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Total cellular protein presence of the transcription factor IRF8 does not necessarily correlate with its nuclear presence

Hans Minderman^{1,4}, Orla Maguire¹, Kieran L. O'Loughlin¹, Jason Muhitch², Paul K. Wallace¹, and Scott I. Abrams³

¹Flow and Image Cytometry Shared Resource, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo NY

²Dept of Urology, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo NY

³Dept of Immunology, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo NY

Abstract

The transcription factor interferon regulatory factor-8 (IRF8) plays an essential role in myeloid differentiation and lineage commitment, based largely on molecular and genetic studies. The detection of IRF8 in specific cell populations by flow cytometry (FCM) has the potential to provide new insights into normal and pathologic myelopoiesis, but critical validation of this protein-based approach, particularly in human samples, is lacking. In this study, the assessment of total cellular IRF8 presence was compared to its specific nuclear presence as assessed by imaging flow cytometry (IFC) analysis. Peptide neutralization of the IRF8-specific antibody that has been predominantly used to date in the literature served as a negative control for the immunofluorescent labeling. Expression of total IRF8 was analyzed by total cellular fluorescence analogous to the mean fluorescence intensity readout of conventional FCM. Additionally, specific nuclear fluorescence and the similarity score between the nuclear image (DAPI) and the corresponding IRF8 image for each cell were analyzed as parameters for nuclear localization of IRF8. IFC showed that peptide blocking eliminated binding of the IRF8 antibody in the nucleus. It also reduced cytoplasmic binding of the antibody but not to the extent observed in the nucleus. In agreement with the similarity score data, the total cellular IRF8 as well as nuclear IRF8 intensities decreased with peptide blocking. In healthy donor peripheral blood subpopulations and a positive control cell line (THP-1), the assessment of IRF8 by total cellular presence correlated well with its specific nuclear presence and correlated with the known distribution of IRF8 in these cells. In clinical samples of myeloid-derived suppressor cells derived from patients with renal carcinoma, however, total cellular IRF8 did not necessarily correlate with its nuclear presence. Discordance was primarily associated with peptide blocking having a proportionally greater effect on the IRF8 nuclear localization versus total fluorescence assessment. The data thus indicate that IRF8 can have cytoplasmic presence and that during disease its nuclear-cytoplasmic distribution may be altered, which may provide a basis for potential myeloid defects during certain pathologies.

⁴Corresponding author: Hans Minderman, PhD, Assistant Director, Flow and Image Cytometry, Cancer Cell Center Rm 311, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo NY 14263, Ph: 716-845 1162, hans.minderman@roswellpark.org.

Keywords

IRF8; imaging flow cytometry intracellular localization

INTRODUCTION

Tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs) and immature or tolerogenic dendritic cells (DCs), have been well-characterized in a number of solid cancers. Importantly, their presence generally correlates with a poorer prognosis largely due to their ability to mediate potent immune suppression [1]. However, the molecular mechanisms, which govern such defective myeloid cell production or behavior, are largely unknown. Alterations in myeloid function or behavior could be associated with perturbations in the expression of key transcription factors, which govern such cellular characteristics. Interferon regulatory factor-8 (IRF8) represents one such example. IRF8, formerly known as interferon consensus sequence binding protein (ICSBP), is a member of the interferon regulatory factor family of transcription factors [2]. In contrast to most IRF family members that are expressed in various cell types, IRF8 is largely restricted to cells of the myeloid lineage. The majority of mechanistic insights gained on IRF8 have been derived from preclinical mouse models rather than ex-vivo human samples.

Several key studies demonstrate that IRF8 is indispensable for adaptive immunity through its ability to control: 1) the development of monocytes/macrophages and DCs into productive antigen-presenting cells (APCs); and 2) the production of critical pro-inflammatory cytokines, such as IL-12, from these APCs [3]. Moreover, recent work from our laboratory has identified IRF8 as a previously unrecognized negative regulator of MDSCs [4]. The fact that IRF8 is essential for normal myeloid cell differentiation and function raises the broad notion that myeloid IRF8 levels will have prognostic significance in various pathologic states, including neoplasia.

