Published in final edited form as:

Toxicol Appl Pharmacol. 2017 December 15; 337: 39–44. doi:10.1016/j.taap.2017.10.021.

Application of gene specific mRNA level determinations in individual cells using flow cytometry-based PrimeFlow™ in immunotoxicology

Joseph Henriquez^{1,4,◆}, Jiajun Zhou^{2,4,◆}, Jinpeng Li^{3,4}, Robert Crawford^{2,4}, and Norbert Kaminski^{1,2,4}

¹Department of Pharmacology and Toxicology, Michigan State University, East Lansing, MI 48824, USA

²Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824, USA

³Department of Genetics, Michigan State University, East Lansing, MI 48824, USA

⁴Institute for Integrative Toxicology, Michigan State University, East Lansing, MI 48824, USA

Abstract

Determining changes in gene expression by measuring mRNA levels is an important capability in biological research. Real-Time Quantitative PCR (RT-qPCR) is the most ubiquitous technique for measuring changes in mRNA transcript levels, but heterogeneity of cell populations and low cell number are serious technical limitations. Recent advances in flow cytometric analytical techniques have enabled the quantification of mRNA levels in individual cells. Here, we present examples demonstrating the strength and challenges of concurrently measuring mRNA using PrimeFlow™ with other endpoints in immunotoxicological studies. Specifically, we demonstrate how measuring gene specific mRNA levels on a per cell basis was used to study: 1) markers of activation and differentiation; 2) cell signaling by measuring intracellular proteins in mature and developing cell types; and 3) a cell type that constitutes a minor population in peripheral blood. We also discuss cell type-specific modifications to the parent technique, which facilitated optimal performance in these cells. While the examples provided are focused on immunotoxicological questions and endpoints, this new strategy can be applied to a wide variety of toxicological research problems.

Keywords

immunotoxicology; mRNA; flow cytometry; PrimeFlow™; B cells; plasmacytoid dendritic cells

◆ Authors contributed equally to the manuscript.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The authors report no conflict of interest.

Introduction

Determining differences in gene expression by way of measuring changes in mRNA is central to mechanistic immunotoxicological investigations. The regulation of gene expression is involved in almost every aspect of immune responses, including : 1) cytokine production and cell surface receptors expression after activation (Chen *et al.*, 2012; Phadnis-Moghe *et al.*, 2015; Henriquez *et al.*, 2017; Li *et al.*, 2017); 2) clonal expansion (Sablitzky *et al.*, 1985; van Stipdonk *et al.*, 2001); and 3) terminal differentiation into mature effector and memory cell types (Rissoan *et al.*, 1999). The most ubiquitous and reliable method of gene transcriptional analysis is Real-Time Quantitative PCR (RT-qPCR). RT-qPCR has become the gold standard for quantifying target gene specific mRNA levels in biological research (Giulietti *et al.*, 2001).

Despite the prevalence and utility of RT-qPCR, this technique has several limitations including: 1) the need for large numbers of cells to obtain adequate amounts of mRNA for accurate quantification; 2) the inability to quantify gene expression in a specific subpopulation of cells within a heterogeneous cell preparation; and 3) the inability to measure protein and mRNA concurrently. In particular, studying transitional cell types undergoing development is a major challenge for immunotoxicological studies. Assessing the expression of the key transcription factors in distinct developmental stages is not feasible using RT-qPCR due to the heterogeneity of the cell population and the inability to concurrently measure mRNA and cell-stage specific markers. Furthermore, the concerns of inadequate cell number and mixed cell preparations are even a consideration when measuring mature immune cell populations. For example, plasmacytoid dendritic cells (pDC) and innate lymphoid cells (ILC) type 2 compose less than 0.5% of peripheral blood mononuclear cells (PBMC) (Chang *et al.*, 2011; Henriquez *et al.*, 2017). Due to their rarity, obtaining an adequate number of cells to reliably quantify mRNA levels using traditional RT-qPCR is challenging and endpoints are often limited.

