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Coordinate Analysis of Murine Immune Cell Surface Markers and Intracellular Phosphoproteins by Flow Cytometry[1]

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Recently, phosphospecific flow cytometry has emerged as a powerful tool to analyze intracellular signaling events in complex populations of cells because of its ability to simultaneously discriminate cell types based on surface marker expression and measure levels of intracellular phosphoproteins. This has provided novel insights into the cell- and pathway-specific nature of immune signaling. However, we and others have found that the fixation and permeabilization steps necessary for phosphoprotein analysis often negatively affect the resolution of cell types based on surface marker analysis and light scatter characteristics. Therefore, we performed a comprehensive profile of >35 different murine surface marker Abs to understand the effects of fixation and permeabilization on surface Ag staining. Fortuitously, ~80% of the Abs tested resolved cell populations of interest, although with decreased separation between positive and negative populations and at very different titers than those used on live cells. The other 20% showed either complete loss of separation between populations or loss of intermediately staining populations. We were able to rescue staining of several of these Ags by performing staining after fixation, but before permeabilization, although with limited fluorophore choices. Scatter characteristics of lymphocytes were well retained, but changed dramatically for monocyte and neutrophil populations. These results compile a comprehensive resource for researchers interested in applying phosphospecific flow cytometry to complex populations of cells while outlining steps necessary to successfully apply new surface marker Abs to this platform. *The Journal of Immunology,* **2005, 175: 2357–2365.**

Phosphospecific flow cytometry is well suited to the analysis of intracellular signaling cascades in heterogeneous cell populations such as those present in immunological organs (peripheral blood, thymus, bone marrow, lymph nodes, and spleen). Recent studies have shown the utility of this technique in discerning signaling events at the single-cell level and highlight the importance of being able to resolve individual cell types in complex populations (1). For instance, the in vitro and in vivo Ag-specific induction of MAPK pathway members (ERK, p38, and JNK) in T cells were measured in murine TCR transgenic models (2, 3). Importantly, Ag-specific T cells comprising only 1–2% of the spleen could be analyzed in the presence of the other cell types by staining with anti-TCR clonotypic Abs (3). In other studies, responsiveness to IL-7 was examined in thymic T cell subsets and was found to vary among early progenitor populations separated by CD4, CD8, CD44, and CD25 staining (4, 5). Our group has used the technique to stratify acute myeloid leukemia patient samples based on their response to cytokine panel profiles (6). We have also shown that it is possible to analyze multiple phosphoproteins inside single cells simultaneously (7). Finally, single-cell phosphoprotein measurements have proven useful in analyzing small molecule kinase inhibitor specificity in peripheral blood (8).

Although these studies were able to discriminate cell types based on surface marker expression, separation between positive and negative populations often decreased, especially when methanol permeabilization was used (5). As we have begun to apply phosphospecific flow cytometry to more immunological cell types, we have encountered difficulties while trying to stain for surface Ags after cells have been fixed and/or permeabilized. In particular, staining of B cell progenitors in the bone marrow has proven difficult due to poor CD43 and CD24 staining, both of which are critical to separation of early B cell populations. Identification of mast cells from splenic and peritoneal populations has also been hindered by poor FcεR1a and c-Kit resolution. Thus, much effort has been put into finding and characterizing Abs that work in the context of phosphospecific flow cytometry. We believe that as researchers increasingly apply the technique to rare cell types and novel organ systems, cell subset identification will be of critical importance. Therefore, we decided to undertake the current study to generate a comprehensive profile of the effects of fixation and permeabilization on surface marker Ab-staining characteristics to provide both a resource of data and a set of guidelines to follow when applying the technique.

Due to the transient nature of phosphorylation events, cells must be fixed rapidly after stimulation to freeze the phosphorylation status of proteins. Also, to analyze phosphorylation at the single-cell level, cells must be permeabilized to allow phosphospecific Abs access to relevant phosphoepitopes inside the cell (1). Fixation and permeabilization are thus absolutely required for the technique in its current state of application (9–11). Although many techniques have been used to analyze phosphoproteins by flow cytometry (2–6, 8, 12–18), we recently showed that formaldehyde fixation followed by methanol permeabilization (F/M)[3] was superior for the analysis of many key signaling molecules of the immune

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[3] Abbreviations used in this paper: F/M, formaldehyde fixation and methanol permeabilization; Ax, Alexa Fluor; FSC, forward scatter; MFI, median fluorescence intensity; pStat, phospho-Stat; SSC, side scatter.

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system, especially those of the STAT protein family (7). In addition to providing high sensitivity and signal-to-noise ratios, methanol has an advantage over saponin permeabilization in that phosphoepitopes are stable at −80°C for prolonged periods (months) when stored in methanol (7). This is especially important for experiments involving clinical samples, where samples can be collected over weeks or months, then stained and analyzed simultaneously.

Because multiple steps are required to prepare cells for analysis by phosphospecific flow cytometry, the simplest procedure would be to stain cells for surface markers and intracellular phosphoproteins simultaneously, after fixation and permeabilization. Therefore, in the context of intracellular detection of phosphoproteins, we set out to characterize the effects of formaldehyde fixation and methanol or saponin permeabilization on the detection of cell surface Ags and the maintenance of light scatter properties. Most Abs worked well after F/M treatment, although the separation between positive and negative populations decreased in nearly all cases. In addition, optimal titration conditions differed significantly after F/M treatment. For those Abs negatively affected by permeabilization, we tested whether a stepwise staining of surface markers after fixation, but before permeabilization, could rescue the staining efficiency for these Ags. After screening >35 Abs, we applied surface staining in combination with intracellular phosphoprotein analysis to characterize the immune cell subsets that were responsive to IL-6 and IL-10 treatment and to analyze $CD4^+$ and $CD8^+$ T cell differences in cytokine response. We show in this study the many parameters that must be taken into consideration when applying the technique to primary cell populations and provide a basic framework for researches interested in applying phosphospecific flow cytometry to their complex tissues of interest, particularly those of the immune system.

Materials and Methods

Abs and reagents

All surface and phosphospecific Abs were provided by BD Pharmingen and are summarized in Table I. Abs were supplied conjugated to FITC, Alexa Fluor 488 (Ax488), PE, Cy5PE, PerCP, Cy5.5 PerCP, allophycocyanin, or Ax647. The Ab concentration was 0.2 mg/ml, except for FITC conjugates, which were at 0.5 mg/ml. Phosphospecific Abs against Stat3 (Y705, clone 4) and Stat5 (Y694, clone 47) were conjugated to Ax647 and were used at optimal titrations.

Formaldehyde was obtained from Electron Microscopy Sciences. Methanol and saponin (from Quillaja bark) were purchased from Sigma-Aldrich. IL-15 was obtained from R&D Systems. IL-2, IL-6, and IL-10 were provided by BD Pharmingen.