Mechanistically, detailed information on how IRF8 activity is regulated is limited [5]. Reports on IFN-gamma-induced expression of IRF8 [6, 7] and PU.1-dependent chromatin restructuring at the IRF8 gene [8] suggest that its activity, at least in part, is regulated at the transcriptional level. Hence, the majority of the correlative studies to date have evaluated total intracellular IRF8 levels as a functional parameter for IRF8, which have been measured by molecular approaches (e.g., qPCR or western blot) or immunophenotyping approaches (flow cytometry or immunohistochemistry). However, additional evidence indicates that intracellular redistribution of IRF8 between the nuclear and cytoplasmic compartments may contribute to regulating its activity [9]. A study on the mobility of IRF8 and its interaction with chromatin in macrophages demonstrated a nuclear as well as cytoplasmic distribution of IRF8 with the majority (80%) of IRF8 being highly mobile and only becoming more stably interacting with chromatin upon binding to PU.1 and or IRF1 [8]. There are a multitude of examples of transcription factors for which their activity is assessed by determining their specific nuclear localization rather than by the assessment of total cellular protein. For example, the activity of the NF-kappaB and NFAT signaling pathways are

commonly evaluated by the nuclear versus cytoplasmic presence of the RelA (p65) and NFATc1 signaling intermediaries, respectively [10, 11].

The main objective of the present study was to evaluate the correlation between total cellular IRF8 versus nuclear localization of IRF8. Due to the increased research interest in the IRF8 signaling pathway, the availability of IRF8 specific polyclonal and monoclonal antibodies has expanded significantly over recent years. The choice for using the specific polyclonal antibody used in this study was based on its early use including for flow cytometry in previous published literature [12–22] but, equally important, because of the availability of the peptide against which this polyclonal reagent was generated. The latter enables blocking experiments in which the antibody is incubated with the peptide prior to staining of the cells. This can be used to determine the specificity of the antibody binding which is of particular relevance in validating its use in the context of the nuclear versus cytoplasmic localization of IRF8.

MATERIALS AND METHODS

All methods are described according to MIFlowCyt 1.0 guidelines [23]. All analysis files mentioned are available upon request.

Cell Line and Study Population

THP-1 human acute myeloid leukemia cell line was purchased from ATCC. Cells were maintained in RPMI-1640 media (Mediatech Inc., Manassas, VA) supplemented with 10 % fetal bovine serum (PAA Laboratories Pty Ltd, Queensland, Australia), 2 mM L-glutamine, 20 U/mL penicillin, and 20 µg/mL streptomycin (Mediatech Inc., Manassas, VA). The cell line was maintained at exponential growth at 37°C in a fully humidified atmosphere of 5 % CO₂ in air.

Healthy donor peripheral blood and tumor samples from renal carcinoma patients used for this study were collected in accordance with protocols approved by the Institutional Review Board at Roswell Park Cancer Institute (IRB protocols I36404 and NHR 015410).

Antibodies and reagents

CD45 (clone H130) - BV605, CD14 (clone MφP9) - PE, CD14 (clone MφP9) - BV510, CD15 (clone D3HL60.251) - APC, CD33 (clone WM53) - PE, HLA-DR (clone G46-6) - PECE594 were purchased from BD Biosciences (San Diego, CA). Goat IgG isotype control, unconjugated goat anti-human IRF8/ICSBP (C-19) and IRF8/ICSBP peptide were purchased from Santa Cruz Biotechnologies (Dallas, TX), rabbit-anti-goat FITC was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). The nuclear stain DAPI (4', 6-Diamidino-2-Phenylindole, Dihydrochloride) was purchased from Life Technologies (Carlsbad, CA).

Sample Preparation and Antibody Staining

Healthy donor peripheral blood—500 µL of whole blood was incubated with fluorochrome-conjugated antibodies for immunophenotyping as listed in figure legends for

20 minutes at room temperature. All antibodies were previously titrated to optimize the signal to noise ratio. Following incubation, red blood cells were lysed with 1× BD Phosflow Lyse/.Fix solution (BD Biosciences). Following lysis and fixation, cells were pelleted by centrifugation at $600 \times g$ for 4 minutes. Supernatants were removed by decanting, and cell pellets were washed with 5 mL of FCM Buffer (1× PBS + 0.5% BSA). Washed cells were centrifuged as above and cell pellets resuspended in residual volume FCM buffer, IRF8 labeling was performed as described below.