Recent advances in flow cytometric analysis have enabled the simultaneous analysis of protein and mRNA on a per cell basis. One of the first commercially available strategies is the Quantigene® PrimeFlow™ RNA assay by Affymetrix/Thermo-Fisher Scientific™ (Waltham, MA/Santa Clara, CA). This flow cytometry-based assay enables simultaneous quantification of gene specific mRNA levels and intracellular/membrane bound proteins and is already considered a valuable tool in immunological studies (Frank *et al.*, 2015). Furthermore, the ability to identify cell populations using well defined surface proteins eliminates the need for cell purification and/or bulk lysis, thereby enabling analysis of gene specific mRNA levels in minor and transitional cell populations. In this way, significant insight into the mechanisms by which xenobiotics alter gene expression can be gained with minimal manipulation of the cells.

The overall objectives of this article are: 1) to raise awareness within the scientific community of this new and powerful technology; 2) to present examples on how this technology can be applied; and 3) to provide generalized guidance on key challenges and limitations of PrimeFlow™ technology that we have experienced. In the following sections, we illustrate how employing this new methodology enabled the study of toxicant-induced

modulation of gene specific mRNA levels, which would not have been feasible using conventional RT-qPCR. Specifically, we show how PrimeFlow™ can be used to quantify: 1) gene specific mRNA level changes in a transitional cell population; 2) simultaneous gene specific mRNA levels and cell surface proteins; and 3) expression of gene specific mRNA levels in a rare population of cells. We also highlight key parameters of the manufacture's protocol that are critical for assay success under "Materials and Methods" and include examples where modifications to the standard protocol facilitated optimum mRNA detection.

Materials and Methods: Critical Conditions for PrimeFlow™ Assay Determinations

The most reliable results were obtained when PBMC were isolated from whole blood via Ficoll-Paque PLUS (GE Healthcare Life Sciences, Pittsburgh, PA) density gradient centrifugation. Cell number was also found to affect mRNA quantification such that 2×10^6 cells (human HSC, naïve B cell, or PBMC)/test produced optimum results.

The PrimeFlow™ assay was performed per the manufacturer's suggested protocol (available through the Thermo-Fisher™ website) and visualized in Figure 1, except where indicated in the sections below. Overall, maintaining a consistent temperature of 40°C to ensure successful amplification for the target mRNA was found to be the most critical condition within the protocol. To this end, placing a heating block in an incubator, both set to 40°C, provided the best results. It is noteworthy that the *RPL13A* internal positive control can also be used as an indicator of cell viability, since standard methods of viability detection may not be compatible with PrimeFlow™.

The combination of PBMC isolation followed by treatment and analysis by PrimeFlow™ typically required more than 11 hours to complete from initiation to completion. To facilitate a more manageable workflow, we found that the best stopping point was after the hybridization step with the target probe (Fig. 1). Stopping the procedure at this stage and storing the cells overnight at 4°C resulted in no loss of mRNA signal quality compared to continuous completion of the protocol.

Measuring changes in gene specific mRNA levels in transitional cell populations

The well-orchestrated development of hematopoietic stem cells into immunocompetent cells is regulated by the sequential expression of cell-stage specific transcription factors. Quantifying perturbations in expression of these transcription factors represents a major advancement toward elucidating the molecular mechanisms underlying the developmental immunotoxicity of xenobiotics. The ability of PrimeFlow™ to measure gene specific mRNA on a per cell basis enables quantification of cell stage specific gene expression in transitional cell populations during hematopoiesis. For instance, we have reported that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) treatment impairs the development of human hematopoietic stem/progenitor cells (HSPC) to lineage committed B cells (Li *et al.*, 2017).

To study the molecular mechanisms underlying the impaired B cell development, PrimeFlow™ was used to measure the changes in mRNA expression of lineage specific genes in the HSPC-derived heterogeneous cell population. We found that treatment with TCDD (1 nM) significantly suppressed the mRNA levels of Early B-cell Factor 1 (*EBF1*), a critical transcription factor that regulates B cell development (Fig 2 A–B).

