ONCOIMMUNOLOGY
2023, VOL. 12, NO. 1, 2212550
https://doi.org/10.1080/2162402X.2023.2212550

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ORIGINAL RESEARCH

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B cells require licensing by dendritic cells to serve as primary antigen-presenting cells for plasmid DNA

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ABSTRACT

DNA vaccines have been an attractive approach for treating cancer patients, however have demonstrated modest immunogenicity in human clinical trials. Dendritic cells (DCs) are known to cross-present DNA-encoded antigens expressed in bystander cells. However, we have previously reported that B cells, and not DCs, serve as primary antigen-presenting cells (APCs) following passive uptake of plasmid DNA. Here we sought to understand the requirements for B cells to present DNA-encoded antigens, to ultimately increase the immunogenicity of plasmid DNA vaccines. Using ovalbumin-specific OT-1 CD8+ T cells and isolated APC populations, we demonstrated that following passive uptake of plasmid DNA, B cells but not DC, can translate the encoded antigen. However, CD8 T cells were only activated by B cells when they were co-cultured with DCs. We found that a cell-cell contact is required between B cells and DCs. Using MHCI KO and re-purification studies, we demonstrated that B cells were the primary APCs and DCs serve to license this function. We further identified that the gene expression profiles of B cells that have been licensed by DCs, compared to the B cells that have not, are vastly different and have signatures similar to B cells activated with a TLR7/8 agonist. Our data demonstrate that B cells transcribe and translate antigens encoded by plasmid DNA following passive uptake, however require licensing by live DC to present antigen to CD8 T cells. Further study of the role of B cells as APCs will be important to improve the immunological efficacy of DNA vaccines.

ARTICLE HISTORY
Received 13 December 2022
Revised 5 May 2023
Accepted 8 May 2023

KEYWORDS
Antigen presentation; B cell; dendritic cell; DNA vaccine; plasmid DNA

Introduction

Given the current success of mRNA vaccines that have been developed for COVID-19, there has been increased interest in understanding the mechanism of action of nucleic acid vaccines[1–4]. Nucleic acid vaccines, using either mRNA or DNA, essentially work on the same principle, that they require pre-processing by antigen-presenting cells (APCs) to translate the encoded antigen into a protein. That protein is then either directly presented or cross-presented by professional APCs to activate antigen-specific T cells[5]. While mRNA vaccines and DNA vaccines are similarly appealing as potential therapeutic strategies for cancer treatment, DNA vaccines in particular have demonstrable safety, easy manipulation, scalability, stability and economical manufacturing.[6] However, while a DNA vaccine has been approved for canine melanoma[7], early phase clinical studies in humans have been generally disappointing[8]. Further studies to understand their mechanism of action, in order to improve their immunogenicity, are therefore needed.

Current delivery approaches for plasmid DNA vaccines use intradermal or intramuscular injections. These are typically delivered alone as naked plasmids, but can be given with or without adjuvants, and with or without particle bombardment or electroporation approaches to improve cell transfection. The majority of administered DNA is encountered by local non-professional APCs such as dermal cells and myocytes[9]. Some of the tissue-resident professional APCs, such as B cells, dendritic cells (DC) and macrophages, can also encounter the DNA vaccine. Studies in murine models have demonstrated that DC are required, but they function primarily to cross present antigens produced by bystander cells that have taken up and expressed DNA-encoded antigens[10]. In fact, studies using DNA plasmids encoding antigens under a DC-specific promoter failed to elicit immune responses in murine studies[11,12].

These observations led us to explore whether subsets of professional APCs could serve as primary APC. We have previously reported that upon passive uptake, professional APC subsets process plasmid DNA differently[13]. We have reported that DCs and macrophages capture the plasmid DNA by phagocytosis after which it undergoes endosomal/lysosomal degradation. On the contrary, B cells capture the DNA by macropinocytosis and translocate it to the nucleus where the encoded antigen is transcribed[13]. This implies that only B cells can effectively process the naked plasmid DNA amongst the professional APC subsets. We further found that B cells could present antigens to T cells, but we could not identify translation of the DNA-encoded antigen within B cells[13]. These findings suggested that targeting DNA vaccines specifically to B cells, and understanding the requirements for antigen presentation by B cells, could be important to improve the immunogenicity of DNA vaccines.