THP-1 cell line— 1×10^6 cells were fixed for 10 minutes in 4 % methanol-free formaldehyde (FA) (Polysciences Inc) at room temperature. Following fixation, cells were washed and cell pellets resuspended in residual volume FCM buffer as above, IRF8 labeling was performed as described below.

Renal carcinoma cells—Tumor biopsies were dissected into small pieces weighing approximately 100ng each, and dissociated by enzymatic digestion with a human enzyme kit (Miltenyi Biotec) in the Miltenyi gentleMacs system. Samples were then passed through a 100 micron filter and cryopreserved in fetal bovine serum with 10 % DMSO. Cryopreserved samples were thawed quickly in the presence of 70 IU/mL of DNase (Sigma) in media. Following thawing, cells were washed in FCM buffer, blocked with normal human IgG for 10 minutes at room temperature and then immunophenotyped for MDSCs surface markers (CD45+/CD33+/CD14-/CD15-/HLA-DR-) for 20 minutes at room temperature. Cells were then fixed for 10 minutes in 4 % methanol-free formaldehyde (FA) (Polysciences Inc) at room temperature. Following fixation, cells were washed and cell pellets resuspended in residual volume FCM buffer as above. IRF8 labeling was performed as described below.

IRF8 labeling—Peptide neutralization of the IRF8 antibody was achieved by incubating the IRF8 antibody with a 10-fold excess (volume) of peptide in permeabilization buffer (PB) consisting of 0.1% Triton-X-100 (Sigma-Aldrich Corp, St Louis, MO) in FCM Buffer for 2 hours at room temperature. IRF8 was detected by indirect labeling. The IRF8 and IRF8 neutralized antibodies were diluted in 1:100 in PB. Samples were incubated for 20 minutes in the dark at room temperature. Primary antibodies were removed by washing with PB and blocked with human IgG for 10 minutes at room temperature). Subsequently, a 1:200 dilution of secondary FITC conjugated F(ab')₂ fragment rabbit anti goat IgG antibody (Jackson ImmunoResearch Laboratories Inc.) was added and incubated at room temperature in the dark for 20 minutes. Secondary antibody was removed by washing with 5 mL FCM Buffer, and resuspended in FCM Buffer.

Flow Cytometry Acquisition and Analysis

Flow cytometry data were acquired between years 2013–2015 on a LSR Fortessa (Becton Dickinson) analyzer equipped with a 405nm laser (50mW), 488nm laser (50mW) and a 640nm laser (40mW) and configured for 18 parameters. The acquisition software used was FACSDiva (Version 7.0). The cytometer was quality controlled daily using BD FACSDiva CS&T research beads (BD Biosciences). A compensation matrix for FITC and PE was constructed based on single color controls and was applied during acquisition. A stopping gate for 200,000 mononuclear events was applied, with all events being collected. A medium

flow rate (60 $\mu\text{L min}^{-1}$ for LSR Fortessa) was used. ListMode data were analyzed using WinList v6 software (Verity Software House).

ImageStream^X-Mk-II Acquisition

Just prior to imaging flow cytometry data acquisition, DAPI was added to all samples (0.5 $\mu\text{g/mL}$ final concentration), to stain the nucleus. Data acquisition was performed on an ImageStream^X Mk-II (Amnis, part of MilliporeSigma, Seattle, WA) between years 2013–2015. Images acquired included a brightfield image (Channel 1 and 9; 430–480 nm), IRF8-FITC (Channel 2; 480–560 nm), CD33-PE or CD14-PE (Channel 3; 560–595 nm), HLA-DR-PECF594 (Channel 4; 595–642 nm), DAPI (Channel 7; 430–505 nm), CD14-BV510 (Channel 8; 505–570 nm), CD45-BV605 (Channel 10; 595–642 nm) and CD15-APC (Channel 11; 660–740 nm). FITC, PE, and PECF594 were excited by a 488 nm laser at 100 mW output, DAPI, BV510, and BV605 were excited by a 405 nm laser at 40mW output, and APC was excited by a 642 nm laser at 50 mW output. The selected laser outputs prevented saturation of pixels in the relevant detection channels as monitored by the corresponding Raw Max Pixel features during acquisition. For each sample, relevant images were simultaneously collected for 50,000 events (cell line) or 200,000 events (peripheral blood and tumor samples). Cell classifiers were set for the lower limit of size of the brightfield image to eliminate debris, the upper limit of size of the brightfield image to eliminate aggregates, and a minimum intensity classifier on the DAPI channel to exclude non-cellular (DAPI negative) images.

