Supplemental information

Biosafety assessment of engineered CCL20 locked dimers *in vivo*

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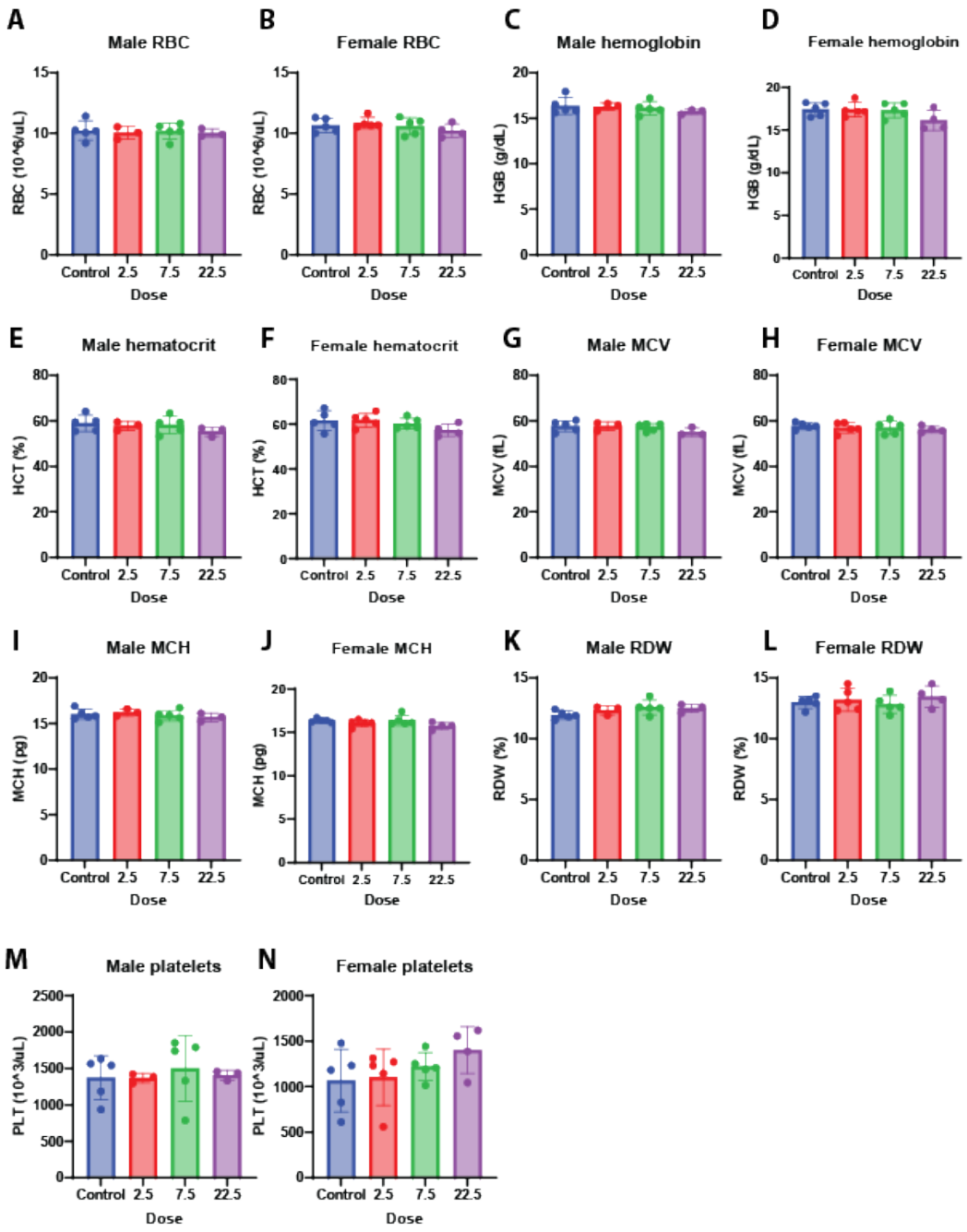
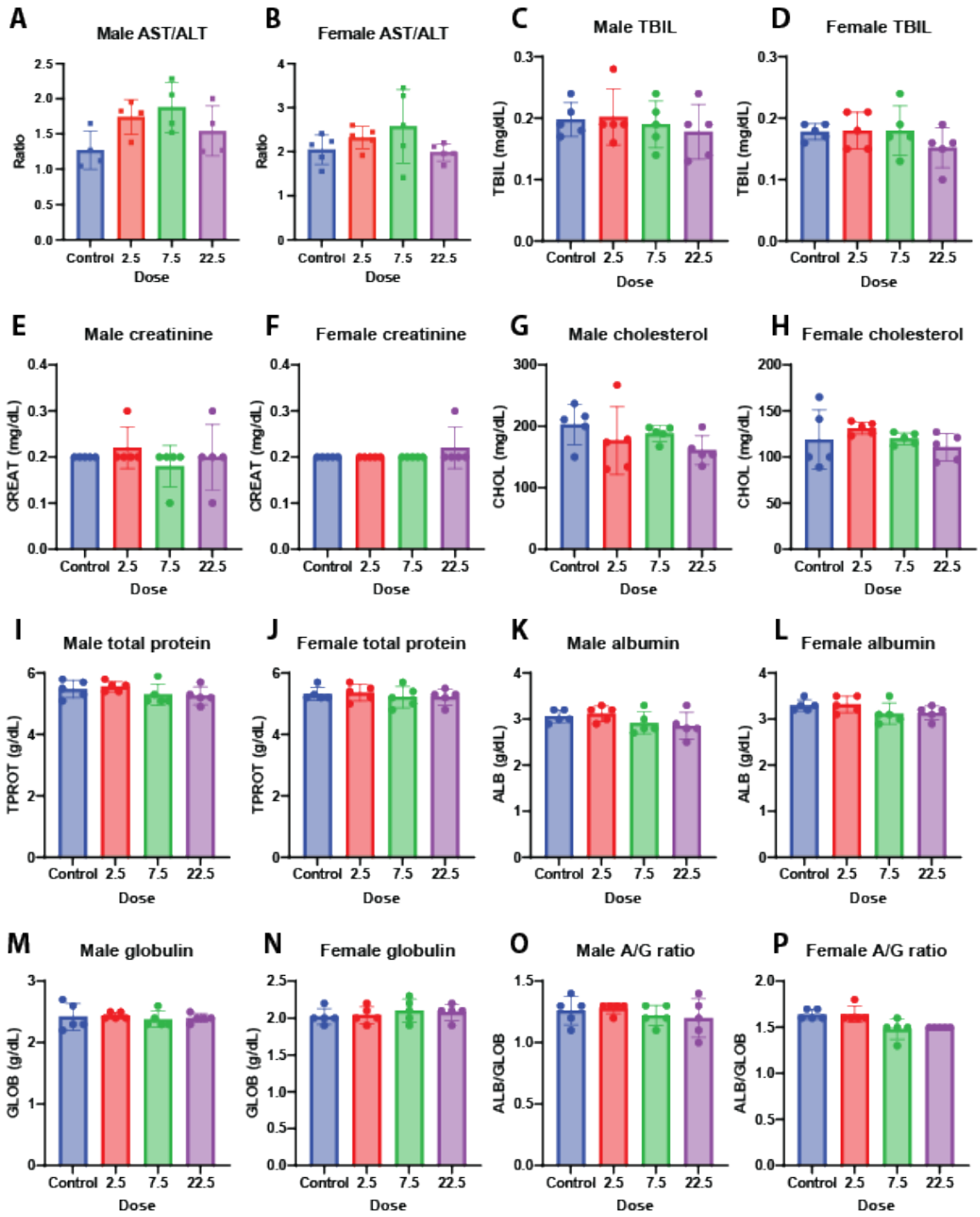
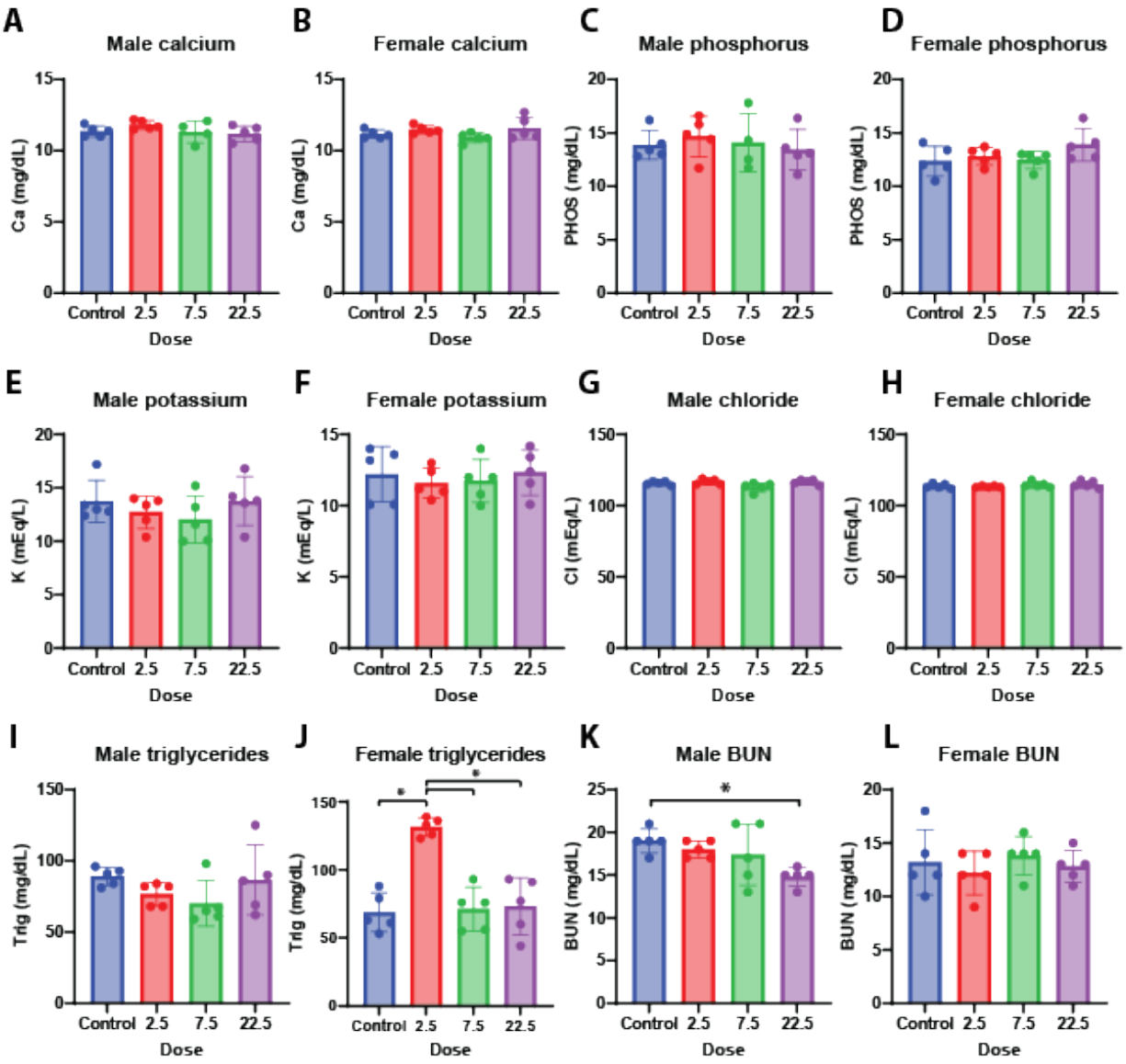


