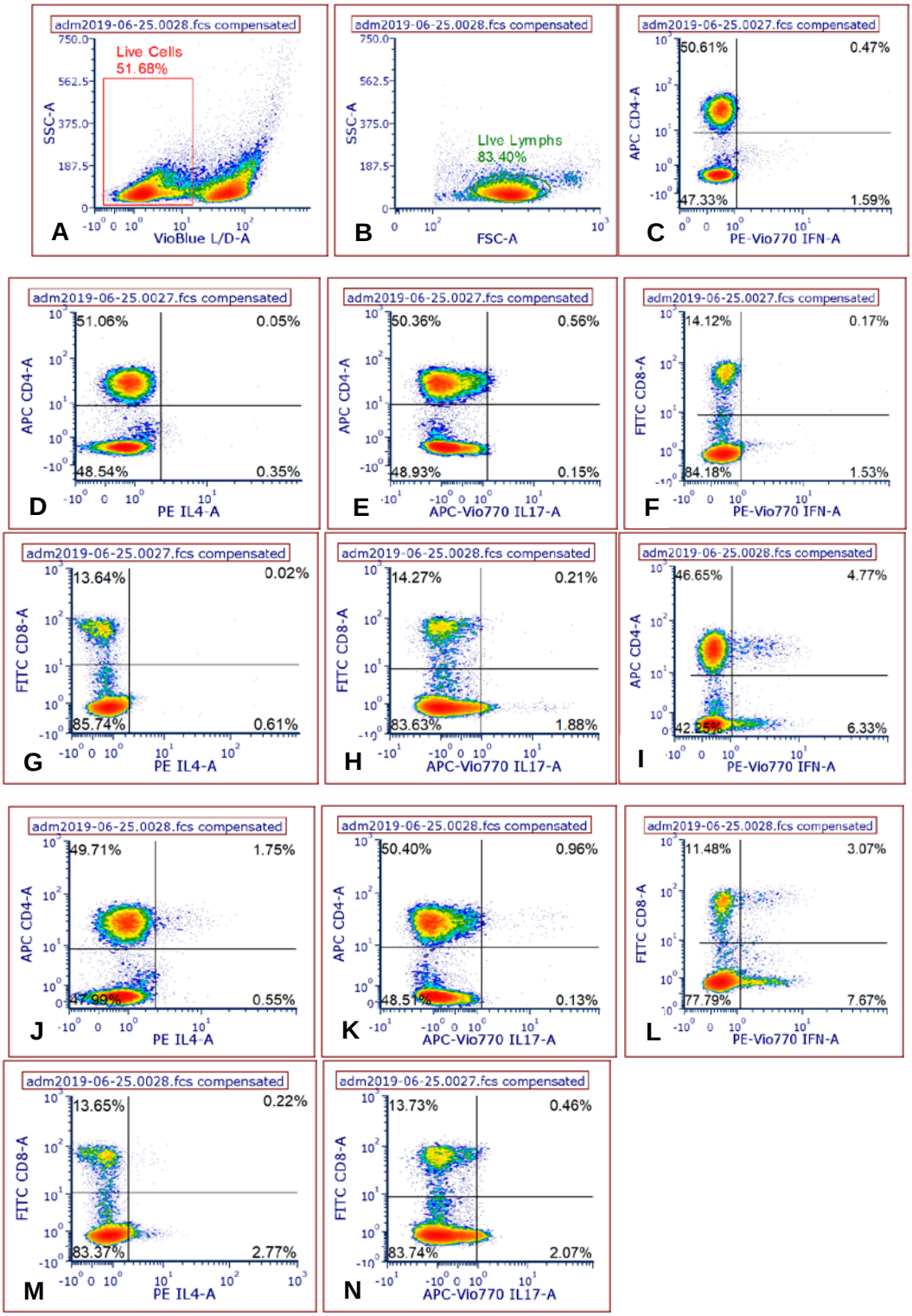
Supplemental Figure II. Flow cytometry gating schemes for CD3+ T, CD19+ B, Natural Killer, and γδ T cells