To further characterize the *EBF1*-expressing cell population, we concurrently measured the intracellular protein level of CD79α, a cell marker demarcating early-B and pro-B cells. During the PrimeFlow™ staining procedure, we noticed that fixation after intracellular staining (Fixation II) was required to minimize the loss of intracellular protein detection during the subsequent RNA staining procedure (Fig 2 C–D). However, loss of mRNA amplification was observed after fixing with Fixation Buffer II (Fig 2 C–D). To obtain both optimal staining of intracellular proteins and retain RNA detection, the protocol was altered by either: 1) reducing the concentration of Fixation Buffer II by half and fix for the recommended amount of time; or 2) fixing the cells with the recommended concentration of Fixation Buffer II for half the incubation time stated in the original protocol (Fig 2 E–F). The co-expression of *EBF1* mRNA and CD79α (Fig 2 E–F) suggests that the expression of *EBF1* is B cell lineage specific. The ability to quantify both mRNA and protein levels on a per cell basis using PrimeFlow™ enables gene expression profiling in heterogeneous cell populations, and facilitates studies of mechanism that involve multiple cell types.

Simultaneous quantification of gene specific mRNA levels and cell surface proteins

Exposure to xenobiotics can alter gene expression and signal cascades in mature immune cells. Using PrimeFlow™, it is possible to measure mRNA and signaling molecules at the same time. For example, PrimeFlow™ was used to understand the effect of aryl hydrocarbon receptor (AHR) activation by TCDD on signaling pathways implicated in impairment of the primary antibody response in B cells (Lu *et al.*, 2010). AHR activation results in direct induction of cytochrome p450-1A1 (*CYP1A1*) transcription. Therefore, induction of *CYP1A1* mRNA was used as a biomarker of AHR activation to study TCDD-mediated effects in only those B cells in which the AHR signal cascade was activated.

Preliminary experiments were first performed using the human liver hepatocellular carcinoma (HEPG2), which express high levels of *CYP1A1* mRNA upon TCDD treatment (Whitlock, 1990). HEPG2 cells were treated with TCDD and the cells were then collected and used for *CYP1A1* mRNA analysis (Fig 3A – C). A major obstacle encountered when quantifying *CYP1A1* mRNA levels in B cells was the extremely low levels of drug metabolizing enzymes and hence mRNA levels expressed for these genes, which were not encountered in HEPG2.

In primary human B cells, *CYP1A1* and CD69, a surface marker of cellular activation (Phadnis-Moghe *et al.*, 2015), were measured simultaneously to determine if activation was preferentially impaired in cells in which the AHR signaling cascade was activated (Fig. 3D and 3E). The expression of CD69 was significantly suppressed by TCDD treatment within

the *CYP1A*^{hi} B cells (Fig. 3F) suggesting that AHR activation occurs concomitantly with impairment of primary human B cell activation.

For these experiments, the idiosyncrasies of B cell biology necessitated several changes to the protocol to enable successful mRNA measurement. Specifically, RNase activity is high in human primary B cells making reliable mRNA quantification difficult. To minimize mRNA degradation, higher levels of RNase inhibitor (2× the recommended amount) were used during the first cell fixation process (Fig. 1). Human primary B cells were also found to be very sensitive to the fixation and permeabilization processes recommended in the standard PrimeFlow™ protocol, which caused B cells to diminish in size and compromise mRNA quantification. By eliminating the second fixation step entirely, B cells retained their morphology and the sensitivity of target mRNA detection was maintained.

Quantifying gene specific mRNA levels in a rare population of cells

Minor cell populations within the immune system can significantly influence immune responses, but measuring changes in mRNA levels within these populations from mixed cell preparations can be a technical challenge. For example, the primary IFNα secreting leukocyte is the Plasmacytoid Dendritic cell (pDC), which represents 0.2–0.5% of circulating peripheral blood mononuclear cells (PBMCs). Interestingly, the primary psychoactive cannabinoid in marijuana, ⁹-Tetrahydrocannabinol (THC), is a potent inhibitor of IFNα secretion (Guterman, 1994).

To determine if IFNα mRNA induction is quantifiable in pDC from a mixed preparation of cells, PBMC were stimulated with CpG-ODN 2216 (CpG), a potent inducer of IFNα secretion (Fig. 4A–C). Under these optimal conditions, CpG stimulation induced only 10–30% of pDC to express *IFNA2* mRNA (Fig. 4E–F), which correlated with previous results (Henriquez *et al.*, 2017). To put this in perspective; starting with 1×10^6 PBMCs, there are approximately 2×10^3 pDCs of which only 200 pDC may secrete IFNα.