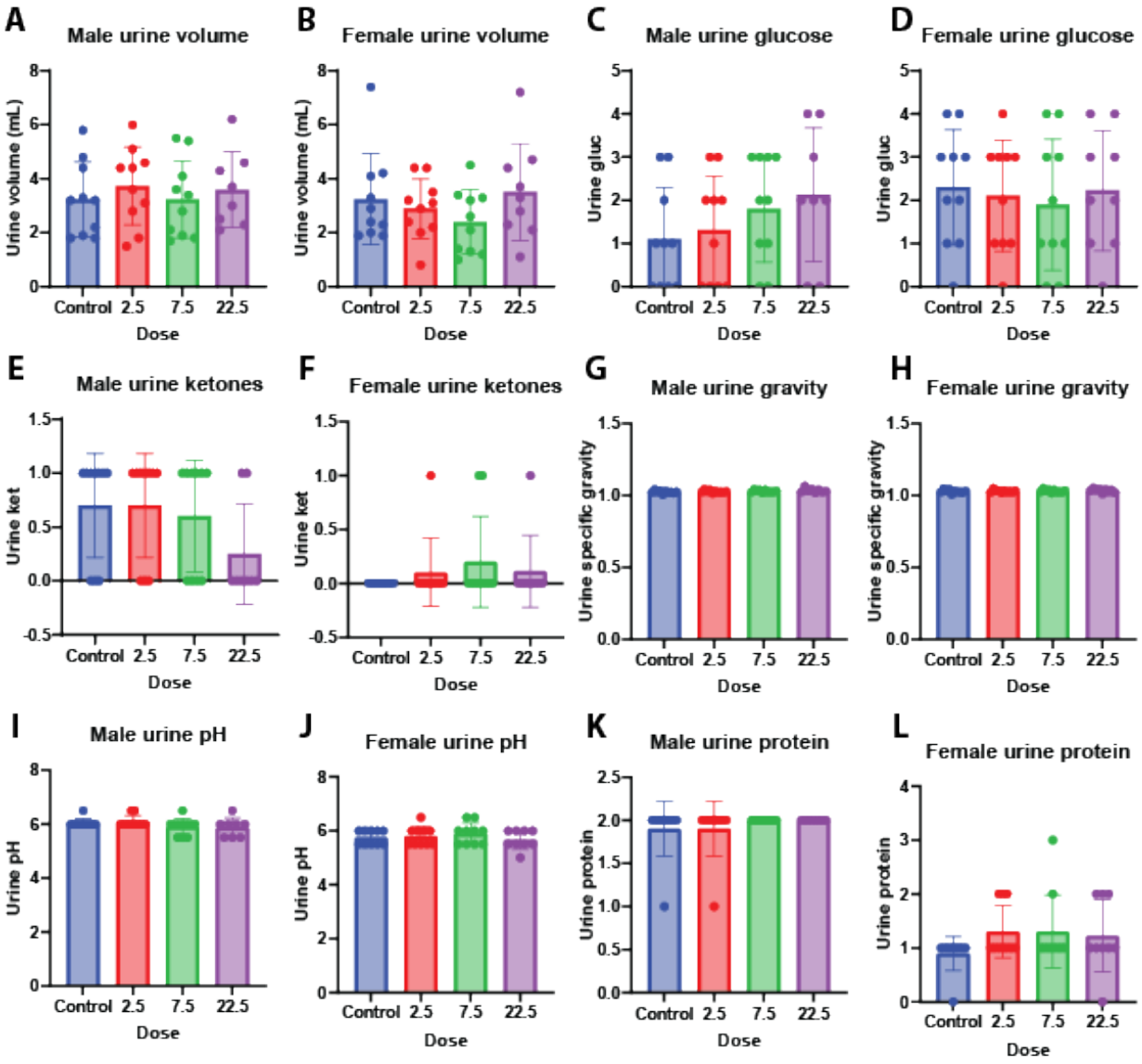
Supplemental Figure 1. Hematological lab values. Vena cava blood was tested following carbon dioxide inhalation after daily 2-week treatment of CD1 male or female mice with the stated concentrations of CCL20LD or the sterile PBS vehicle negative control (control). The following parameters were measured in males and females: (**A, B**) Red blood cells (RBC, (10^6/μL), (**C, D**) hemoglobin (HGB, g/dL) (**E, F**) hematocrit (HCT, %), (**G, H**) mean corpuscular volume (MCV, fL), (**I, J**) mean corpuscular hemoglobin (MCH, pg), (**K, L**) red blood cell distribution width (RDW, %), and (**M, N**) platelets (PLT, 10^3/μL). No values tested had $P \leq 0.05$ by one-way Anova with Tukey's multiple comparisons test.