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/2162402X.2023.2212550.

In this article we analyzed B cells and the requirements for their antigen presentation capability following passive uptake of plasmid DNA, most analogous to direct delivery of plasmid DNA when administered as a vaccine. We demonstrate that B cells transcribe, translate, and present encoded antigen to CD8+ T cells, but require DCs to license their antigen-presentation capacity via cell-cell interaction(s). This licensing function appears to be at least partially dependent on a CD40/CD40L interaction. Following DNA uptake and exposure to DC, the phenotype of B cells changed dramatically, with gene expression signatures similar to those of B cells activated by TLR7/8 agonists or through the B-cell receptor. Future studies will explore the specific receptors on B cells that become activated by DCs, as this may enable next-generation DNA vaccine approaches using DNA-loaded autologous B cells as APCs.

Materials and methods

Plasmid DNAs

pCI-neo-sOVA plasmid (Cat.# 25098) was purchased from Addgene (Watertown, MA) and the ovalbumin-encoding gene was subcloned into the pTVG4 vector[14]. As a negative control we used either pTVG4 (empty vector) or pTVG4-SSX2 (nonspecific antigen-encoding plasmid DNA). For protein expression studies, pcDNA3-EGFP plasmid (Cat.# 13031-DNA.cg) was purchased from Addgene (Watertown, MA).

Mouse models

C57Bl/6 mice (stock no. 000664), OT1 mice (stock no. 003831), OT2 mice (stock no. 004194), MHC I knockout mice (Stock no. 002087) and CD40 knockout mice (stock no. 002928) were obtained from the Jackson laboratory (Bar Harbor, ME) and were housed and monitored by the Wisconsin Institute of Medical Research vivarium facility. All mice were maintained under aseptic conditions, and all experiments were conducted under an IACUC-approved protocol.

Materials

Flow cytometry antibodies

Anti-mouse CD19-PE-Cy7 (Cat.# 561739), anti-mouse CD80-APC (Cat.# 560016), anti-mouse CD86-BV421 (Cat.# 564198), anti-mouse CD4-BUV395 (Cat.# 563790), anti-mouse CD8-BV786 (Cat.# 563332), anti-mouse MHCI-BV711 (Cat.# 749707), and anti-mouse MHCII-BUV805 (Cat.# 748844) were purchased from BD Biosciences (Franklin Lakes, NJ). Anti-mouse CD11c-PE (Cat.# 50–0114-U100) and Ghost780 live dead dye (Cat.# 13–0865-T500) were purchased from Tonbo Biosciences (San Diego, CA). Anti-mouse SIRP1α (Cat.# 144032) antibody was purchased from Biolegend (San Diego, CA). All antibodies were used at a dilution of 1:100 when staining for flow cytometry.

ELISA antibodies

Purified anti-mouse IFN-γ (Cat.# 551216) and biotinylated anti-mouse IFN-γ (Cat.# 554410) were purchased from Thermo Fisher Scientific (Waltham, MA) and both were used at 1:250 dilution. Avidin-HRP (Cat.# 170–6528) was purchased from Bio-Rad laboratories (Hercules, CA) and used at 1:3000 dilution.

Other antibodies

Purified anti-mouse CD23 (Cat.# 101602) was purchased from BioLegend (San Diego, CA), anti-mouse CD70 (Cat.# BE0022) was purchased from BioXCell (Lebanon, NH) and anti-mouse CD40 (Cat.# 553721) was purchased from BD Biosciences (Franklin Lakes, NJ)

Tetramer

BV421-labeled SIINFEKL tetramer was provided by the NIH tetramer core facility at Emory University (Atlanta, GA), used at 1:250 dilution when staining for flow cytometry.