Compensation—In each experiment single color controls were stained for each fluorochrome. Five hundred events were collected for each individual single color control with all relevant lasers on at the outputs used for the experimental conditions, and with the brightfield illumination and scatter laser off to accurately quantify spectral overlap in all channels. Only events exhibiting a positive signal in the channel of interest were collected. Each single color control file was then merged to generate a compensation matrix and all sample files were processed with this matrix applied.

Imaging Flow Cytometry Data Analysis

Following compensation for spectral overlap based on single color controls, image analysis was performed with IDEAS[®] software (Amnis, part of MilliporeSigma). Cell populations were hierarchically gated for events in focus that represent single cells, and were DAPI positive as described previously [10, 24]. Expression levels of each relevant parameter in the entire cell are represented as ‘Intensity’. Intensity in the IDEAS[®] software is calculated as the sum of the pixel values in the software-generated ‘combined mask’ minus the background pixel values (i.e., those not in the combined mask).

The spatial relationship between the IRF8 and nuclear images was measured using the ‘Similarity’ feature in the IDEAS[®] software, as described previously [10, 24]. Briefly, a ‘Morphology’ mask is created to conform to the shape of the nuclear DAPI image, and a ‘Similarity Score’ (SS) feature is defined. The SS is a log-transformed Pearson’s correlation coefficient between the pixel values of two image pairs, and provides a measure of the degree of nuclear localization of a factor by measuring the pixel intensity correlation

between the IRF8 images and the DAPI images within the masked region. Cells with a low SS exhibit poor correlation between the images (corresponding with a predominant cytoplasmic distribution of IRF8), whereas cells with a high SS exhibit positive correlation between the images (corresponding with a predominant nuclear distribution of IRF8). To determine the cytoplasmic distribution of IRF8 a combined mask was created that subtracted the nuclear morphology mask from the default system mask for the brightfield image of the cell using the Boolean logic of 'system mask AND NOT nuclear mask'.

RESULTS

Specificity of the IRF8 polyclonal antibody

In order to validate the specificity of the IRF8 polyclonal antibody, its staining of human healthy donor monocytes was compared with isotype controls, secondary only controls or blocking by the peptide to which the polyclonal was generated (peptide blocking step). According to the manufacturer's recommendation, an incubation of the antibody for up to 2h with a 5-fold excess (by volume) of blocking peptide will effectively neutralize the antibody reactivity. To ensure complete neutralization, for all peptide-based blocking experiments blocking was performed with 10-fold excess. The analysis of human monocytes (based of SSC and CD33 expression) by conventional flow cytometry demonstrated that, based on the geometric mean fluorescence intensity (MFI), a strong signal of IRF8 is observed as would be predicted by the known high expression levels of IRF8 in this cell type (Figure 1A). Peptide blocking reduced the intensity of IRF8 staining but did not reduce the MFI to the concentration-matched isotype or secondary only control levels. The effect of peptide blocking on healthy donor monocytes was also assessed by imaging flow cytometry and was found to be in agreement with the conventional flow cytometry measurement and reproducible among 3 healthy donors samples (Figure 1B).

Peptide blocking indicates a nuclear as well as cytoplasmic presence of IRF8

The effect of peptide blocking on the nuclear and cytoplasmic staining of the antibody was then assessed by imaging flow cytometry by determining the changes in fluorescent intensity in the nuclear and cytoplasmic regions of the cell as defined in Figure 2A. On an individual cell level, each nuclear area was defined by the DAPI stain, while the cytoplasmic area was defined as described in the methods section. Figure 2B and C demonstrate that the peptide blocking not only affected the nuclear staining by the IRF8 antibody but also the cytoplasmic staining in both THP1 cells (Figure 2B) and healthy donor monocytes (Figure 2C). In the THP-1 cells in particular, the nuclear staining is affected to a greater extent.