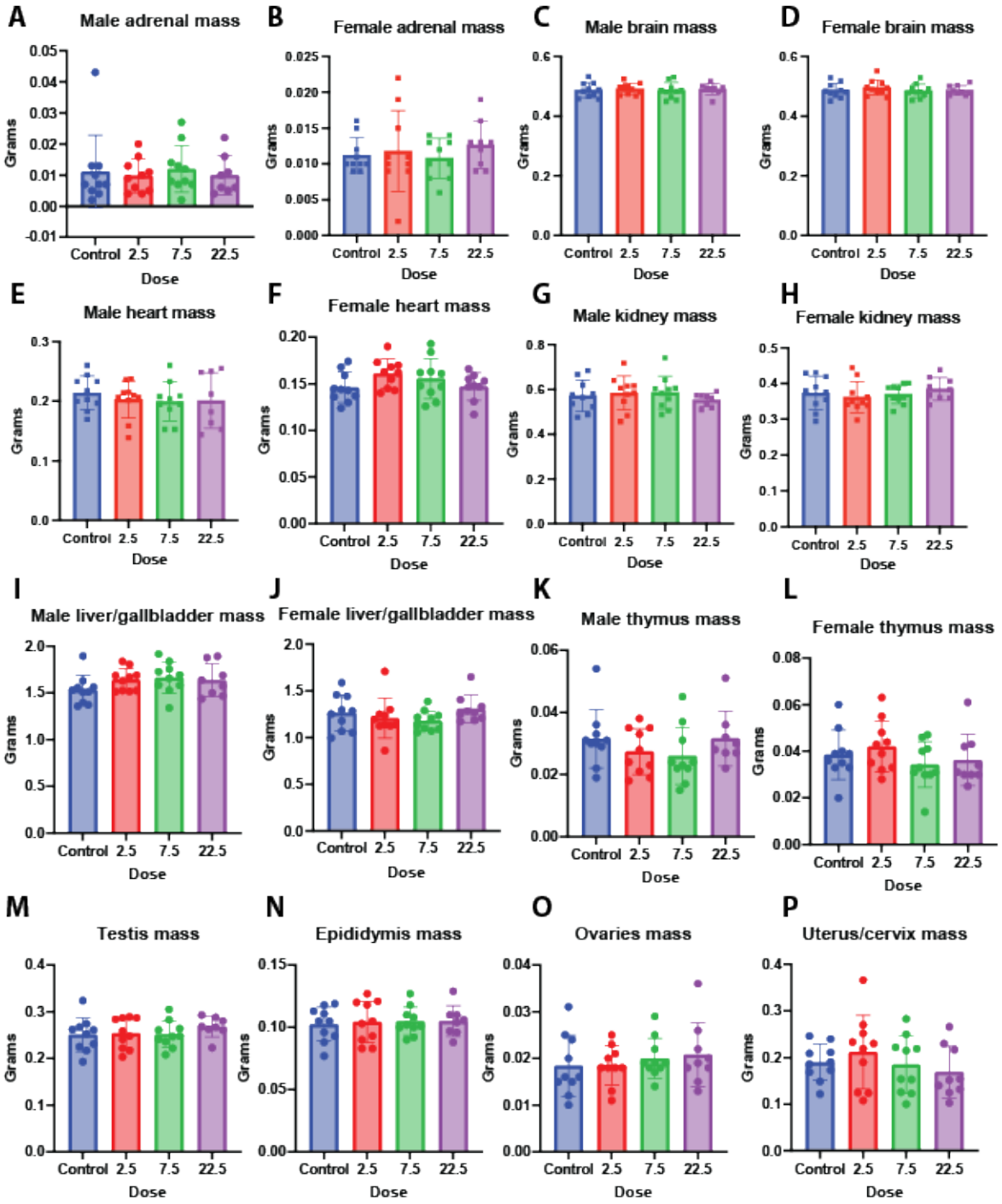
Supplemental Figure 2. Clinical chemistry measurements. Clinical chemistry values obtained from CD1 mice treated daily with CCL20LD or sterile PBS as a vehicle control. (**A, B**) The AST/ALT, or De Ritis Ratio. (**C, D**) Total bilirubin (TBIL, mg/dL). (**E, F**) Creatinine (mg/dL). (**G, H**) Cholesterol (mg/dL). (**I, J**) Total protein (g/dL). (**K, L**) Albumin (g/dL). **(M, N**) Globulin (g/dL). (**O, P**) The albumin to globulin ratio (A/G). None of the displayed clinical chemistry lab values achieved statistical significance ($P \leq 0.05$) by one-way Anova with Tukey's multiple comparisons test.