Surface marker Ab analysis

Male BALB/c and C57BL/6 mice were obtained from the Stanford Animal Core Facility in-house breeding colony and were used at 6–12 wk of age. Animals were treated in accordance with university and Administrative Panels on Laboratory Animal Care guidelines. After death, peritoneal cavity cells were obtained by flushing the peritoneum with MEM containing 5% FBS. Spleens were removed and homogenized in MEM and 5% FBS; bone marrow cells were obtained by flushing tibia and femurs with the same buffer. All cells were then passed through a 70-μm pore size mesh, pelleted, and resuspended at room temperature in RPMI 1640 containing 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine at 5×10^6 cells/ml. Cells were then cultured at 37°C for 2 h before surface marker analysis or stimulation with cytokines for combined surface and intracellular phosphoprotein analysis.

For surface marker analysis alone, staining with Abs was performed at multiple points. For unfixed samples, cells were pelleted out of the culture medium and resuspended in staining medium (PBS with 0.5% BSA and 0.02% sodium azide) at 10^7 cells/ml. Staining was performed in 100-μl volumes (10^6 cells). Abs were added at various dilutions, and cells were stained for 25–30 min. The cells were washed with 15 vol of staining medium, pelleted, and resuspended for flow cytometric analysis. For formaldehyde fixation, formaldehyde was added directly to culture medium to a final concentration of 1.6% and incubated for 10 min at room temperature. Cells were pelleted, and then resuspended in staining medium and stained as described above. For samples that were permeabilized with methanol after this staining step, cells were washed with PBS instead of staining medium and resuspended directly in ice-cold 100% methanol after pelleting. Cells were incubated at 4°C for 10–30 min before washing twice with staining medium and analyzing by flow cytometry. Finally, for cells that were both fixed and permeabilized before staining, the formaldehyde-fixed cells were pelleted and resuspended directly in 100% methanol. They were incubated for 10–30 min at 4°C before being washed twice with staining medium and stained with Abs as described above. When cells were permeabilized with saponin, 0.1 or 0.5% solutions of saponin in staining medium were substituted for methanol. Also, saponin was present during staining to maintain cell permeability, because saponin permeabilization is reversible.

Table I. *Results of surface Ab testing before and after formaldehyde/methanol (F/M) treatment*

Ag	Clone	Fluorophore[a]	Unfixed Stain[b,c]	Post F/M Stain
B220	RA3–6B2	PerCP	+++	+++
CD19	1D3	PE	+++	+(+)
IgM	II/41	Allophycocyanin	+++	++
IgD	11–26.c2a	FITC	+++	++
CD21	7G6	FITC	++	++
CD23	B3B4	PE	+++	+++
CD40	3/23	PE	+++	+
TCR	H57–597	PE	+++	++
CD3	145–2C11	Ax488	++	+
CD4	RM4–5	FITC	+++	+++
CD4	GK1.5	FITC	+++	++
CD4	RM4–4	FITC	+++	++
CD4	H129.19	FITC	+++	++
CD5	53–7.3	Allophycocyanin	+++	++
CD8	53–6.7	Allophycocyanin	+++	++
CD25	PC61	PE	+++	+++
CD25	7D4	FITC	+++	++
CD25	3C7	PE	+++	++
CD44	IM7	Allophycocyanin	+++	++
CD45RA	14.8	PE	+++	+++
CD45RB	16A	FITC	+++	++
CD45RC	DNL-1.9	PE	+++	+++
CD69	H1–2F3	FITC	+++	++
CD11b	M1/70	FITC	+++	+++
CD11c	HL3	Allophycocyanin	+++	++
CD16	2.4G2	FITC	++	+
MHC I-A^d	AMS-32.1	FITC	++	++
MHC I-A^b	AF6–120.1	FITC	++	−
CD86	GL1	PE	−	++
Gr-1	RB6–8C5	PE	+++	+(+)[d]
Ly6C	AL-21	FITC	+++	−(+)[d]
NK1.1	PK136	FITC	++(+)	++
CD49b	DX5	Allophycocyanin	+++	+
CD27	LG.3A10	PE	+++	+++
CD24	M1/69	FITC	+++	−[d]
CD43	S7	PE	+++	+
c-*kit*	2B8	PE	++	+

[a] Other fluorophores were tested for many of these clones and found to generally provide similar results, although absolute separation does vary.

[b] All Abs were tested on BALB/c or C57BL/6 splenocytes, except CD25 (thymocytes) and CD69 (activated splenic T cells).

[c] Scoring: −, no separation of positive and negative populations; +, <0.5 log separation; ++, 0.5–1.5 log separation; +++, >1.5 log separation, see text for further explanation.

[d] Unfixed cells show broad positive populations that are lost after F/M, although some positives remain, and scores reflect maintenance of these positives.

Simultaneous phosphospecific and surface Ab staining

Phosphospecific flow cytometry was performed as previously described (7). Briefly, splenocytes in RPMI 1640 with 10% FBS were stimulated with the indicated cytokines for 15 min at 37°C before fixation with 1.6% formaldehyde for 10 min. The cells were then pelleted, resuspended in ice-cold methanol, and incubated for 15–30 min at 4°C. The cells were washed twice with staining medium, resuspended at 10^7 cells/ml, and

stained with a mixture of Abs including CD11b, TCRβ, B220 and phospho-Stat (pStat3)(Y705), or TCRβ, CD4, CD8, and pStat5(Y694). Cells were stained for 30 min, then washed with staining medium and resuspended before FACS analysis.

Flow cytometry

All flow cytometry was performed on a FACSCalibur instrument equipped with a 633-nm helium-neon laser for allophycocyanin and Ax647 excitation. After acquisition, data were analyzed with a FlowJo (TreeStar). Three-dimensional plots were generated by exporting the scale values of 2000 cells to Spotfire DecisionSite8.0. Median fluorescence intensity (MFI) values were determined for both positive and negative populations of cells. The efficacy of Abs was assessed by determining the ratio between the MFI of the positive population and the MFI of the negative population. Larger ratios indicate better separation of populations. The percent loss upon fixation and permeabilization was calculated as % loss = $100 - 100 \times (MFI_{pos}/MFI_{neg})^{fixed}/(MFI_{pos}/MFI_{neg})^{unfixed}$. For phosphoprotein analysis, the fold change upon stimulation was calculated as the $MFI_{stimulated}/MFI_{unstimulated}$ ratio for the phosphospecific Ab fluorescence channel (FL4).