Specific nuclear localization of IRF8 has previously been quantified by the so-called similarity score analysis [10, 24]. This analysis assigns, on an individual cell basis, a value of 'similarity' between two spectrally separated, spatially correlated images of a cell within a defined region of interest. In this case, the spectrally separated images are that of the IRF8 and DAPI images and the region of interest is the nucleus as defined by the DAPI fluorescent image. Figure 3 shows the relationship between the similarity scores and the intracellular IRF8 distribution. The effects of peptide blocking on the reduction of total cellular IRF8 measurements and the reduction of its specific nuclear localization as

determined by the similarity score parameter in monocytic cells with different levels of IRF8 activity (healthy donor monocytes and three monocytic cell line models) has been published previously [28] and was found correlate well.

Nuclear IRF8 expression in healthy donor peripheral blood

In subsequent experiments, nuclear IRF8 expression was assessed by the similarity score analysis using the peptide blocking as the negative reference control (Figure 4). Subpopulations were gated, based on their scatter and CD33 expression profiles. Neutrophils and eosinophils were negative for nuclear IRF8; monocytes were positive; lymphocytes were predominantly negative, with the tailing of the nuclear IRF8 staining likely presenting B cells. The small population of cells with intermediate SSC and CD33 expression levels are most likely basophils or plasmacytoid DCs but more detailed immunophenotyping would be required for a definitive identification of these cells or the B cells. The evidence of nuclear IRF8 in DCs, basophils and B cells, however is in agreement with the literature [25–27].

Clinical samples are heterogeneous with regards to the correlation between IRF8 assessment by total versus nuclear presence

The immunosuppressive properties of MDSCs represents an example of a pathologic myeloid response and we have previously shown [4] IRF8 to be a negative regulator of MDSCs. The availability of procured surgical resections of 10 renal carcinoma patients without any prior cancer treatment presented the opportunity to apply and compare the two analysis approaches for expression of IRF8 on MDSCs derived from these samples. Tumor-derived MDSCs were phenotypically defined as CD45+/CD14–/CD33+/CD15–/HLA-DR–. The expression of IRF8 was analyzed by total IRF8-specific cellular fluorescence analogous to the MFI readout of conventional flow cytometry (Figure 5). In this case, the % mean intensity change between the peptide blocked and non-peptide blocked IRF8 intensity distributions was used as the metric for total IRF8 expression ($tIRF8$). Additionally, the similarity score between the nuclear image (DAPI) and the corresponding IRF8 image for each cell was analyzed as a parameter for nuclear localization of IRF8. The Rd value between the similarity score distributions of the peptide blocked and non-peptide blocked IRF8-stained populations was then used as the metric for nuclear IRF8 expression ($nIRF8$). In 5/10 samples (Group A), the $nIRF8$ was > 10% of the $tIRF8$ (range 23–75%). The high $nIRF8$ in these cases indicate a predominantly specific IRF8 presence in the nucleus while the low $tIRF8$ indicates a relatively high contribution of non-specific binding of the antibody in these cases.

In 3/10 cases (Group B) the $nIRF8$ was within 10% of the $tIRF8$ indicating an equal contribution of the IRF8-specific and non-specific binding of the antibody in the nucleus and the total cell.

In 2/10 samples (Group C), high $nIRF8$ was observed concomitant with a high $tIRF8$. In these cases $tIRF8$ was >10% of the $nIRF8$ (range 11–23%) indicating a relatively low non-specific binding of the antibody in the total cell.

Discussion

The objective of the present study was to determine if IRF8 expression assessed by immunofluorescence measurements of total cellular intensity would necessarily correlate with its nuclear presence. The incentive for this study was the known property of IRF8 having a low binding affinity to chromatin unless it associates with coregulatory factors, such as PU.1 or IRF1 [5]. In addition, there has been a report that suggest a cytoplasmic presence of IRF8 [9]. If IRF8 indeed could shuttle between the cytoplasm and the nucleus, then this could be a yet underappreciated regulatory mechanism and would indicate that its specific nuclear localization, assessed by imaging flow cytometry, would be a better parameter of activity compared to assessment of total cellular IRF8 presence, analogous to other transcription factors such as NF- κ B and NFAT [10, 11].