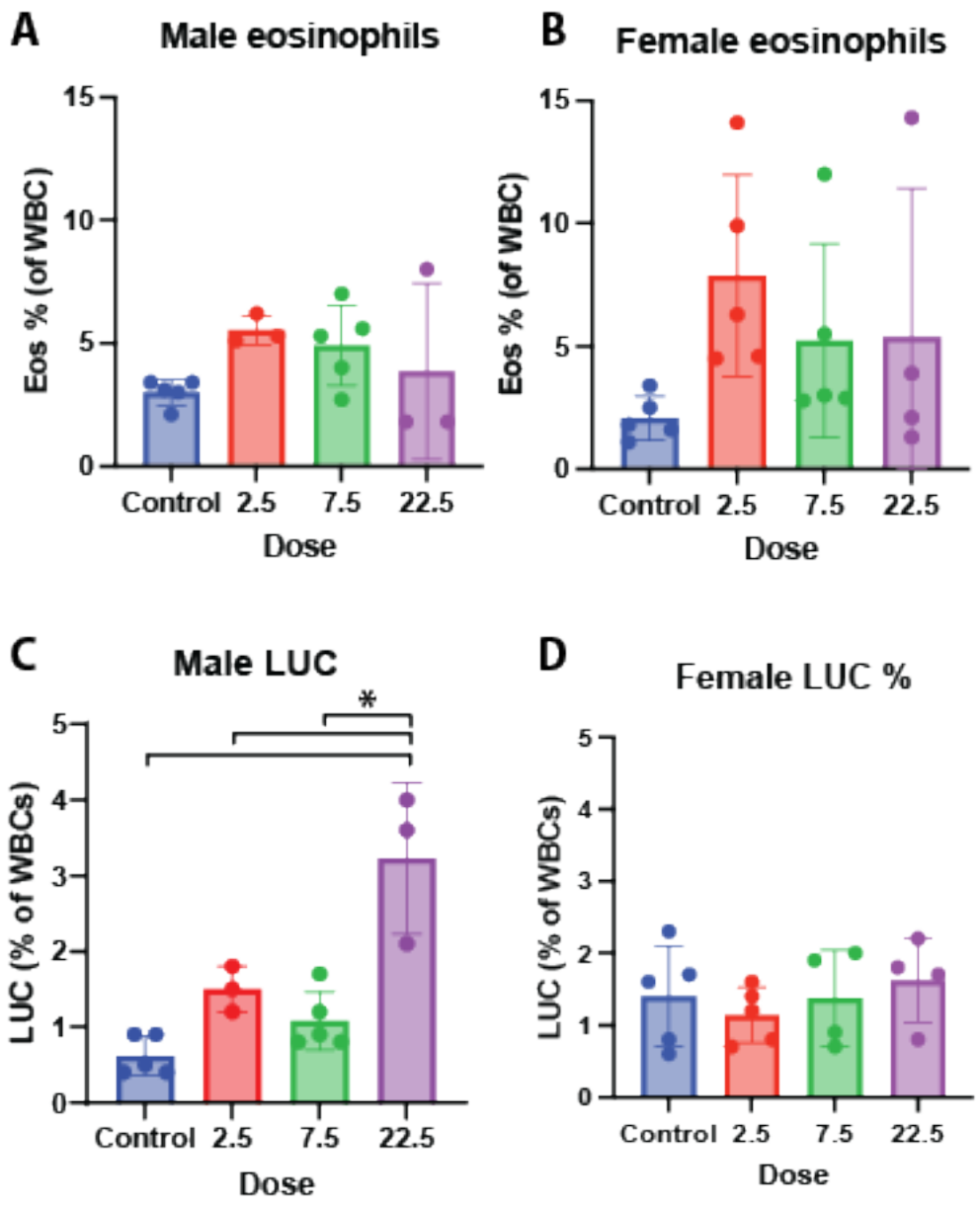
Supplemental Figure 3. Renal function after CCL20LD administration. Clinical chemistry values obtained from CD1 mice treated daily with CCL20LD or sterile PBS as a vehicle control. (**A, B**) Calcium, (**C, D**) phosphorus, (**E, F**) potassium, (**G, H**) chloride, (**I, J**) triglycerides, and (**K, L**) blood urea nitrogen (BUN). * denotes $P \leq 0.05$ by one-way Anova with Tukey's multiple comparisons test.