Results

Simultaneous measurement of protein phosphorylation and surface makers

To highlight the utility of phosphospecific flow cytometry in analyzing immune cell signaling, we applied the technique to analyze IL-2, IL-6, IL-10, and IL-15 signaling in murine splenocytes. First, we treated splenocytes with IL-6 and IL-10 ex vivo and performed four-color flow cytometry with CD11b, TCRβ, B220, and pStat3(Y705) Abs (Fig. 1). After careful screening, we have found these surface Abs to maintain staining characteristics after F/M treatment (see Table I).

Levels of pStat3 are plotted against B220, TCRβ, and CD11b to highlight the importance of the multidimensional aspect of flow cytometry (Fig. 1*A*). The B220 vs pStat3 plot reveals that B cells have a heterogeneous response to IL-6, but respond relatively uniformly, and strongly, to IL-10 with pStat3 induction. However, for IL-6, it is clear that $B220^-$ cells also respond, and do so with a larger increase in pStat3 levels. Plotting TCRβ vs pStat3 shows that T cells make up the majority of these $B220^-$ cells that are responsive to IL-6. The $CD11b^{int}$ population appears to contain two distinct subsets, one that is responsive to both IL-6 and IL-10 (the intermediate high cells, just above the quadrant line), and one that responds only to IL-10 (intermediate low cells, below the quadrant line). These intricacies are not accessible to standard biochemical techniques and require the analysis of samples at the single-cell level.

In addition, we sought to characterize the difference between $CD4^+$ and $CD8^+$ T cells in response to IL-2 and IL-15. Thus, we stained cells with TCRβ, CD8, CD4, and pStat5(Y694) (Fig. 1*B*). The plot of CD4, CD8, and pStat5 shows that a fraction of $CD4^+$ cells respond to IL-2 and IL-15, whereas $CD8^+$ cells uniformly respond to IL-15, but not to IL-2. Some $CD4^-CD8^-TCR^-$ cells also respond to IL-15 (most likely macrophages and dendritic cells). By resolving cell types on a single-cell basis, one begins to appreciate the underlying heterogeneity that is often present in seemingly uniform cell types. However, these forms of analysis require careful optimization of surface markers, such that cell types analyzed with phosphoprotein staining techniques correspond to those analyzed by standard staining and biochemical techniques.

Effects of fixation and permeabilization on surface marker Ab binding

To evaluate the effects of formaldehyde fixation and methanol permeabilization on surface marker analysis, we compared the staining characteristics of the tested Abs on live, intact cells and F/M-treated cells. Two parameters were used to assess Ab efficacy: the separation between positive and negative populations (e.g., the ratio of the MFIs of the two populations) and the percentage of cells that are positive. To obtain optimal separation, the surface Ab concentration during staining must be titrated. Thus, we titrated 36 different Abs against multiple classes of Ags on murine splenocytes, from functional receptors such as TCRβ and MHC to less well-characterized Ags such as CD24 and Ly6C, on both live cells and F/M-treated cells. The results of this extensive profile are summarized in Table I. It should be noted that although we use the term surface marker analysis for both live cells and fixed/permeabilized cells, addition of Abs against surface Ags to permeabilized cells will also measure intracellular stores of the Ag. We have found that most typical surface markers are present at very low concentrations inside cells, although some markers, such as IgG and CD69, may be detected before they are actually expressed on the cell surface. One must always be aware of this caveat when staining surface markers after permeabilization.

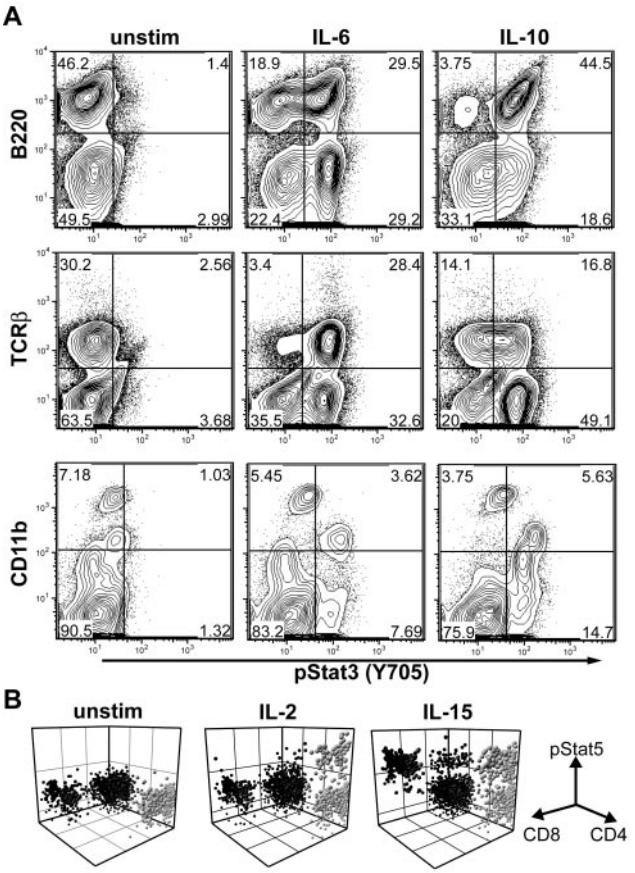

FIGURE 1. Simultaneous surface marker and intracellular phosphoprotein staining reveals subtle differences in immune cell subsets. *A*, BALB/c splenocytes were left untreated or were treated with IL-6 (100 ng/ml) or IL-10 (100 ng/ml) for 15 min in vitro before F/M treatment. Cells were then stained with CD11b-FITC, TCRβ-PE, B220-Cy5.5PerCP, and pStat3(Y705)-Ax647. Contour plots were generated showing each surface marker against pStat3 levels. Increases in Stat3 phosphorylation are indicated by shifts to the right in each plot. *B*, Splenocytes were left untreated or were stimulated with IL-2 or IL-15 for 15 min before F/M treatment and staining with TCRβ-FITC, CD8-PE, CD4-Cy5.5PerCP, and pStat5(Y694)-Ax647. Three-dimensional plots were generated in Spotfire, with 2000 cells being shown on each plot. Samples were gated to eliminate debris and dead cells. CD4 and CD8 are shown on the *x*- and *y*-axes, with pStat5 on the *z*-axis, such that increases in phosphorylation are indicated by upward shifts on each plot. Each of the 2000 cells is indicated by a sphere whose size correlates with its pStat5 level.