The choice of the antibody used in these studies was based on its widespread use in previous publications, as well as by the availability of the peptide against which the antibody was raised which enabled the applied blocking strategy as a negative reference control. Driven by the increasing research efforts on IRF8, the availability of both polyclonal and monoclonal antibodies against IRF8 has significantly increased recently. It is important to note that although the results presented herein are specific to the antibody employed, the validation strategy, in particular with regards to the nuclear versus whole cell distribution, is applicable to any of the available IRF8 antibodies and is recommended based on the results presented.

In healthy donor peripheral blood monocytes, a good correlation was found between the effect of peptide blocking on total cellular IRF8 presence and its nuclear presence (figure 1). This correlation is in agreement with a previous study that studied these parameters in monocytic cells with different levels of IRF8 activation [28]. In this previous work, the relationship between MFI and similarity score analysis as applied in this study was also in agreement with IRF8 mRNA levels [28]. The imaging flow cytometry analysis showed that peptide blocking eliminated binding of the IRF8 antibody in the nucleus. It also reduced cytoplasmic binding of the antibody, but not to the extent observed in the nucleus and it did not completely eliminate cytoplasmic binding (Figure 2). These data thus suggest that IRF8 expression is not limited to the nucleus and that the polyclonal IRF8 antibody used in this and previously published studies, in addition to having specificity for IRF8 as evidenced by the reagent product data sheet data, has cross-reactivity with another unidentified primarily cytoplasmic component. The latter issue could be addressed by the use of a monoclonal as opposed to a polyclonal antibody provided that the monoclonal antibody does not have specific cross-reactivity with another protein. In fact, since the generation of the current data, additional monoclonal antibodies have become commercially available including from the same vendor (Santa Cruz Biotechnology) who now also has included a statement on their website that the polyclonal antibody that was used in this study and many studies before will be discontinued as of December 31, 2016 “due to the availability of a much superior monoclonal antibody alternative”. The importance of the data presented herein is therefore the documentation of the extent of this specific reagent’s limitations that now have been revealed by imaging flow cytometry and the use of the peptide blocking control. It is important to stress that this does not imply that the previously published studies using this polyclonal antibody are incorrect. For example, flow cytometric studies that quantified IRF8

by the relative change between the IRF8 staining with and without the peptide block do indeed determine the differences of the total cellular IRF8-specific binding of the polyclonal antibody, regardless of its nuclear or cytoplasmic localization. However, the current data do offer an alternative interpretation for a lack of change in total IRF8 signal intensity (using this specific antibody) in that it does not necessarily imply that IRF8 activity is unaffected. This is an important realization when reconciling results between forthcoming studies that may use different IRF8-specific antibodies with the published literature.

The presence of IRF8 in a healthy donor peripheral blood subpopulation with intermediate scatter and CD33 expression profiles (Figure 4) is noteworthy. The IRF8 distribution in the gated cell population appears to be bimodal with a negative and positive population present. IRF8 has been implicated in regulating DC [27] and basophil [26] maturation. These results warrant a more detailed immunophenotyping of the gated cells to determine any potential correlation between IRF8 expression and maturation of these cells.

The analysis of IRF8 expression in clinical samples was performed on renal cell carcinoma-derived MDSCs. The presentation of these data is to demonstrate that assessment of total cellular IRF8 in clinical samples does not necessarily correlate with its nuclear presence. To our knowledge, this is the first evidence presented of its kind in regard to IRF8 in human myeloid biology. In the current analysis, evidence is found that the two analysis approaches can be proportionately concordant as well as discordant. Discordance was primarily associated peptide blocking having a proportionally greater effect on the nuclear localization versus total fluorescence assessment, although evidence for the reciprocal correlation was also found. The patient sample size in this cohort is too small to determine any clinical correlates with regard to the impact of total vs nuclear IRF8 assessment. We previously showed that total cellular IRF8 measured by FCM did carry significance in MDSCs of breast cancer patients compared to healthy controls [4]. The current preliminary clinical data warrant investigations to determine whether a more detailed focus on IRF8 nuclear presence would provide added value by solidifying the observed clinical correlates even further.

