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OMIP-032: Two Multi-Color Immunophenotyping Panels for Assessing the Innate and Adaptive Immune Cells in the Mouse Mammary Gland

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Additional Supporting Information may be found in the online version of this article.

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Purpose and Appropriate Sample Types

A multi-color antibody panel was designed and optimized to identify and characterize 10 leukocyte subpopulations from both the innate and adaptive arms of the immune system. Markers to detect B-cells ($CD45.2^+$ $CD19^+$), T-cells ($CD45.2^+$ $TCR\beta^+$), natural killer cells ($CD45.2^+$ $TCR\beta^-$ $CD49b^+$ $NKp46^+$), and myeloid cells ($CD45.2^+$ $CD11b^+$) were employed. Myeloid cells were further differentiated as dendritic cells ($CD45.2^+$ $CD11b^+$ $CD11c^+$ $MHCII^+$), neutrophils ($CD45.2^+$ $CD11b^+$ $Ly6G^+$), macrophages ($CD45.2^+$ $CD11b^+$ $Ly6G^-$ $Ly6C^{low}$), and monocytes ($CD45.2^+$ $CD11b^+$ $Ly6G^-$ $Ly6C^{high}$). T-cell populations were sub-divided into T-helper cells ($CD45.2^+$ $TCR\beta^+$ $CD8^-$ $CD4^+$), and cytotoxic T-cells ($CD45.2^+$ $TCR\beta^+$ $CD4^-$ $CD8^+$). T-cell memory/effector status was determined using CD62L and CD44 to distinguish between effector ($CD44^+$ $CD62L^-$), memory ($CD44^+$ $CD62L^+$) and naïve ($CD44^-$ $CD62L^+$) T cells. This panel was established for the analysis of collagenase digested mouse (Balb/C) mammary gland, spleen and tumor samples, as well as RBC-lysed whole blood (Table 1).

Background

Immune cells have been shown to play a role in various stages of mammary gland development. Macrophages in particular have been shown to assist with ductal elongation, estrus cycle regulation, mammary gland differentiation during pregnancy, and wound healing during mammary involution, post weaning (1). Considering the specific role immune cells play during development, it is not surprising that they also have been correlated with disease progression (2) and drug efficacy (3) within the breast. Most studies to date have used techniques such as immune deficient mouse models to determine the role of the immune cells in the mammary gland, immunohistochemistry to characterize the immune contexture of the breast, or gene arrays to establish which immune pathways may be activated during different stages of mammary gland development (1,4,5). However, these methods allow for analysis of only one or two specific immune cell subtypes at any one time, or are a more general analysis on homogenized whole mammary glands (gene array). Assessment of the distribution and function, and interplay of the immune cells with normal and diseased mammary tissue remains poorly studied.

With growing interest in the role of the immune microenvironment in influencing tissue development, disease progression and drug efficacy, there is an increasing need for structured methodologies to examine the immune composition of the breast.

Our flow cytometry immunophenotyping panels were developed to quantitate frequency and differentiation status of a broad range of innate and adaptive immune cells within mouse mammary tissue (Fig. 1; Supporting Information Methods and tables).