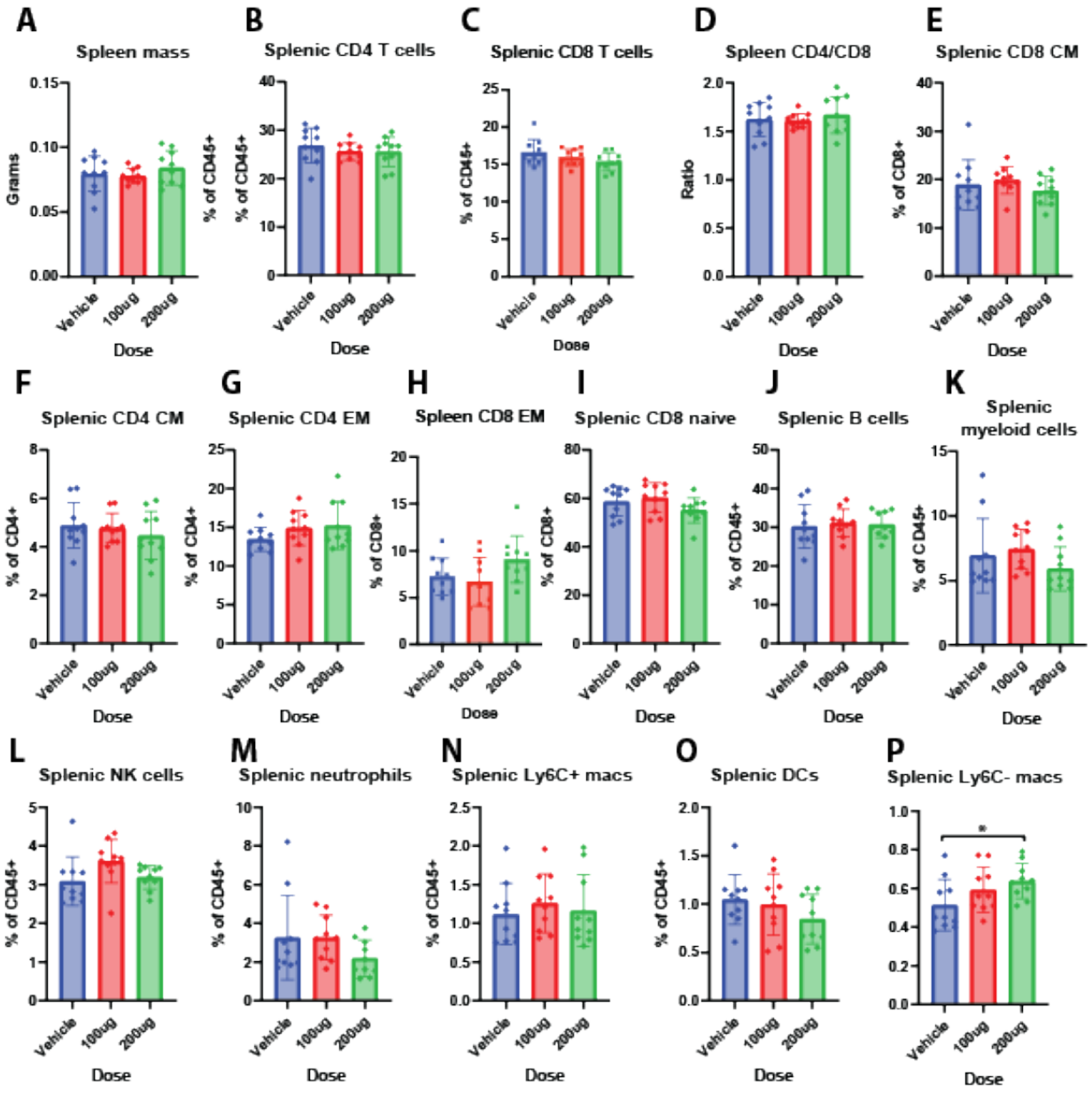
Supplemental Figure 4. Kidney function assessment. Values obtained were from urine collected from treated or control CD1 mice for a period of at least 12 hours. (**A, B**) Urine volume, (**C, D**) urine glucose, (**E, F**) urine ketones, (**G, H**) urine gravity, (**I, J**) urine pH, and (**K, L**) urine protein. None of the values tested were statistically different ($P \leq 0.05$) by one-way Anova with Tukey's multiple comparisons test.