Abs were scored on a functional basis for the ability to resolve positive and negative populations as follows: negative (−) indicates no separation and cells cannot be discriminated based on this marker alone; single positive (+) indicates a <0.5 log difference, such that positive cells can be discriminated, but not clearly from this marker alone, and gating requires extra markers for accurate analysis; double positive (++) indicates a 0.5–1.5 log difference, such that positive cells can be discriminated based on this surface marker alone as long as a significant portion of the total population is positive, e.g., >5%; and triple positive (+++) indicates a >1.5 log difference, so that positive cells can be gated clearly on this marker alone even when they are present at very low numbers, e.g., <1% of the total cells. We used this scoring scheme to emphasize which Abs were effective after F/M (++ or +++) vs those that were difficult to use or were completely unusable (+ or −). Many Abs that scored +++ for both unfixed and F/M-treated cells actually showed a large decrease in separation after F/M (see B220 below). However, this loss does not affect simple identification of positive cells and therefore does not decrease the utility of the Ab for subsetting complex populations. Gr-1, Ly6C, and CD24 were difficult to score because of their broad positive populations. In some cases, only portions of positive subpopulations were lost, whereas other subpopulations were maintained. Particular attention should be paid to such Abs. The differential results displayed in Table I suggest careful analysis of these Abs and their titration levels before application to phosphoprotein characterization.

To highlight the differences between effective and ineffective Abs, we examined three Abs of each type more closely: B220, TCRβ, and MHC I-A^d (Fig. 2*A*) and CD24, Ly6C, and MHC I-A^b (Fig. 2*B*), respectively. Among the more effective Abs (Fig. 2*A*), TCRβ recognition is largely unchanged after F/M, with the optimal titration remaining at 1 μl, the percentage of positive cells at 35%, and only 50% loss in the positive to negative ratio. B220 recognition is also maintained, but ~20-fold less Ab is required to obtain optimal separation between positive and negative populations after fixation and permeabilization. In this study, >80% of the separation is lost after fixation, yet positive cells are still 30-fold brighter than negative cells. Most of the reduction in separation is caused by a large increase in the MFI of negative cells, because the positive population has nearly identical MFI values on both fixed and unfixed cells at any given Ab titer. We attempted to decrease background binding levels by using BSA, FBS, and Ab isotypes as blocking reagents, but none of these reagents significantly enhances surface marker analysis (data not shown). Therefore, it appears that even markers that remain effective are negatively affected by F/M treatment and require careful titration for optimal separation.

For other surface markers, the effects of fixation and permeabilization render certain Ab clones largely unusable (Fig. 2*B*). Ly6C shows three populations when cells are stained live: high, intermediate, and negative populations. When fixed and permeabilized, the high population is still present at 16% and is distinguishable from negative cells, but the intermediate population is no longer discernible. For MHC I-A^b (C57BL/6 haplotype of MHC class II), fixation and permeabilization with methanol completely eliminate the separation between positive and negative cells. It is interesting that recognition of MHC I-A^d (BALB/c haplotype) is well maintained after treatment, indicating that Abs against the same class of surface marker can be affected differently depending on the particular epitope that they recognize. CD24 staining behaved similarly to Ly6C, although intermediate populations were slightly separable at optimal titrations after formaldehyde/methanol treatment. The importance of titration was particularly evident for CD24 staining, because 0.5 μl was optimal on unfixed cells, but provided almost no separation of the intermediate population on fixed cells. Only at a 10-fold lower concentration did the intermediate population become slightly distinguishable.

Intracellular Ags as source of background staining

It appeared that F/M treatment tended to increase background staining levels of nearly all Abs tested. This could be due to specific effects of methanol on intracellular and surface epitopes or to general surface Ab nonspecificity when introduced to the interior of cells. Therefore, we tested the effects of saponin permeabilization on surface marker Ab binding characteristics (Fig. 3).

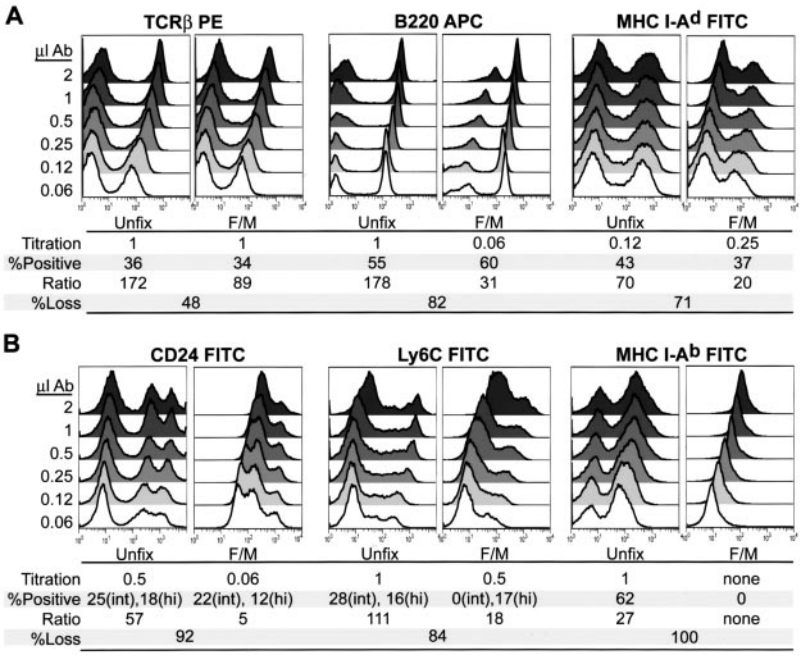

FIGURE 2. Fixation and permeabilization alter surface marker Ab binding characteristics. Murine splenocytes were left unfixed (*left* histograms) or were fixed with 1.6% formaldehyde and permeabilized in 100% methanol (F/M; *right* histograms) before staining with 2-fold serial dilutions of TCRβ-PE (0.2 mg/ml), B220-allophycocyanin (0.2 mg/ml), MHC I-A^d-FITC (0.5 mg/ml), or CD24-heat-stable Ag-FITC (0.5 mg/ml) on BALB/c splenocytes and Ly6C-FITC (0.5 mg/ml), or MHC I-A^b-FITC (0.5 mg/ml) on C57BL/6 splenocytes. Histograms are ordered with decreasing amounts of Ab applied from *top* to *bottom* in each set. Below each histogram set is a summary of four parameters for each Ab for unfixed or F/M conditions: titration indicates the amount of Ab required for the optimal ratio between positive to negative cells, %Positive indicates the percentage of positive cells at the optimal titer, ratio indicates the MFI ratio of positive to negative cells, and %loss indicates the loss in ratio after F/M treatment. *A*, Representative Abs that maintain proper staining characteristics after fixation and permeabilization. *B*, Representative Abs that are negatively affected, or become unusable, after F/M treatment. Data shown are representative of three or more titrations with each Ab.