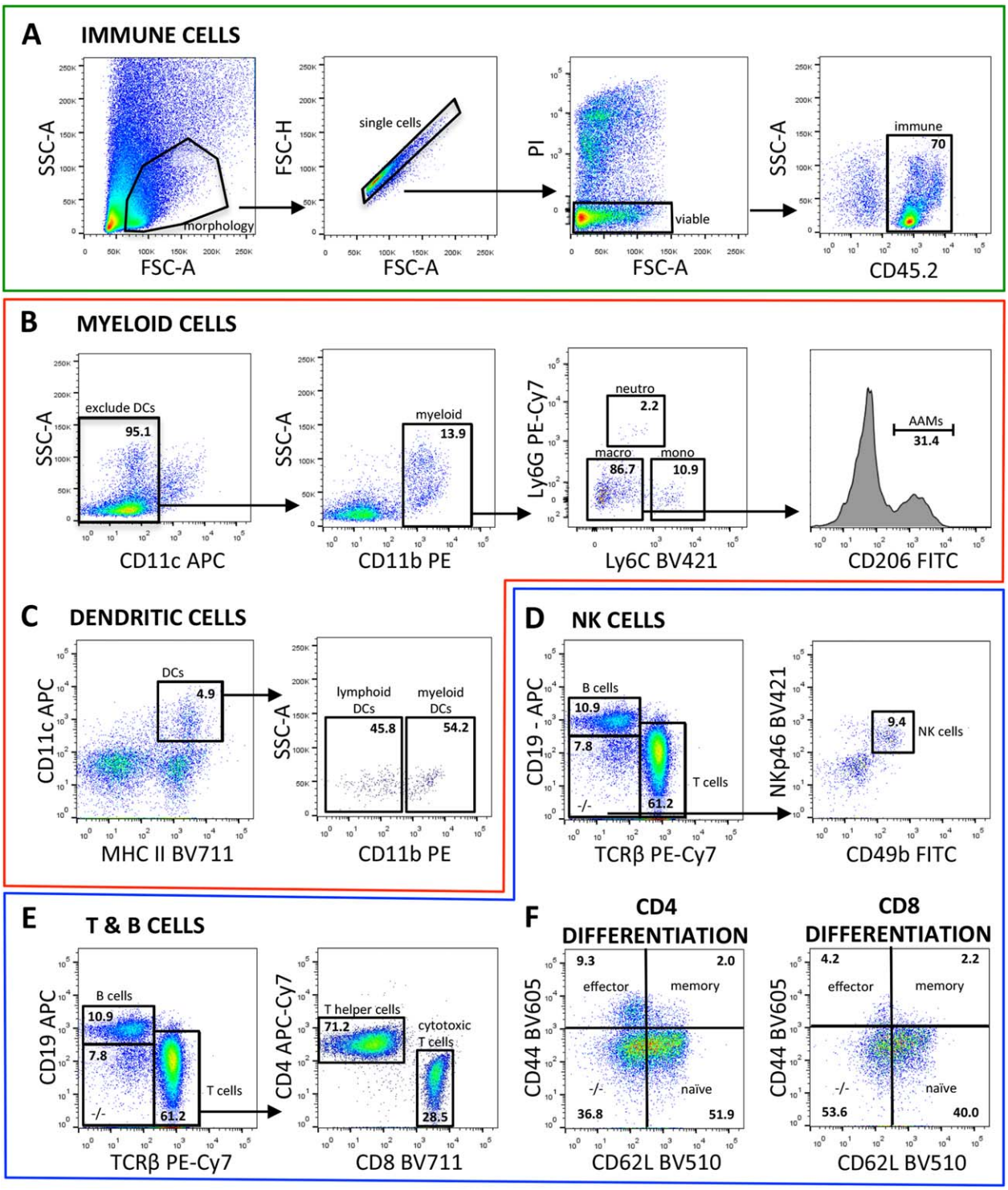

Figure 1. Flow cytometry gating scheme for mouse mammary glands. Green box = panel 1 + 2 antibodies. Red box = panel 1 antibodies. Blue box = panel 2 antibodies. **A**: Leukocytes were gated according to morphology (SSC-A vs. FSC-A), doublets were excluded (FSC-H vs. FSC-A) and live immune cells were selected using PI and CD45.2. This gating strategy was used as a base for all following gates. **B**: Dendritic cells were excluded from the myeloid cell analysis by gating on $CD11c^{-}$ cells. Myeloid cells were then identified as $CD11b^{+}$. This population could be further dissected into macrophages (macro: $CD11b^{+}$ $Ly6G^{-}Ly6C^{-/low}$), monocytes (mono: $CD11b^{+}$ $Ly6G^{-}Ly6C^{high}$), and neutrophils (neutro: $CD11b^{+}$ $Ly6G^{+}$ $Ly6C^{-/low}$) using Ly6C vs. Ly6G. The population described as macrophages in this figure is macrophage enriched, but may also contain up to 25% of other myeloid cells including eosinophils and monocytes. The alternatively activated macrophages (AAMs) were subsequently identified as $CD11b^{+}$ $Ly6G^{-}Ly6C^{-/low}CD206^{+}$. **C**: A CD11c vs. MHC II plot was used to identify the dendritic cells (DCs: $CD11c^{+}$ MHC II^{+}), while CD11b was subsequently used to distinguish between lymphoid derived DCs ($CD11c^{+}$ MHC II^{+} $CD11b^{-}$) and myeloid derived DCs ($CD11c^{+}$ MHC II^{+} $CD11b^{+}$). **D**: T cells were excluded from the NK cell analysis by isolating $TCR\beta^{-}$ cells. NK cells were then identified as being $NKp46^{+}$ $CD49b^{+}$. **E**: A CD19 vs. $TCR\beta$ plot was used to discriminate between T cells ($TCRB^{+}$ $CD19^{-}$) and B cells ($TCR\beta^{-}CD19^{+}$). CD4 vs. $CD8\alpha$ was then used to further differentiate T cells into T helper cells ($TCR\beta^{+}CD19^{-}CD4^{+}$ $CD8^{-}$) and cytotoxic T cells ($TCR\beta^{+}CD19^{-}CD4^{-}CD8^{+}$). **F**: The memory/effector status of both $CD8^{+}$ and $CD4^{+}$ T cells could be determined using a CD62L vs. CD44 plot on both populations, to characterize naïve ($CD62L^{+}$ $CD44^{-}$), memory ($CD62L^{+}$ $CD44^{+}$), and effector ($CD62L^{-}CD44^{+}$) CD4 and CD8 T cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