Reagents

Recombinant mouse GM-CSF (Cat.# 576304) and recombinant mouse BAFF (Cat.# 591202) were purchased from BioLegend (San Diego, CA). Recombinant mouse IL-4 (Cat.# 21–8041-U0020) was purchased from Tonbo Biosciences (San Diego, CA). Recombinant mouse CD40L (Cat.# 8230-CL-050/CF) was purchased from R&D systems (Minneapolis, MN). TLR7/8 agonist, R848 (Cat.# vac-R848) and TLR9 agonist, CpG (Cat.# trlr-2395) were purchased from InvivoGen (San Diego, CA). LPS (Cat.# L4516-1 mg) was purchased from Sigma Aldrich (St. Louis, MO). RPMI-1640 (Cat.# 10–040-cv) and penicillin/streptomycin solution (Cat.# 15140122) were purchased from Thermo Fisher Scientific (Waltham, MA). BenchMark FBS (Cat.# 100–106 500 ml) was purchased from Gemini Bio (Sacremento, CA), TMB-substrate (Cat.# 50-76-00) was purchased from Sera Care Life Sciences (Milford, MA)

B cell, DC and T cell isolations

Mouse spleen(s) were acquired at necropsy and processed to single cell suspension following red blood cell lysis. B cells were isolated using a negative selection kit (Cat.# 12210–110) from Akadeum technologies (Ann Harbor, MI) following the manufacturer's protocol, or by positive selection using CD19-PE as previously described[13]. CD8 and CD4 T cells were isolated using negative selection kits (Cat.# 19853 and Cat.# 19852) from Stemcell technologies (Vancouver, Canada) following the manufacturer's protocol. B16/Flt3-L cell line was implanted in C57Bl/6 mice for generation of primary DCs *in vivo*, as previously described[15]. DCs were isolated by either CD11c positive selection (Cat.# 17684) or negative selection enrichment (Cat. # 19763) from Stemcell technologies (Vancouver, Canada) following the manufacturer's protocol.

In vitro antigen presentation assay

In general, APCs were isolated as described above, re-suspended in PBS at 10^7 cells/ml and incubated with plasmid DNA (25 μg/ml) for 60 minutes, with gentle mixing every 15 minutes. RPMI media supplemented with 10% fetal calf serum and 1% penicillin/streptomycin was then added to the culture, and the cells were incubated

overnight at 37°C. The following day, other cell populations (such as CD4 T cells, CD8 T cells or DCs, each at a ratio of 1:2 B cells) were added to the culture, as were GM-CSF (25 ng/ml) and/or IL-4 (20 ng/ml). In some studies, CD8 T cells added to culture were labeled with either PKH67 (Cat#. PKH67GL-1KT, Sigma Aldrich, St. Louis, MO) or CFSE (Cat# 15530597, Invitrogen, Waltham, MA) following the manufacturer's protocol. Additional treatments, such as activation agents and blocking antibodies, were also added on the second day of the culture where indicated. After three to five days of further incubation, the cells and media supernatant from the culture were collected separately for analysis. Cells were analyzed by flow cytometry, and media supernatants were analyzed for secreted IFNγ via ELISA as described previously[14]. Flow cytometry was performed using a BD-Fortessa instrument. Data obtained were analyzed using FlowJo software (version 10.8). For GFP expression analysis, cell images were recorded using Amnis ImageStream imaging flow cytometer and analyzed using IDEAS software (version 6.2). Expression of cytokines and chemokines in media supernatant was analyzed using the Proteome Profiler Mouse Cytokine Array (Cat.# ARY006, R&D Systems, Minneapolis, MN), following the manufacturer's protocol.