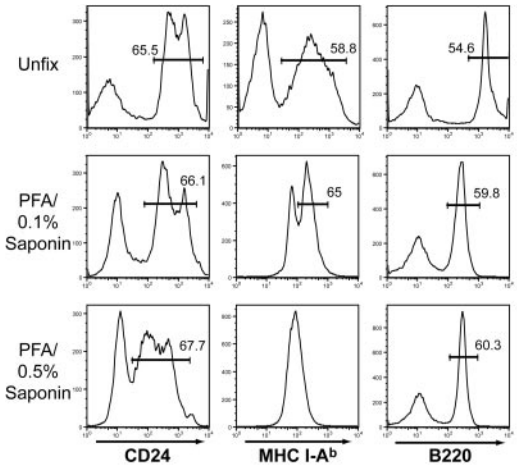

FIGURE 3. Exposure to intracellular Ags affects surface marker Ab binding characteristics. C57BL/6 splenocytes were left unfixed (*first row*) or were fixed with formaldehyde and then permeabilized in 0.1 or 0.5% saponin (*second* and *third rows*, respectively). The cells were then stained with various concentrations of MHC I-A^b-FITC, CD24-PE, and B220-allophycocyanin (in the presence of saponin for permeabilized cells). Shown are histograms of the optimal Ab staining concentration. The histograms are representative of two or three experiments for each Ab.

Although saponin permeabilization is not as effective for phosphoprotein analysis, we sought to determine whether cell permeabilization alone was sufficient to increase surface Ab background binding levels (19). Similar to methanol permeabilization, TCRβ, B220, and MHC I-A^d staining was maintained after saponin permeabilization, but showed increased background, decreased positive signals, decreased positive to negative ratios, and generally a reduction in optimal titrations.

For CD24-heat-stable Ag, permeabilizing with low concentrations of saponin (0.1%) had mild effects on cell separation and percentage of positive cells (Fig. 3). However, with 0.5% saponin, separation decreased dramatically, indicating that the degree of permeabilization may correlate with the loss in separation. Saponin permeabilization allowed for efficient analysis of Ly6C populations, including the intermediates that were lost after methanol treatment (data not shown). Finally, MHC I-A^b was essentially unusable, as it was for methanol (see sequential staining section). Thus, it appears that in some cases, permeabilizing cells and exposing new intracellular epitopes for surface Ab binding/interaction are sufficient to reduce the separation between positive and negative cells. The use of any permeabilization reagent may cause similar losses in population separation, although the effects may vary in intensity and character.

Rescue of poorly resolved surface markers by sequential staining techniques

Abs were first tested by staining after F/M permeabilization, because that is the simplest technique, requiring that cells be stained only once for both surface and intracellular Ags. In most cases, it appears this technique works with appropriate titration, but there are clearly instances (CD24, Ly6C, and MHC I-A^b) where the staining is ineffective after F/M. Thus, we tested whether sequential staining techniques could rescue recognition of these Ags (Fig. 4). As is often used for cytokine analysis, we first fixed cells with formaldehyde, stained them with appropriate titrations of surface Abs conjugated to FITC, then permeabilized them with methanol (Fig. 4, *third column*). As a comparison, cells were left unfixed (*first column*), fixed, and stained without subsequent permeabilization (*second column*) or were fixed, permeabilized, and then stained as described in Fig. 2 (*fourth column*).

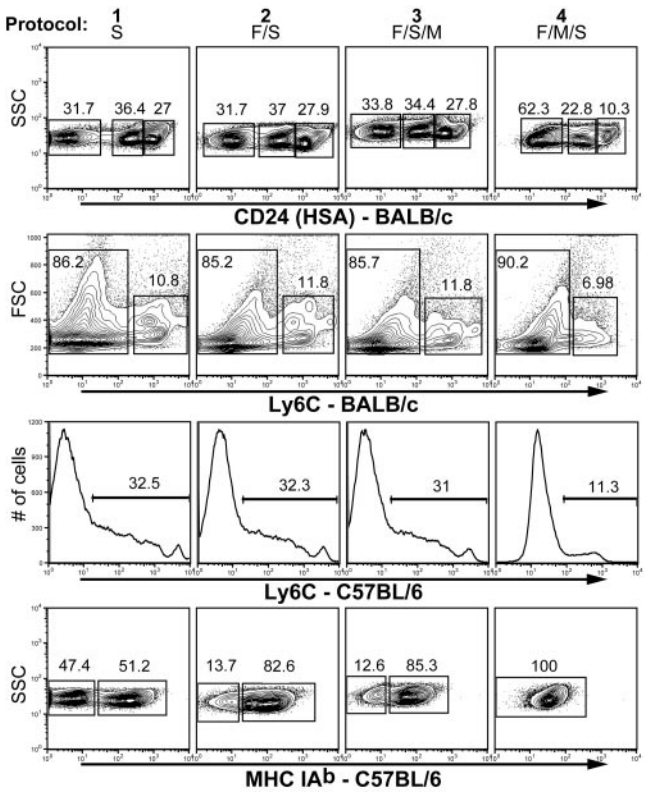

FIGURE 4. Sequential staining techniques rescue some Ags lost after fixation and permeabilization. Splenocytes were stained with the following protocols: 1) cells were stained without treatment (S); 2) cells were fixed with 1.6% formaldehyde and then stained (F/S); 3) cells were fixed, stained, then permeabilized with methanol (F/S/M); or 4) cells were fixed with formaldehyde and permeabilized with methanol and then stained (F/M/S). CD24 (0.05 μl), Ly6C (0.5 μl), and MHC I-A^b (0.5 μl) were tested in BALB/c or C57BL/6 splenocytes as indicated. All Abs used were conjugated to FITC to avoid the effects of methanol (see Fig. 5). Numbers indicate the percentage of total cells represented in each gate. For MHC staining with protocol 4, only one population was discernible. Data shown are representative of at least three experiments with each Ab.

For CD24 and Ly6C, staining after fixation, but before permeabilization, yielded separation and percentage of positive cells similar to unfixed cells (Fig. 4). After fixation and permeabilization, CD24 staining produced high background and incorrect percentages of positive cells. However, sequential CD24 staining reduced background and restored positive percentages to untreated levels. In the case of Ly6C, staining before permeabilization not only restored subpopulation percentages to untreated values, it also enabled intermediate cells to be clearly discriminated in C57BL/6 mice. These results indicate that staining after fixation, but before permeabilization, may be particularly well suited to resolving intermediate populations and surface staining with broad characteristics, such as CD24, Ly6C, and Gr-1.

Surprisingly, MHC I-A^b staining could not be restored by sequential staining techniques (Fig. 4). In this study, formaldehyde fixation alone was sufficient to hamper detection and separation of positive from negative cells. Permeabilization with either saponin or methanol did not affect the separation.