By measuring IFNA2 mRNA in CpG-stimulated pDC, the influence of THC on *IFNA2* could be studied and to also determine whether the effects occurred upstream or downstream of transcription. Toward this end, PBMC were treated with THC, stimulated with CpG, and *IFNA2* mRNA was then measured by PrimeFlow™. These experiments demonstrated that THC suppressed the transcription of *IFNA2* (Fig. 4D – 4H) and indicated that the level of THC-mediated suppression of IFNα secretion is upstream of transcription. No alterations to the standard protocol were required for quantification of mRNA levels in pDC despite the remarkably minor population these cells represent within PBMC.

Discussion

Recent advancements in flow cytometric analysis enable the measurement of gene-specific-mRNAs in single cells. These advancements provide evaluation of changes in individual cell mRNA expression at a very high rate of acquisition. In addition, this new method enables the identification of differences in gene expression across cell types in a mixed population of cells. Furthermore, the expression of specific genes can be correlated with surface or

intracellular markers, which would have, heretofore, been very difficult to achieve using more traditional methods.

In this article, we have focused on the application of PrimeFlow™ assay as a tool for mechanistic toxicological investigations. We have highlighted a few examples using PrimeFlow™ in measuring toxicant-induced changes of mRNA levels in: a key transcription factor in differentiating cells (*EBF1* in early/pro B cells); a metabolizing enzyme as a biomarker of toxicant exposure in mature cells (*CYP1A1* in B cells); and an early response gene in a minor population of cells (*IFNA2* in plasmacytoid dendritic cells). We have also provided critical aspects for successfully applying PrimeFlow™ including: 1) The recommendation for freshly isolated cells; 2) a higher than suggested minimum cell number; 3) Higher RNase concentration for specific cell types; and 4) alterations to the second fixation step for optimum intracellular staining or complete omission.

Specifically, we have shown that while PrimeFlow™ can measure mRNA in $1 - 5 \times 10^6$ cells/test, a minimum of 2×10^6 freshly isolated cells/test is ideal to account for cell loss. We have also found that there is a need for optimization based on cell type and tissue of origin. Specifically, high constitutive RNase activity in tissues such as the spleen (Eichel and Roth, 1962), pancreas (Dickman *et al.*, 1960), and lungs (Aho *et al.*, 1983) makes isolation of intact mRNA a challenge. Likewise, we discovered that the elevated levels of B cell RNase activity can be mitigated by simply increasing the concentration of RNase inhibitor. This alteration to the protocol enables broader application of PrimeFlow™ in cells typically known to express high levels of RNase activity.

The strength of PrimeFlow™ is that it provides the tools and reagents to conduct high speed gene specific mRNA quantification in individual cells, but in many cases the application is not strictly “off the shelf”. Indeed, our experience has been that reagent optimization is often essential, situational and can be cell type specific. For example, based on the application we found that it was absolutely critical to modify the fixation protocols, in some instances reducing fixation time or fixative concentration, to facilitate intracellular staining. Fixation protocols may need to be modified for different cell types, especially where a second fixation is required for intracellular marker detection while not compromising the mRNA signal. To this point, we found that when intracellular markers are not a priority, the second fixation step can be completely omitted without compromising mRNA detection. The omission of the second fixation step also retained B cell morphology. This practice is therefore applicable for cell types that are sensitive to the harsh, double fixations as recommended by the standard protocol.

Viewed in this light, the PrimeFlow™ assay is a powerful and robust tool for measuring toxicant-induced changes in gene specific mRNA levels in individual cells in combination with a variety of there simultaneous endpoints in a high throughput manner. Importantly, PrimeFlow™ can tolerate and often requires standard protocol amendments to accommodate optimized intracellular protein staining and the idiosyncrasies of cell specific physiology. Therefore, regardless of the model, the application of this technology extends broadly when utilizing individual cell preparations. Specifically, this technology is most useful where the

problems of cell heterogeneity and small population size previously limit investigations of cell-type specific changes following toxicant exposure.