In conclusion, the combined data herein provides evidence that the nuclear presence of IRF8 can be quantified by means of imaging flow cytometry. It is further demonstrated that IRF8 can have a cytoplasmic presence but to a much lesser extent compared to its nuclear presence. Since the biological activity of IRF8 is in the nucleus, the quantitative measurement of nuclear IRF8 as enabled by imaging flow cytometry may be a more relevant correlate to its activity than whole cell assessments. The nuclear versus cytoplasmic distribution of IRF8 should be considered in the interpretation of IRF8 activity data that are based on immunofluorescent measurements of total cellular fluorescence. Finally, when reconciling forthcoming flow- or imaging flow cytometry-based IRF8 data with the current literature, the cross-reactivity of the polyclonal antibody that has been used in many of the studies to date needs to be taken into consideration.

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Figure 1. Effect of peptide blocking on immunofluorescent detection of IRF8 in healthy donor monocytes

A: Healthy donor peripheral blood was labeled with CD33-PE and either IRF8-FITC (blue), peptide blocked IRF8 (yellow), isotype-FITC (red), secondary only (black) or left unstained (green). Monocytes were gated based on SSC and CD33-PE expression. Histogram of FITC height of the gated cells indicates that peptide blocking decreases IRF8 expression in entire cell, but not to the levels of isotype or unstained controls. Data acquired on a LSRIIFortessa flow cytometer. B: Effect of peptide blocking on IRF8 intensity measurements by imaging flow cytometry. Standard error bars represent standard deviations of 3 separate assessments.

Figure 2. Masking strategy of the nuclear and cytoplasmic regions and the effects of peptide blocking on the IRF8 antibody staining intensity in THP1 cells and healthy donor monocytes
 Figure 2A. masking strategy used to determine both the nuclear and cytoplasmic areas of the cell. All masks are shown in blue. The left image shows the default system mask over the cell, or total cell mask. The middle image shows the nuclear mask based on the fluorescence intensity of the DAPI image. The right image shows the resulting mask when the nuclear mask is subtracted from the default total cell mask. When this mask is applied to the IRF8-FITC signal it determines the cytoplasmic fluorescence intensity distribution of IRF8. For this example, THP-1 cells were used. Figure 2B: the IRF8-FITC intensity in either the total cell, the nucleus or cytoplasm of THP-1 cells. Figure 2C: the IRF8-FITC intensity in either the total cell, the nucleus or cytoplasm of healthy donor monocytes. Error bars represent standard deviations of 3 separate assessments.

Figure 3. The IRF8-Nucleus similarity score

In this figure, the relationship between the IRF8-nucleus similarity score (SS) and the cell imagery for IRF8 immunofluorescent staining distribution is shown. In this example, the SS distribution is shown for human healthy donor monocytes after staining with (black) and without (grey) peptide blocked IRF8 antibody. Below the similarity score distributions are examples of cell images of monocytes with corresponding SS. Images from left to right include the Brightfield (BF) image, IRF8-FITC, nucleus-DAPI, and a composite of the two fluorescent images with the similarity score shown on the right.

Figure 4. Nuclear IRF8 expression levels in healthy donor peripheral blood sub-populations
 Following hierarchical gating for single, focused, viable events, peripheral blood sub-populations were identified based on CD33 -PE Intensity versus scatter intensity (upper, far left, scatter plot). The nuclear IRF8 was determined based on similarity score with non-peptide blocked (grey line) or peptide blocked sample (black line). A decrease in nuclear IRF8 can be observed in monocytes, and CD33 dim cells which are most likely basophils. A small change upon blocking can be observed in lymphocytes, likely due to B cell IRF8 staining. No change is observed in neutrophils or eosinophils which do not express IRF8.

Figure 5. Correlation between IRF8 assessment by total cellular presence and nuclear presence in MDSCs derived from renal carcinoma tumors

MDSCs were defined as CD45+/CD14-/CD33+/CD15-/HLA-DR-. The reduction in peptide block-induced total cellular IRF8 intensity was normalized to the highest change in intensity observed ($\Delta tIRF8$, black bars). Likewise, the reduction in peptide block induced nuclear localization was normalized to the highest change in Rd observed ($\Delta nIRF8$, grey bars).