Thus, it appears that both formaldehyde fixation and methanol permeabilization can act separately or together in negatively affecting the discrimination of immune cell types via surface markers. However, sequential staining of cells before permeabilization

but after fixation can rescue the binding characteristics of many of these Abs and provides a method for analysis of "difficult" surface Ags. In addition, this technique avoids staining of intracellular Ag stores and may therefore provide results closer to those obtained with live, intact cells.

Fluorophore choices for sequential staining

Because sequential staining techniques were able to rescue two of three Ags that could not be analyzed after methanol treatment, the technique may become more widely used as phosphospecific flow cytometry techniques are applied to novel cell types. However, we were concerned that due to the dehydrating and denaturing effects of methanol, some fluorescent proteins, such as PE, PerCP, and allophycocyanin, might be destroyed if added before methanol. Therefore, we performed sequential staining of murine splenocytes with a B220-specific Ab conjugated to the small molecules Ax488, FITC, and Ax647; to the protein fluorophores PE, PerCP, and allophycocyanin; and to a tandem of small molecules on a protein fluorophore Cy5.5 PerCP (Fig. 5).

It is readily apparent that B220 Abs labeled with the small molecule fluorophores FITC, Ax488, and Ax647 were resistant to the detrimental effects of methanol permeabilization (Fig. 5, *third column*). The protein fluorophores, in contrast, responded differently to methanol. The fluorescence of PE, PerCP, and Cy5.5 PerCP was almost completely lost after methanol treatment, whereas allophycocyanin fluorescence levels were great enough to allow discrimination of positive from negative cells (although fluorescence decreased >10-fold). Therefore, when performing staining before methanol permeabilization, the choice of fluorophores is restricted to small molecules or possibly allophycocyanin and its tandems.

Ab clone selection

We next tested whether particular Ags, such as CD4 or CD25, are altered by F/M treatment in such a way that all Abs directed against them are equally affected, or whether different Ab clones might be superior after fixation and permeabilization (Fig. 6). Four different FITC-conjugated Ab clones against CD4, RM4-5, GK1.5, H129.19, and RM4-4, were compared before and after F/M treatment. Clone RM4-5 was superior to all other clones, with only a 35% loss in signal, whereas RM4-4 was worst, with about an 80% loss (Fig. 6*B*). GK1.5, H129.19, and RM4-5 are competitive for their epitopes when added simultaneously, whereas RM4-4 binding is not affected by these other mAbs (according to the manufacturer's data sheets). It therefore appears that the location of the RM4-4 epitope on the CD4 molecule may be modulated to a greater extent than the epitope recognized by the other clones.

Additionally, three clones against CD25 (the IL-2R α-chain) were tested (Fig. 6*C*). Clone PC61 was tested conjugated to both

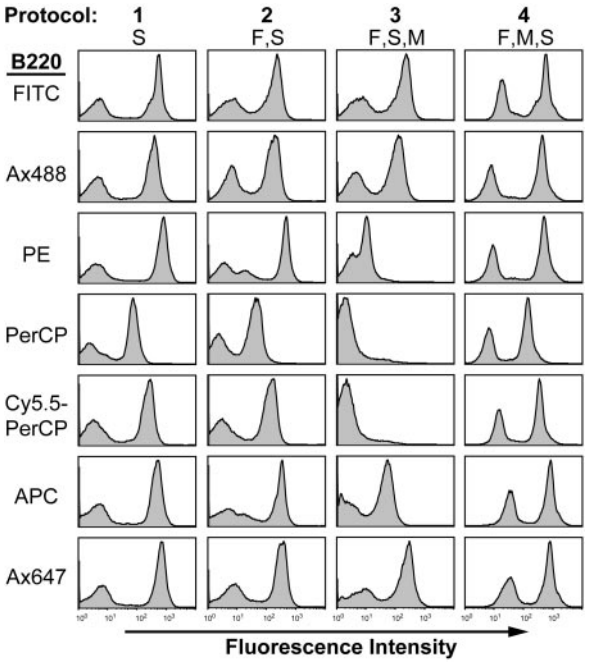

FIGURE 5. Small molecule fluorophores withstand methanol treatment. BALB/c splenocytes were stained with B220 conjugated to FITC, Ax488, PE, PerCP, Cy5.5PerCP, allophycocyanin, or Ax647. Surface staining protocols were the same as in Fig. 3. S, stain; F, formaldehyde fixation; M, methanol permeabilization. All Abs were used at 0.1 μl/stain (10^6 cells) for all protocols. Data are representative of three independent experiments.

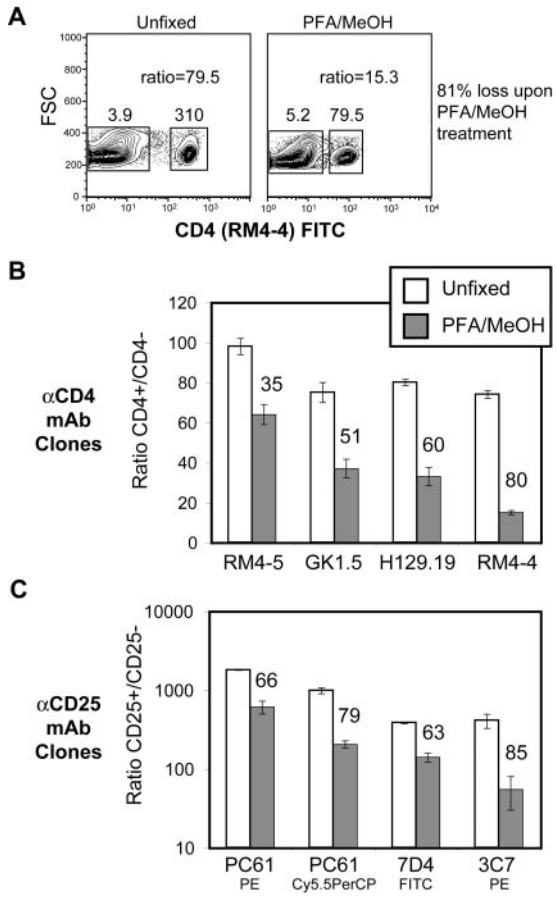

FIGURE 6. Multiple Ab clones against the same cell surface Ag are affected differently by formaldehyde/methanol treatment. Cells were left unfixed or were treated with F/M before staining with four different mAb clones against CD4 or three clones against CD25. Gates were drawn around positive and negative populations, and the ratio of the MFI of the two populations was obtained. The percent loss was calculated as described in *Materials and Methods*. In each experiment, the Abs were titrated to obtain optimal separation between positive and negative cells. *A*, Shown is a representative plot with clone RM4-4 against CD4. *B*, Anti-CD4 clones RM4–5, GK1.5, H129.19, and RM4-4 (all conjugated to FITC) were tested in BALB/c splenocytes. The MFI ratio of positive to negative cells is shown for each clone before and after F/M treatment (□, unfixed cells; ■, F/M-treated cells). Numbers over the bar graphs indicate the percent loss upon F/M treatment. *C*, Anti-CD25 clones PC61, 7D4, and 3C7 were tested in BALB/c thymocytes. PC61 was used conjugated to PE and Cy5.5PerCP to compare the effects of the fluorophore on Ab accessibility and binding capacity. Note that the *y*-axis has a logarithmic scale for CD25. The mean and SD for each clone were calculated from at least three experiments.