Acknowledgments

The authors would like to thank Mrs. Kimberly Hambleton for the final revision and submission of this article.

Funding

This work was funded by grants from the National Institute for Environmental Health Sciences (NIEHS) – ES004911; the National Institutes of Health (NIH) – 2R01ES002520-25A1; and the National Institute on Drug Abuse (NIDA) – 07908.

References

- Aho SA, Lehtinen PA, Viljanen MK, Kulonen EI. Antifibrogenic Effects of Antiserum against the Macrophage RNase 1–3. *American Review of Respiratory Disease*. 1983; 127:180–184. [PubMed: 6830034]
- Chang Y-J, Kim HY, Albacker LA, Baumgarth N, McKenzie AN, Smith DE, DeKruyff RH, Umetsu DT. Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. *Nature immunology*. 2011; 12:631–638. [PubMed: 21623379]
- Chen W, Kaplan BL, Pike ST, Topper LA, Lichorobiec NR, Simmons SO, Ramabhadran R, Kaminski NE. Magnitude of stimulation dictates the cannabinoid-mediated differential T cell response to HIVgp120. *Journal of leukocyte biology*. 2012; 92:1093–1102. [PubMed: 22899554]
- Dickman SR, Morrill G, Trupin K. Extraction and chromatography of mouse and bovine pancreatic ribonucleases. *Journal of Biological Chemistry*. 1960; 235:169–174. [PubMed: 13816604]
- Eichel HJ, Roth JS. Intracellular Localization Of Enzymes in Spleen. *The Journal of cell biology*. 1962; 12:263–276. [PubMed: 13889545]
- Frank R, Scott B, Lai C, Sias L, Funatake C. PrimeFlow™ RNA assay as a method to elucidate the effect of protein transport inhibitors on cytokine gene expression in whole blood (TECH3P. 941). *The Journal of Immunology*. 2015; 194:207.211–207.211.
- Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods*. 2001; 25:386–401. [PubMed: 11846608]
- Gutterman JU. Cytokine therapeutics: lessons from interferon alpha. *Proceedings of the National Academy of Sciences*. 1994; 91:1198–1205.
- Henriquez JE, Rizzo MD, Schulz MA, Crawford RB, Gulick P, Kaminski NE. 9-Tetrahydrocannabinol Suppresses Secretion of IFN α by Plasmacytoid Dendritic Cells From Healthy and HIV-Infected Individuals. *JAIDS Journal of Acquired Immune Deficiency Syndromes*. 2017; 75:588–596. [PubMed: 28692581]
- Li J, Phadnis-Moghe AS, Crawford RB, Kaminski NE. Aryl hydrocarbon receptor activation by 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin impairs human B lymphopoiesis. *Toxicology*. 2017; 378:17–24. [PubMed: 28049042]
- Lu H, Crawford RB, Suarez-Martinez JE, Kaplan BL, Kaminski NE. Induction of the aryl hydrocarbon receptor-responsive genes and modulation of the immunoglobulin M response by 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin in primary human B cells. *Toxicological Sciences*. 2010:kfq234.
- Phadnis-Moghe AS, Crawford RB, Kaminski NE. Suppression of human B cell activation by 2,3,7,8-tetrachlorodibenzo-p-dioxin involves altered regulation of B cell lymphoma-6. *Toxicol Sci*. 2015; 144:39–50. [PubMed: 25543051]
- Rissoan M-C, Soumelis V, Kadowaki N, Grouard G, Briere F, de Waal Malefyt R, Liu Y-J. Reciprocal control of T helper cell and dendritic cell differentiation. *Science*. 1999; 283:1183–1186. [PubMed: 10024247]
- Sablitzky F, Wildner G, Rajewsky K. Somatic mutation and clonal expansion of B cells in an antigen-driven immune response. *The EMBO journal*. 1985; 4:345. [PubMed: 3926481]

- van Stipdonk MJ, Lemmens EE, Schoenberger SP. Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nature immunology*. 2001; 2:423. [PubMed: 11323696]
- Whitlock JP Jr. Genetic and molecular aspects of 2,3,7,8-tetrachlorodibenzo-p-dioxin action. *Annual review of pharmacology and toxicology*. 1990; 30:251–277.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Highlights

- Toxicant-induced changes of mRNA are quantified on a per-cell basis using PrimeFlow
- Measurement of target proteins and mRNA in developing and mature B cells
- Measured changes of mRNA in a minor population of cells from a mixed preparation
- Recommendations and modifications of the PrimeFlow assay are presented

Figure 1.