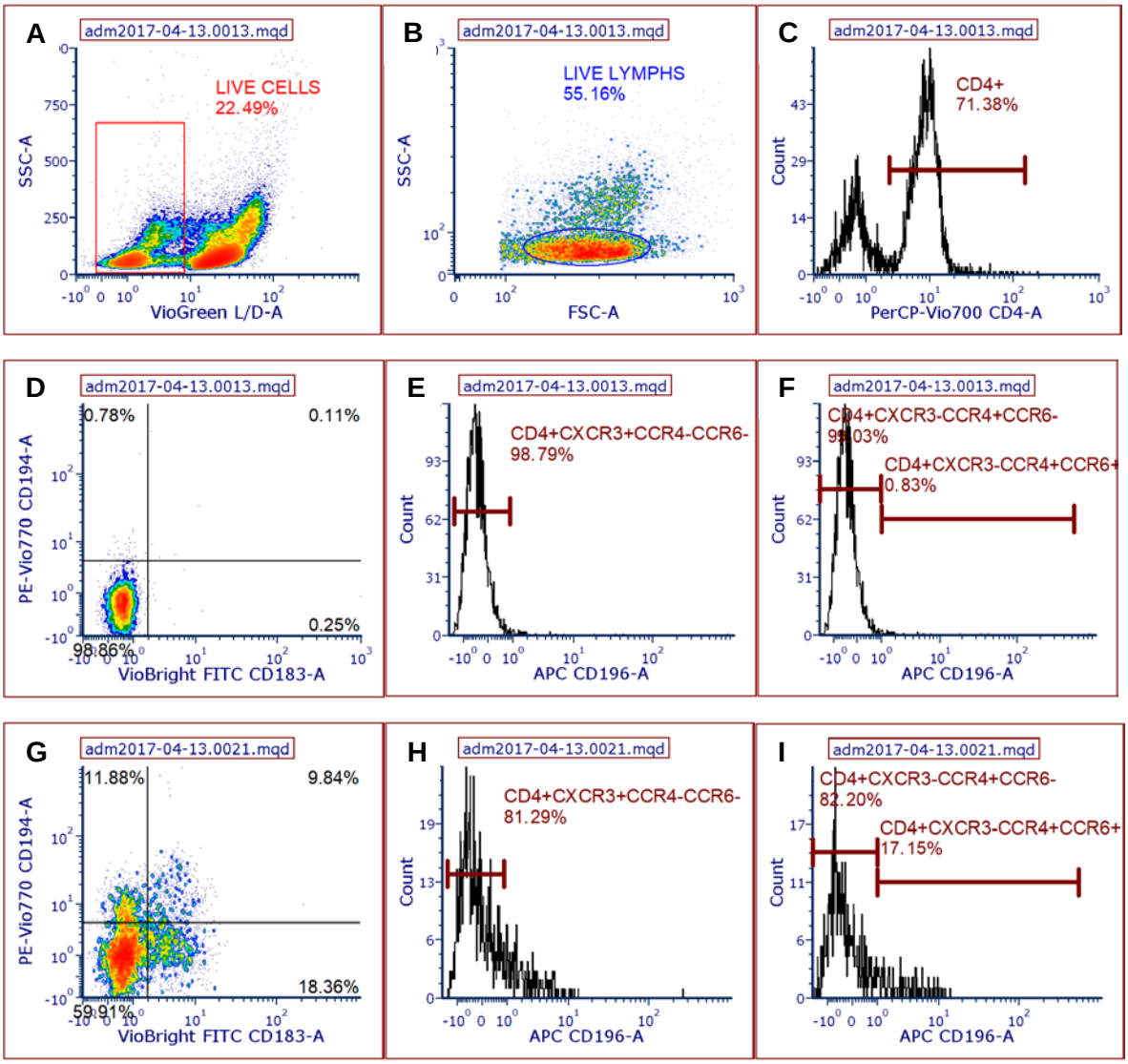
A.) PBMCs gated on live cells; B.) Gate on lymphocytes within the live cell gate; C.) T cells (CD3+) vs. B cells (CD19+) isotypes within the lymphocyte gate; D.) γδ T cell receptor vs. CD3 isotypes within the lymphocyte gate; E.) CD56 vs. CD16 isotypes within the CD3- gate; F.) CD5 vs. CD27 isotypes; G.) CD3+ (upper left quadrant) vs. B cells (lower right quadrant) within the lymphocyte gate (the CD3- gate is highlighted); H.) γδ T cells (upper right quadrant) within the lymphocyte gate; I.) Natural Killer cells (upper right quadrant) within the CD3- gate; J.) CD5 vs. CD27 within the B cell gate.