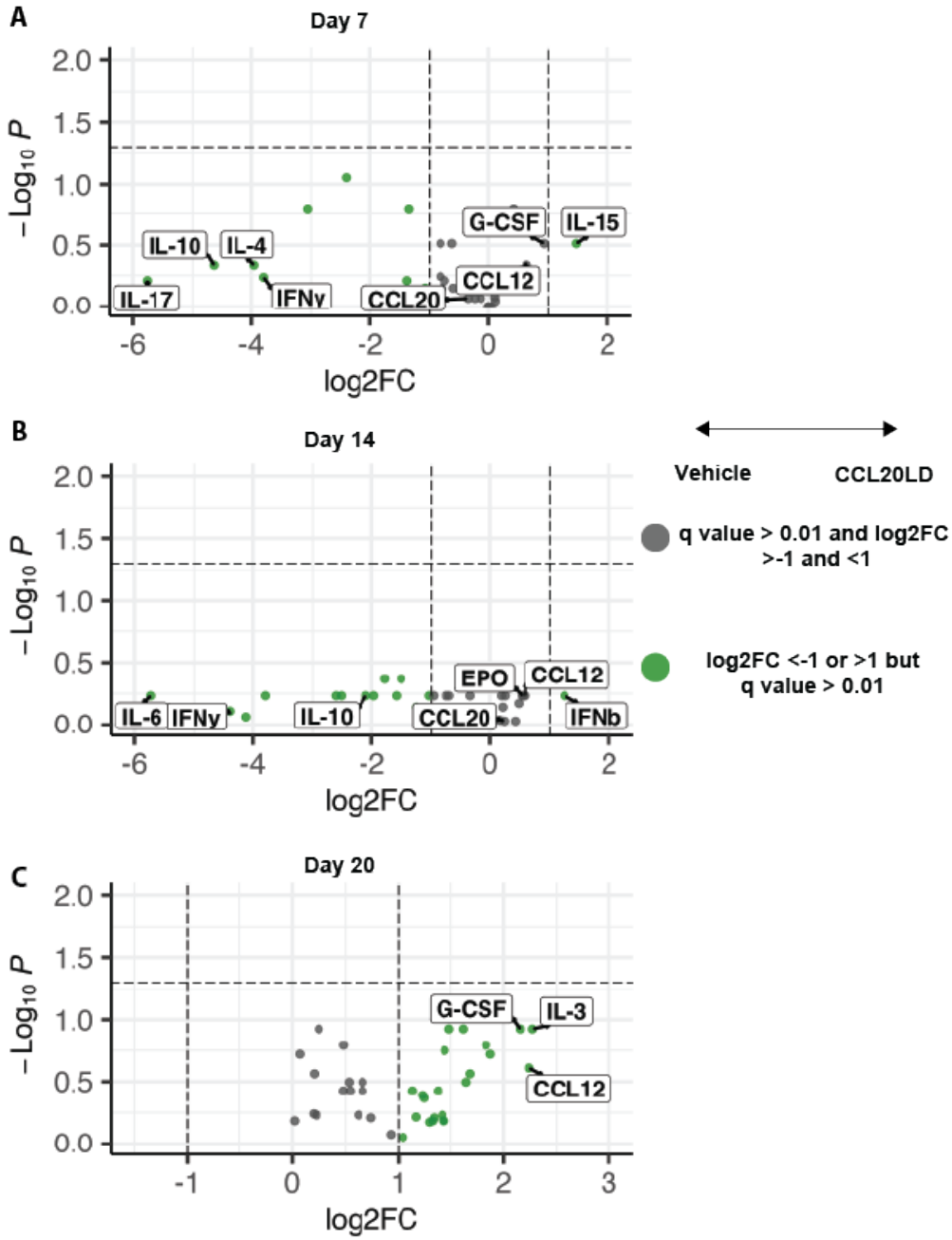
Supplemental Figure 5. Measurement of organ size in treated and control mice. Organs were weighed following euthanasia after daily 2-week treatment of CD1 mice. (**A, B**) Adrenal glands (paired), (**C, D**) brain, (**E, F**) heart, (**G, H**) kidneys (paired), (**I, J**) liver and gallbladder together, (**K, L**) thymus, (**M**) testis, (**N**) epididymis, (**O**) ovaries, and (**P**) uterus and cervix together. None of the values measured achieved statistical significance ($P \leq 0.05$) by one-way Anova with Tukey's multiple comparisons test.



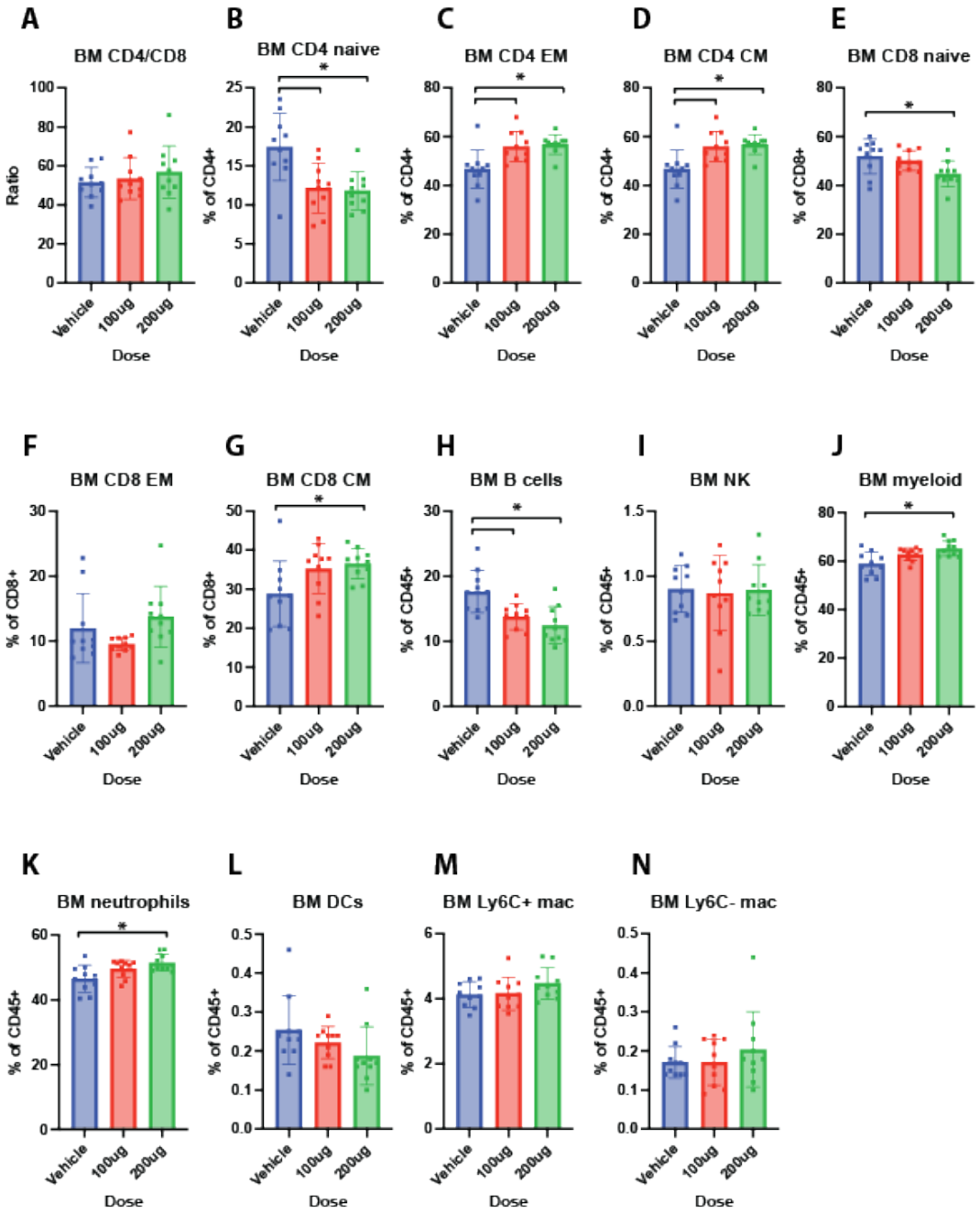
Supplemental Figure 6. Serum eosinophil and large unstained cell populations. (**A, B**) Eosinophil levels relative to total leukocytes in male or female CD1 mice. (**C, D**) Large unstained cells (LUC), an undefined population that may include large lymphocytes, activated lymphocytes, atypical lymphocytes, blast cells or plasma cells, relative to total leukocytes. * denotes $P \leq 0.05$ by one-way Anova with Tukey's multiple comparisons test.