Overview of the standard PrimeFlow™ assay protocol. Cells are first fixed with fixation buffer I, stained for surface markers, then permeabilized and treated with RNase inhibitors. Cells are then stained for intracellular markers and further fixed with fixation buffer II. To amplify the target mRNA, target probes are hybridized to the mRNA of interest. A preamplifier (PreAmp) oligonucleotide is then bound to the target probe/mRNA dimer followed by the binding of amplification (Amp) probes. Finally, fluorescent dye labeled probes are bound to the Amp probes and detected through flow cytometry.

Figure 2.

Measuring changes in gene specific mRNA levels in transitional cell populations. **A–B)** Human cord blood-derived HSPCs were treated with vehicle (0.02% DMSO) or TCDD (1 nM) on day 0 and cultured for 21 days. The *EBF1* mRNA levels were quantified by PrimeFlow™. **A)** FACS scatter plot of *EBF1* mRNA levels in HSPC-derived cells. **B)** Changes in the percentage of *EBF1* mRNA expressing cells by TCDD treatment. Data are presented as mean $\pm$ SE for triplicate measurements. ** indicating $p < 0.01$ compared to VH by Student T test. Data are representative of three independent experiments with similar results. **C–F)** Human cord blood derived HSPCs were cultured for 28 days. The mRNA levels of *EBF1* and the protein levels of intracellular CD79 α were measured by PrimeFlow™. **C)** PrimeFlow™ staining without the Fixation II step failed to detect intracellular CD79 α . **D)** Standard PrimeFlow™ staining using Fixation Buffer II at the recommended concentration and incubation time detected CD79 α , but showed reduced *EBF1* mRNA detection as compared to (C). **E–F)** The loss of *EBF1* mRNA detection was recovered by either reducing the incubation time (**E**) or the concentration of fixation buffer II (**F**) by half.

Figure 3. Simultaneous quantification of gene specific mRNA levels and cell surface proteins. B cells were isolated via magnetic assisted cell sorting (MACS) and treated with either 0.02% DMSO (Vh) or 30 nM of TCDD. **A)** Flow plots of induction of *CYP1A1* with AHR activation in HEPG2. **B)** TCDD-mediated activation of AHR significantly induced expression of *CYP1A1* mRNA expression in HEPG2 cells. **C)** The mean florescence intensity (MFI) of *CYP1A1* mRNA expression with AHR activation in HEPG2. **D)** Flow plots of *CYP1A1* mRNA with AHR activation in primary human B cells. **E)** TCDD-mediated activation of AHR-induced expression of *CYP1A1* mRNA expression. **F)** Suppression of CD69 in *CYP1A1*^{hi} population from **E**. * $p < 0.05$, **** $p < 0.00001$ significant to Vh group by Student T test.

Figure 4.

Quantifying gene specific mRNA levels in a rare population of cells. Human PBMC were treated with Vehicle Control (0.028% EtOH) or 15 μ M THC for 30 min and then stimulated with 15 μ g/ml CpG-ODN 2216 (CpG) for 5 hours. *IFNA2* gene expression was determined using PrimeFlow™. A–C) FACS scatter plot of pDC (CD303⁺ cells) undergoing CpG induced *IFNA2* expression and suppression by THC while visualizing all PBMC. D) Changes of mean fluorescence intensity (MFI) of *IFNA2* in pDC following stimulation by CpG and suppression by THC. pDC were identified as CD303⁺/123⁺ cells. E–G) FACS scatter plot of pDC undergoing CpG-ODN-induced *IFNA2* expression and suppression by THC while visualizing only pDC. H) Changes in the number of *IFNA2*⁺ pDC following stimulation by CpG and suppression by THC. (* indicates $P < 0.05$ in % *IFNA2*⁺ pDCs compared to the VC+CpG group using 1-Way ANOVA with Dunnett's posttest)