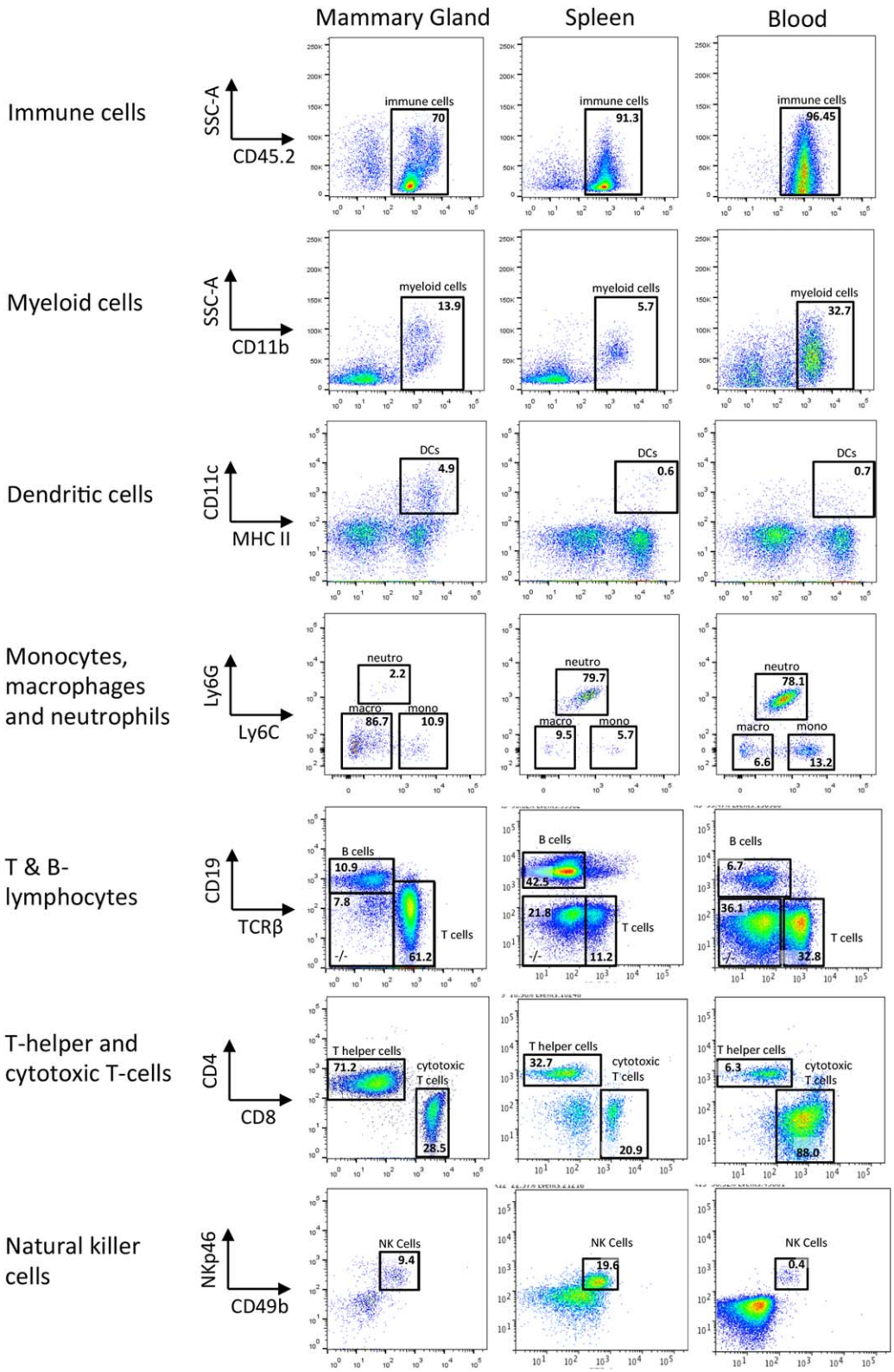

Figure 2. Comparison of immune cell FACS plots in mammary glands, spleens, and blood. While optimized for use in the mouse mammary gland, we also wanted to use this same OMIP on spleen and blood samples. Approximately 50,000 immune cells per spleen or blood sample were stained according to this OMIP and analyzed live on the BD LSRFortessa X20. Major gates for specific immune populations are displayed for mammary gland, spleen, and blood for direct comparison. The gates used prior to this for all were SSC and FSC for morphology, FSC-H and FSC-A for single cells, and PI for viable cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table 1. Summary table for the application of OMIP-032

Purpose	In-depth immunophenotyping of innate and adaptive immune cells
Species	Mouse
Cell types	RBC-lysed whole blood
	Collagenase IV digested spleen
	Collagenase IV digested mammary gland
	Collagenase IV digested tumor
Cross-references	None

Table 2. Summary table for the antibodies of the panel OMIP-032

	LASER	mAb	CLONE	FLUOROCHROME	SOURCE
Panel 1	405 nm	Ly6C	AL-21	BV421	BD
		MHC II	M5/114.15.2	BV711	BD
	488 nm	CD206	C068C2	FITC	Biolegend
	561 nm	CD11b	M1/70	PE	BD
		Ly6G	1A8	PE-Cy7	BD
	640 nm	CD11c	HL3	APC	BD
		CD45.2	104	APC-Cy7	eBioscience
Panel 2	405 nm	NKp46	29A1.4	BV421	BD
		CD62L	MEL-14	BV510	BD
		CD44	IM7	BV605	BD
		CD8	53-6.7	BV711	BD
	488 nm	CD49b	DX5	FITC	BD
	561 nm	CD45.2	104	PE	BD
		TCRβ	H57-597	PE-Cy7	BD
	640 nm	CD4	GK1.5	APC-Cy7	BD
Both	Violet (405 nm)	Live/Dead	–	Fluoro-Gold	Sigma-Aldrich
	Yellow/Green (561nm)	Live/Dead	–	PI	Sigma-Aldrich

APC, allophycocyanin; BV, Brilliant Violet; Cy, cyanin; FITC, fluorescein isothiocyanate; PE, R-phycoerythrin; PI, Propidium Iodide.