Supplemental Figure III. Flow cytometry gating for CD4+ Th1/Th2/Th17 and CD8+ Tc1/Tc2/Tc17 cells



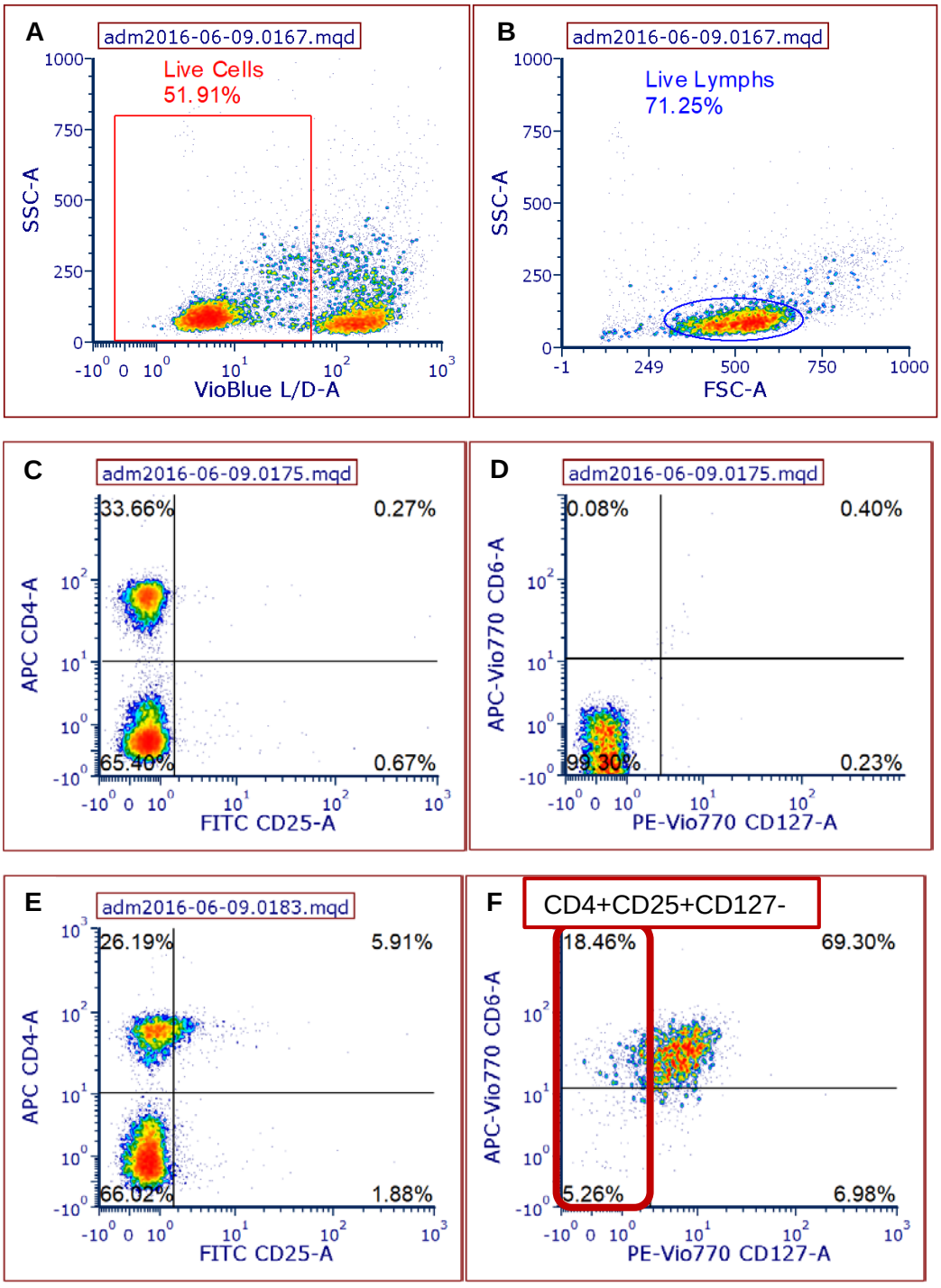
A.) PBMCs gated on live cells; B.) Gate on lymphocytes within the live cell gate; C.) CD4+ vs. interferon-gamma (labeled in the plots as IFN-A) isotype within the lymphocyte gate; D.) CD4+ vs. IL-4 isotype within the lymphocyte gate; E.) CD4+ vs. IL-17A isotype within the lymphocyte gate; F.) CD8+ vs. IFN-γ isotype within the lymphocyte gate; G.) CD8+ vs. IL-4 isotype within the lymphocyte gate; H.) CD8+ vs. IL-17A isotype within the lymphocyte gate; I.) CD4+IFN-γ+ (upper right quadrant) within the lymphocyte gate; J.) CD4+IL-4+ (upper right quadrant) within the lymphocyte gate; K.) CD4+IL-17A+ (upper right quadrant) within the lymphocyte gate; L.) CD8+IFN-γ+ (upper right quadrant) within the lymphocyte gate; M.) CD8+IL-4+ (upper right quadrant) within the lymphocyte gate; N.) CD8+IL-17A+ (upper right quadrant) within the lymphocyte gate. These assays were performed in the Multi-Ethnic Study of Atherosclerosis (MESA) cohort only.