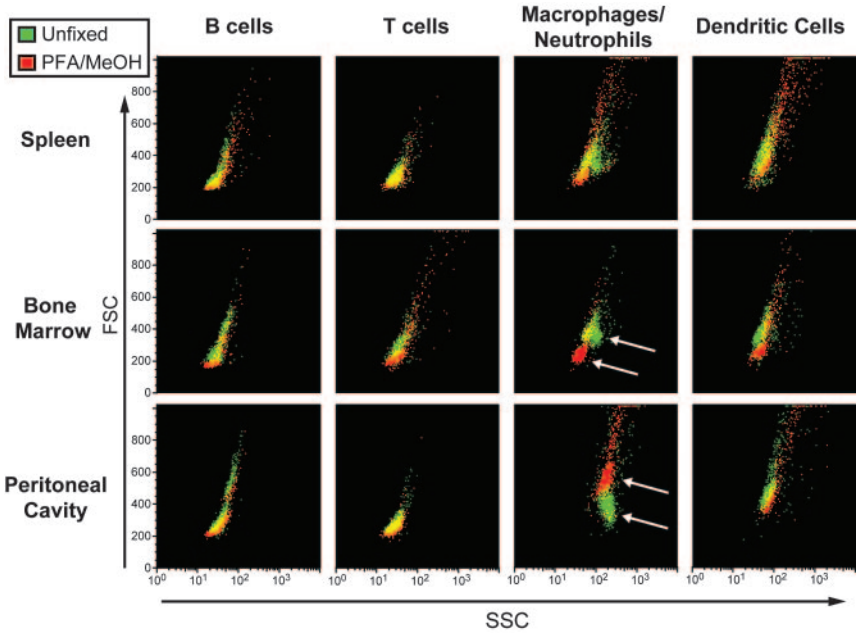

FIGURE 7. Cell scatter characteristics are partially conserved after F/M treatment. BALB/c splenocytes, bone marrow, or peritoneal cavity cells were left unfixed or fixed with formaldehyde and permeabilized in methanol (F/M) before staining with B220-FITC, TCRβ-PE, CD11b-Cy5.5PerCP, and CD11c-allophycocyanin. All of these Abs were validated previously for use after F/M treatment. Shown are the FSC and SSC characteristics for B cells (B220$^+$); T cells (TCRβ^+); monocytes, macrophages, and neutrophils (CD11b$^+$); and dendritic cells (CD11c$^+$) both before (green dots) and after (red dots) F/M treatment. One thousand cells are shown for each population. Yellow areas indicate overlap of the two samples. Arrows indicate significant shifts induced by F/M treatment.

PE and Cy5.5PerCP, whereas clone 7D4 was conjugated to FITC, and clone 3C7 to PE. As was the case for CD4, the clones against CD25 responded differently to F/M treatment. PC61 was the least affected, although the Cy5.5PerCP-conjugated Ab was slightly worse than the PE-conjugated version, and 3C7 was particularly poor.

Therefore, different clones against the same Ag should be tested when initial attempts at surface marker analysis fail. Testing other clones may yield better separations, especially if the clones are directed against different epitopes on the Ag.

Scatter properties of cells in immune organs after F/M treatment

Although most discrimination of immunological cell types is accomplished via surface marker analysis, many gating strategies use initial gates based on cell size and granularity, measured by forward scatter (FSC) and side scatter (SSC) properties, respectively. We therefore examined how F/M treatment affected scatter properties in multiple immunological organs, including the spleen, peritoneal cavity, and bone marrow (Fig. 7). B cells (B220$^+$), T cells (TCRβ^+), monocytes/macrophages/neutrophils (CD11b$^+$ cells), and dendritic cells (CD11c$^+$) were gated according to surface markers. These populations were then analyzed for FSC and SSC characteristics both before and after F/M treatment.

B cells and T cells were largely unaffected by fixation and permeabilization, although a very small decrease in FSC was present in B and T cells of the bone marrow. Dendritic cells have higher SSC properties than lymphocytes and retain this separation after fixation and permeabilization. However, a decrease in FSC was evident, especially in bone marrow dendritic cells. CD11b$^+$ cells, including monocytes, macrophages, and neutrophils, showed highly variable changes in FSC/SSC depending on their source organ (highlighted by arrows in Fig. 7). CD11b$^+$ splenocytes showed both a decrease in SSC and a small decrease in FSC after F/M treatment. The same cells from bone marrow also showed a decrease in SSC, but a more pronounced decrease in FSC. In bone marrow, the obvious separation between monocytes and lymphocytes was lost after F/M, requiring the use of surface markers for cell discrimination. Surprisingly, CD11b$^+$ cells from the peritoneal cavity showed almost no change in SSC, but a large increase in FSC. Thus, it is not predictable how cell size will be affected by F/M treatment and requires empirical testing for proper analysis.

Discussion

Compared with traditional Western blotting techniques, two additional parameters are readily resolved in phosphospecific flow cytometry: the fold change in single-cell phosphoprotein levels and the percentage of cells responding. Although Western blotting can quantify bulk population changes, one cannot distinguish between a small increase in phosphorylation levels of all cells of the bulk population and a large increase in the percentage of responding cells (1). As an example, B cells show an increase in Stat3 phosphorylation in response to both IL-6 and IL-10 (Fig. 1). However, the IL-6 response is restricted to ~60% of B cells. By typical biochemical techniques, the response to IL-6 would appear about half that to IL-10. Yet, such an assessment would be incomplete and inaccurate, because it is clear that ~60% of the cells show a maximal response, equivalent to the response to IL-10. Such detailed analysis will help reveal differences in subsets never before identified and will lead to a better understanding of the specific role of each cell type in the immune system signaling network. To this end, we have recently used these techniques to characterize the subset-specific differences in murine immune cell signaling both in vitro and in vivo and found striking comparisons between the two.