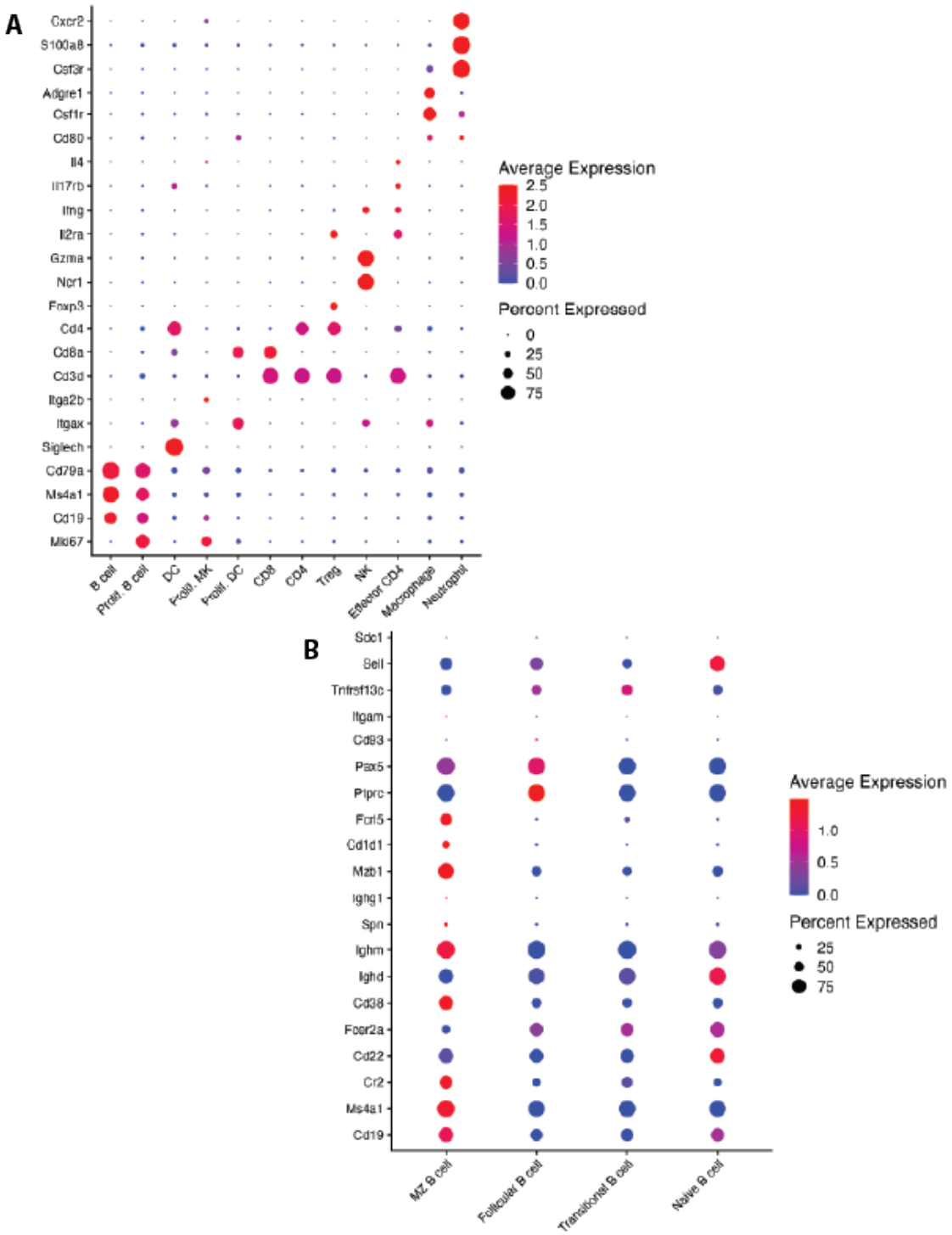
Supplemental Figure 7. CCL20LD treatment effects on spleen immune cell populations. C57/BL6 mice were treated with either sterile PBS vehicle control, 100 μg, or 200 μg CCL20LD every other day for 3 weeks. (**A**) No increase in spleen mass was observed. No changes were seen in the following populations in the spleen: (**B**) CD4+ T cells, (**C**) CD8+ T cells, (**D**) the CD4 to CD8 ratio, (**E**) CD8+ central memory (CM) cells, (**F**) CD4+ CM cells, (**G**) CD4+ effector memory (EM) cells, (**H**) CD8+ EM cells, (**I**) naïve CD8+ cells, (**J**) B cells, (**K**) myeloid cells, (**L**) natural killer (NK) cells, (**M**) neutrophils, (**N**) Ly6C+ macrophages (macs), and (**O**) dendritic cells (DCs). (**P**) The percentage of Ly6C- macrophages increased with 200 μg, $P \leq 0.05$ by one-way Anova with Tukey's multiple comparisons test. No other immune populations listed had significant changes in their relative frequency by one-way Anova with Tukey's multiple comparisons test.



Supplemental Figure 8. Serum cytokine changes in CCL20LD or control mice. Serum from vehicle and 200 μg CCL20LD treated C57BL/6J mice was collected at day (**A**) 7, (**B**) 14, and (**C**) 20. Serum was analyzed by the EVE Mouse 44-plex Discovery Assay (MD44). The volcano plots shown are from three mice per treatment analyzed by multiple unpaired t tests with a Benjamini, Krieger, and Yekutieli false discovery rate (FDR) approach with a desired FDR of 1%. Cytokines greater in the vehicle mice are on the left (negative log_2FC values) and cytokines greater in the CCL20LD treated mice are on the right (positive log_2FC values). Significance bars shown are for log_2FC >1 or <-1 and a value <0.01. None of the measured cytokines achieved q <0.01 at any timepoint.