Supplemental Figure IV. Flow cytometry gating scheme for Th1/Th2/Th17 phenotyping using cell-surface labeling of chemokine receptors



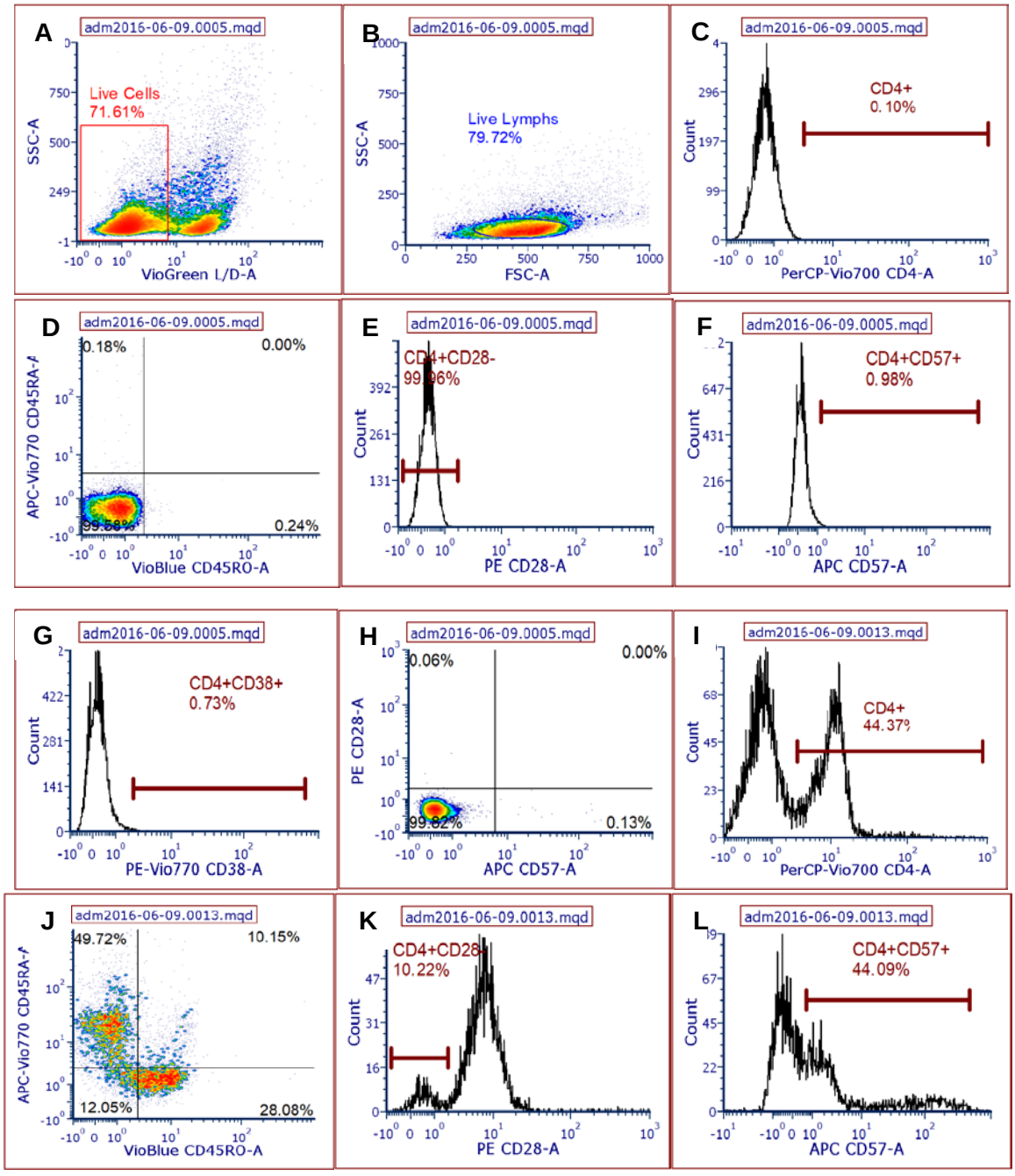
A.) PBMCs gated on live cells; B.) Gate on lymphocytes within the live cell gate; C.) Gate on CD4+ cells within the lymphocyte gate; D.) CCR4 (CD194) vs. CXCR3 (CD183) isotypes within the CD4+ gate; E.) CCR6- (CD196) isotype within the CD4+CXCR3+CCR4- gate (lower right quadrant of panel D); F.) CCR6+ and CCR6- (CD196) isotypes within the CD4+CXCR3-CCR4+ gate (upper left quadrant of panel D); G.) CCR4 (CD194) vs. CXCR3 (CD183) isotype within the CD4+ gate; H.) CCR6- (CD196) isotype within the CD4+CXCR3+CCR4- gate (lower right quadrant of panel D); I.) CCR6+ and CCR6- (CD196) isotype within the CD4+CXCR3-CCR4+ gate (upper left quadrant of panel D). These assays were performed in the Cardiovascular Health Study (CHS) cohort only.