Thus, phosphospecific flow cytometry is a powerful tool for analyzing immune cell signaling events, but has three major requirements for successful application (1). First, transient signaling events must be rapidly terminated or frozen at the time interval of choice (because many of the events decay on the time scale of minutes). Second, cells must be permeabilized efficiently for analysis with phosphospecific Abs. Third, to apply the technique to heterogeneous cell populations, surface Ags must also be stained to identify cell subsets of interest. Although several studies have used surface stains in parallel with phosphoprotein detection, they have primarily used saponin permeabilization and stained the surface Ags before saponin treatment (2, 3, 17). As mentioned previously, saponin permeabilization is limiting to the technique, because samples must be processed immediately after treatment, thereby making studies that require sample collection over several days less consistent. In addition, saponin is inferior to methanol

permeabilization for several key phosphoproteins of immunological signaling pathways, particularly the STAT protein family (7).

However, our studies and others that have examined cytokine signaling using methanol permeabilization have found that it was more difficult to identify cell types based on surface marker characterization (4, 5, 20). It appeared that separation between positive and negative populations was greatly reduced when cells were stained after permeabilization, and fluorophore choices were limited when surface stains were applied before methanol treatment. Therefore, as with many techniques, one must assess which aspect of the experiment is more critical: the phosphoprotein analysis or the cell subset identification.

In this work we sought to answer whether surface markers can still be effectively analyzed after F/M treatment or if the majority of surface Abs are rendered unusable. In Table II, we summarize the critical features of Ab staining that were used to validate Abs for this platform. To our surprise, after screening >35 different murine surface marker Abs, we found that ~80–85% identified the appropriate cell subsets with proper percentages and adequate separation. However, nearly all Abs tested suffered decreased separation between positive and negative populations, typically ~80–90% (e.g., about 1 log on a standard FACS plot). In addition, the optimal titer usually decreased from that used on live cells, sometimes 5- to 10-fold (Fig. 2). We found that the decrease in resolution was primarily caused by an increase in background of negative cell populations, and that this increase was evident in all permeabilized cells regardless of the agent used (Fig. 3). However, it appears that the degree of permeabilization may affect Ab staining characteristics, with more harsh reagents such as methanol leading to larger increases in background. Because surface marker Abs are screened on live cells with intact membranes, it is impossible to predict a priori what the effect of exposing intracellular Ags will be. For several Abs that still worked after treatment with <0.5 log separation between populations (e.g., CD3, CD40, CD43, and CD49b), resolution was greatly enhanced if used in parallel with other markers to generate two-dimensional plots (e.g., CD3 vs B220).

The 15–20% of Abs that did not stain satisfactorily after F/M treatment generally displayed two characteristics. The first, shared by Abs that did work, was a loss in separation between positive and negative populations, however to a point where populations could no longer be resolved (e.g., MHC I-A^b and CD24; see Table I). The second was the loss of intermediate or broadly staining populations, typically leaving only the most positive population as staining above background (e.g., Ly6C and Gr-1). There was also one Ab, CD86, that only resolved a subset of cells in permeabilized splenocytes, most likely indicating intracellular stores of the Ag or nonspecific binding events.

For the Abs with poor staining characteristics, we were able to restore adequate resolution of populations by staining for surface markers after formaldehyde treatment, but before methanol permeabilization (Fig. 4). However, this technique is limited, in that only small molecule fluorophores (Ax 488, Ax647, FITC, etc.) and possibly allophycocyanin can be used. PE and PerCP and their tandems were destroyed by methanol (Fig. 5) (4). Because the small molecule dyes are optimal for conjugation and sensitivity for phosphospecific Abs, using these dyes for surface markers hinders multidimensional staining, especially in rare cell types that require several markers to identify (7). Finally, staining before methanol limits later staining combinations, because one channel will effectively be removed, eliminating a key advantage of storage in methanol for later analysis with several Ab mixtures.

After screening >100 phosphospecific Abs for this platform, we believe that obtaining optimal phosphoprotein staining is most critical to successful application of the technique. Although several Ags were difficult to stain after F/M treatment, we believe that with screening of more Ab clones against multiple epitopes on the same Ag, Abs against all of the critical surface markers can be found

Table II. *Parameters critical to testing of new surface marker Abs in the context of phosphospecific flow cytometry*

Parameter	Steps/Methods for Surface Ab Validation
Population percentage	● Compare surface marker Ab-staining patterns before (unfixed) and after fixation and permeabilization for any new system of interest. Cell populations (e.g., $CD3^+$) should have the same percentage/number of cells before and after fixation. Changes may indicate cell loss or loss/gain of surface marker detection in a subset of cells. Sequential staining techniques can be used to monitor the effects of methanol (Figs. 2–4).
Population separation	● If populations retain the proper proportions, assess whether the difference between the MFI of positive and negative cells is large enough for accurate population identification. Separation decreases for nearly all surface Abs after fixation. Inadequate separation of positive and negative cells may lead to heterogeneous gates and nonuniform phosphoprotein analyses. Use of multiple markers and analysis in more than one dimension promotes accurate gating (Fig. 2, Table I).
Titration	● Titrate all Abs before and after fixation/permeabilization to determine optimal staining concentrations. Much less Ab is often required after fixation to reduce background that is normally not present when staining intact cells (Fig. 2).
Intracellular marker expression	● When staining after fixation and permeabilization, intracellular stores of surface markers can be detected in addition to those on the surface. This is especially important for activation markers such as IgG , CD69, CD86 that may be detected before actual surface presentation.
Sequential staining	● If staining after fixation/permeabilization does not yield proper percentages or separation, attempt to stain cells after formaldehyde treatment, but before methanol permeabilization (Fig. 4). Be cognizant of fluorophore selection in this case.
Fluorophore selection	● Some surface marker Abs show better staining characteristics when conjugated to particular fluorophores. Test multiple conjugates such as FITC, PE, PerCP, and allophycocyanin. If using sequential staining techniques, small molecules such as FITC and the Alexa dyes or allophycocyanin must be used (Fig. 5).
Ab clones	● If staining properties are not improved by sequential staining techniques or different fluorophore selection, another Ab clone against the same Ag should be tested (Fig. 6).
Scatter properties	● Compare scatter properties of the cell type of interest before and after fixation/permeabilization to ensure proper size gating, especially when analyzing large cell types such as myeloid cells. Scatter properties should not be relied upon as the sole identifying feature of cells of interest, unless performing broad analyses (Fig. 7).

within the available commercial Ab pool (see Fig. 6). It should be noted that our group has found human Ags to be more difficult to analyze after F/M treatment (data not shown). This may be due to differences between murine anti-human and rat anti-mouse Abs and the epitopes they tend to recognize.

In this study we provide a comprehensive profiling of most major surface molecules present on murine immune cells for analysis in conjunction with intracellular phosphoprotein staining. This serves as a starting point for researchers interested in applying phosphospecific flow cytometry to novel, complex populations of cells that require surface marker analysis. By understanding the parameters critical to successful surface staining and validation of new Abs, we expect that these results will simplify application of the technique as an alternative system for biochemical analysis of signaling networks in complex populations of immune lineage cells.

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