These panels were also developed so that they were effective on spleen and blood samples (Fig. 2). Due to the limited number of immune cells present within the mammary gland, we began by designing four antibody immunophenotyping panels to be used on pooled 4th inguinal mammary glands from 4+ mice. These four panels were later condensed to just two immunophenotyping panels, allowing an equivalent level of immune analysis from only the 4th inguinal mammary glands of a single mouse. The development and optimization of these immunophenotyping panels will hence be discussed further.

We tested a range of commercially available fluorochrome-conjugated antibodies on collagenase-digested mammary glands. The frequency of immune cells within a pair of 4th inguinal mammary fat pads is very low (~200,000 $CD45^+$ cells in 25-week old mice and ~60,000 $CD45^+$ cells in 6-week- old mice) and hence mice were initially pooled for immune analysis. Due to the need to analyze each mouse individually, our panels underwent further optimization and were condensed to two immunophenotyping panels (Table 2).

The first cocktail delineates cells within the myeloid compartment, including: neutrophils, macrophages, monocytes, and dendritic cells. The second cocktail identifies cells within the lymphocyte compartment, including B-cells, T-cells, and natural killer (NK) cells. Two additional markers CD44 and CD62L are also included to determine the memory/effector status of the T-cell subsets. This panel was optimized for mouse mammary glands, however it has also been shown to work effectively in spleen and blood.

Similarity to Published OMIPs

None to date.

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