Supplemental Figure 9. Relative frequency of specific immune subsets in the bone marrow. C57BL/6J mice were treated with either sterile PBS, 100 μg, or 200 μg CCL20LD every other day for 3-weeks. The relative frequency or ratios of the following immune cells in the bone marrow (BM) were measured by flow cytometry: (**A**) the CD4 to CD8 ratio, (**B**) naïve CD4+ cells, (**C**) CD4+ effector memory (EM) cells, (**D**) CD4+ central memory (CM) cells, (**E**) naïve CD8+ cells, (**F**) CD8+ EM cells, (**G**) CD8+ CM cells, (**H**) B cells, (**I**) natural killer (NK) cells, (**J**) myeloid cells, (**K**) neutrophils, (**L**) dendritic cells (DCs), (**M**) Ly6C+ macrophages (mac), (**N**) Ly6C- mac. * denotes $P \leq 0.05$ by one-way Anova with Tukey's multiple comparisons test.



Supplemental Figure 10. Markers used to identify immune cell populations in the single cell RNA dataset. (**A**) The panel of markers used to identify the listed general cell populations. (**B**) The panel of markers used to identify specific B cell subsets.

Parameter Analyzed	Mouse Strain	Concentration	Change	Sex Specific
Injection site scabbing	CD1	Increasing titrations	Increased	Males only
Reticulocyte count	CD1	22.5mg/kg/day	Increased	Males only
Liver AST	CD1	2.5mg/kg/day	Increased	Males only
Female AST	CD1	7.5mg/kg/day	Increased	Females only
Liver ALP	CD1	22.5mg/kg/day	Decreased	Males only
Blood bicarbonate	CD1	2.5 & 7.5mg/kg/day	Increased	Females only
Triglycerides	CD1	2.5mg/kg/day	Increased	Females only
Blood urea nitrogen	CD1	22.5mg/kg/day	Decreased	Males only
Spleen mass	CD1	22.5mg/kg/day	Increased	Both sexes
Neutrophils	CD1	22.5mg/kg/day	Increased	Males only
Lymphocytes	CD1	22.5mg/kg/day	Decreased	Males only
Basophils	CD1	22.5mg/kg/day	Increased	Males only
Large unstained cells	CD1	22.5mg/kg/day	Increased	Males only
Immune Profile: Spleen (flow cytometric enumeration)				
Splenic Ly6C- macrophages	C57/BL6	200μg QOD	Increased	Not determined
Splenic naïve CD4	C57/BL6	200μg QOD	Decreased	Not determined
Immune Profile: Bone Marrow (flow cytometric enumeration)				
Bone marrow CD4	C57/BL6	100μg, 200μg QOD	Decreased	Not determined
Bone marrow CD8	C57/BL6	100μg, 200μg QOD	Decreased	Not determined
Bone marrow hematopoietic stem cells	C57/BL6	100μg, 200μg QOD	Increased	Not determined
Bone marrow common myeloid progenitors	C57/BL6	200μg QOD	Increased	Not determined
Bone marrow naïve CD4	C57/BL6	100μg, 200μg QOD	Decreased	Not determined
Bone marrow effector memory CD4	C57/BL6	100μg, 200μg QOD	Increased	Not determined
Bone marrow central memory CD4	C57/BL6	100μg, 200μg QOD	Increased	Not determined
Bone marrow naïve CD8	C57/BL6	200μg QOD	Decreased	Not determined
Bone marrow central memory CD8	C57/BL6	200μg QOD	Increased	Not determined
Bone marrow B cells	C57/BL6	200μg QOD	Decreased	Not determined
Bone marrow myeloid	C57/BL6	200μg QOD	Increased	Not determined
Bone marrow neutrophils	C57/BL6	200μg QOD	Increased	Not determined
Immune profiling (cell frequency from scRNA analysis)				
B cells	C57/BL6	200μg QOD	Decreased	Not determined
Regulatory T cells	C57/BL6	200μg QOD	Increased	Not determined
Macrophages	C57/BL6	200μg QOD	Increased	Not determined
Neutrophils	C57/BL6	200μg QOD	Increased	Not determined
Proliferating B cells	C57/BL6	200μg QOD	Increased	Not determined
Effector CD4 T cells	C57/BL6	200μg QOD	Increased	Not determined
Dendritic cells	C57/BL6	200μg QOD	Increased	Not determined
Proliferating megakaryocyte	C57/BL6	200μg QOD	Increased	Not determined
Proliferating dendritic cells	C57/BL6	200μg QOD	Increased	Not determined
Follicular B cells	C57/BL6	200μg QOD	Decreased	Not determined
Marginal zone B cells	C57/BL6	200μg QOD	Increased	Not determined
Naïve B cells	C57/BL6	200μg QOD	Increased	Not determined

Supplemental Table 1. Summary of statistically significant changes measured.

Target	Fluor	Source	Catalog number	Research Resource Identifier (RRID)
Fixable viability dye	eFluor 506	Invitrogen	65-0866-14	
CD3	APC-Fire 750	Biolegend	100248	RRID:AB_2572118
CD4	BUV496	BD	612952	RRID:AB_2813886
CD8a	BUV805	BD	612898	RRID:AB_2870186
CD11b	PE	Invitrogen	12-0112-82	RRID:AB_2734869
CD11c	BUV737	BD	612796	RRID:AB_2870123
CD19	Alexa fluor 700	Biolegend	152414	RRID:AB_2922474
CD44	BUV395	BD	568507	RRID:AB_3677624
CD45	BV786	BD	564225	RRID:AB_2716861
CD62L	Super Bright 702	Invitrogen	67-0621-82	RRID:AB_2717151
F4/80	PE-Cy7	Biolegend	123114	RRID:AB_893478
Ly-6C	PE-Dazzle 594	Biolegend	128044	RRID:AB_2566577
Ly-6G	FITC	Biolegend	127606	RRID:AB_1236494
MHCII	eFluor 450	Invitrogen	48-5321-82	RRID:AB_1272204
NK1.1	APC	Invitrogen	17-5941-82	RRID:AB_469479
Lineage	eFluor 450	Invitrogen	88-7772-72	RRID:AB_10426799
CD16/32	PE-Cy7	Biolegend	101318	RRID:AB_2104156
CD34	PE	Biolegend	128609	RRID:AB_2074602
CD127	Super Bright 645	Invitrogen	64-1271-82	RRID:AB_2744868
cKIT	APC-eFluor 780	Invitrogen	47-1172-80	RRID:AB_1582227
SCA-1	APC	Invitrogen	17-5981-81	RRID:AB_469486

Supplemental Table 2. List of antibodies using for the flow cytometry analyses.