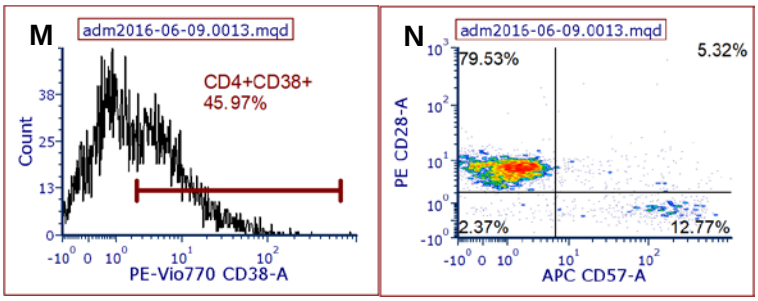
Supplemental Figure V. Flow cytometry gating scheme for T regulatory cells



A.) PBMCs gated on live cells; B.) Gate on lymphocytes within the live cell gate; C.) CD4+ vs. CD25 isotype within the lymphocyte gate; D.) CD6 vs. CD127 isotypes within the CD4+CD25+ gate; E.) CD4+CD25+ cells (upper right quadrant) within the lymphocyte gate; F.) CD6 vs. CD127 within the CD4+CD25+ gate with the CD4+CD25+CD127- gate highlighted.

Supplemental Figure VI. Flow cytometry gating for differentiated CD4+ T cell subsets





A.) PBMCs gated on live cells; B.) Gate on lymphocytes within the live cell gate; C.) Gate on CD4+ isotype within the lymphocyte gate; D.) CD45RA+ vs. CD45RO+ isotype within the CD4+ gate; E.) CD28- isotype within the CD4+ gate; F.) CD57+ isotype within the CD4+ gate; G.) CD38+ isotype within the CD4+ gate; H.) CD28 vs. CD57 isotype within the CD45RA+ gate; I.) CD4+ population; J.) CD45RA+ (upper left quadrant) vs. CD45RO+ (lower right quadrant); K.) CD28- cells within the CD4+ gate; L.) CD57+ cells within the CD4+ gate; M.) CD38+ cells within the CD4+ gate; N.) CD4+CD28-CD57+CD45RA+ (TEMRA) cells (lower right quadrant) within the CD4+CD45RA+ gate. Flow cytometry for CD8+ subsets followed the same gating strategy with CD8 substituted for CD4.