RNA Seq

B cells isolated from C57Bl/6 mice splenocytes were incubated with OVA plasmid DNA overnight as described above, and then cultured with or without DCs (2:1 ratio). DCs had been pre-cultured in the presence of GM-CSF (25 ng/ml) and IL-4 (20 ng/ml) for five days before the addition of B cells to the co-culture. After three days of co-culture, B cells or DC were then sorted by flow cytometry using CD19 or CD11c surface expression and total RNA was isolated. cDNA was prepared, amplified and indexed using SMART-seq v4 ultra low RNA whole transcriptome kit (Cat.# 634890, Takara Bio USA, San Jose, CA). cDNA was then sequenced using NovaSeq6000 for 30 million reads per sample (DNA sequencing facility, University of Wisconsin-Madison Biotech Center). Raw files were processed using Galaxy analysis interface (usegalaxy.org)[16], gene ontology search and functional profiling were performed using gProfiler (https://biit.cs.ut.ee/gprofiler/gost), and gene set enrichment analysis (GSEA) was performed.

Statistical analysis

IFNγ ELISA data presented are pooled from independent experiments, and proliferation data plots are representative of replicates of each experiment/assay. Data are expressed as mean ± standard deviation. A one-way analysis of variance (ANOVA) was used to calculate statistical significance for all data presented that had more than two groups for comparison. Two-way ANOVA was used for experiments that had only two experimental groups. For all analyses $p < 0.05$ was considered statistically significant.

Results

B cells translate antigen encoded by plasmid DNA when co-cultured with DCs

We previously reported that if enriched B cells, DCs or macrophages isolated from C57Bl/6 mice spleens were individually incubated with plasmid DNA encoding ovalbumin or SSX2, only B cells were able to transcribe the encoded antigen. B cells were also able to present antigen to T cells, however we could not detect translation of the gene product within B cells[13]. Based on these results, we wished to evaluate the requirements for B cells to present antigen to CD8 T cells, and whether known B-cell activating agents might further augment antigen expression and presentation. After overnight incubation of B cells with ovalbumin-encoding DNA (OVA DNA), CFSE-labeled CD8 T cells were added to the culture with or without activation agents. These agents included CD40L, anti-CD40, BAFF, CpG, LPS, IL-4, GM-CSF and/or CD4 T cells. As shown in Figure 1a, B cells were unable to activate antigen-specific CD8 T cells, as demonstrated by the absence of OT-1 CD8 T cell proliferation or secretion of IFNγ. These results were surprising, given our previous findings[13]. B cells had increased expression of surface markers associated with B cell activation, including CD80, CD83, and MHCII when treated with these agents, however no significant increases in MHCI or CD86 expression were observed after treatments (Supplemental Figure 1b).

Because we did not detect CD8 T-cell activation, despite B cell activation, we next evaluated the purity of B cells obtained after either negative selection (as used in this manuscript) or after enrichment using PE positive selection (as in our previous manuscript). We achieved higher purity of B cells using negative selection (Supplemental Figure 1a), suggesting that contaminating cells might affect B cell uptake and/or antigen presentation. Consequently, we next evaluated whether the antigen encoded by DNA was translated in different APC subsets. We cultured B cells and DCs, either individually or together, with DNA encoding GFP. As shown in Figure 1b, we identified B cells that expressed GFP, but exclusively in the group where B cells and DCs were in co-culture. On the contrary, DCs did not express GFP, either when cultured alone or with B cells. We were unable to confirm the GFP expression in B cells via conventional flow cytometry or via western blotting. This can be attributed to the low number of B cells that express GFP which would be below the detection threshold of these assays. Collectively, our results indicated that upon passive uptake of plasmid DNA, B cells were the only subset of professional APCs that would transcribe and translate the encoded antigen. However, for translation of the encoded antigen, B cells required co-culture with DCs.

DNA loaded B cells activate antigen specific CD8 T cells when co-cultured with DCs

We next tested this co-culture of B cells and DC for *in vitro* antigen presentation using a plasmid DNA encoding ovalbumin. Purified B cells and DCs were incubated with OVA DNA either individually or in co-culture. The following day, ovalbumin-specific PKH67-labeled CD8 T cells (from OT1 mice)

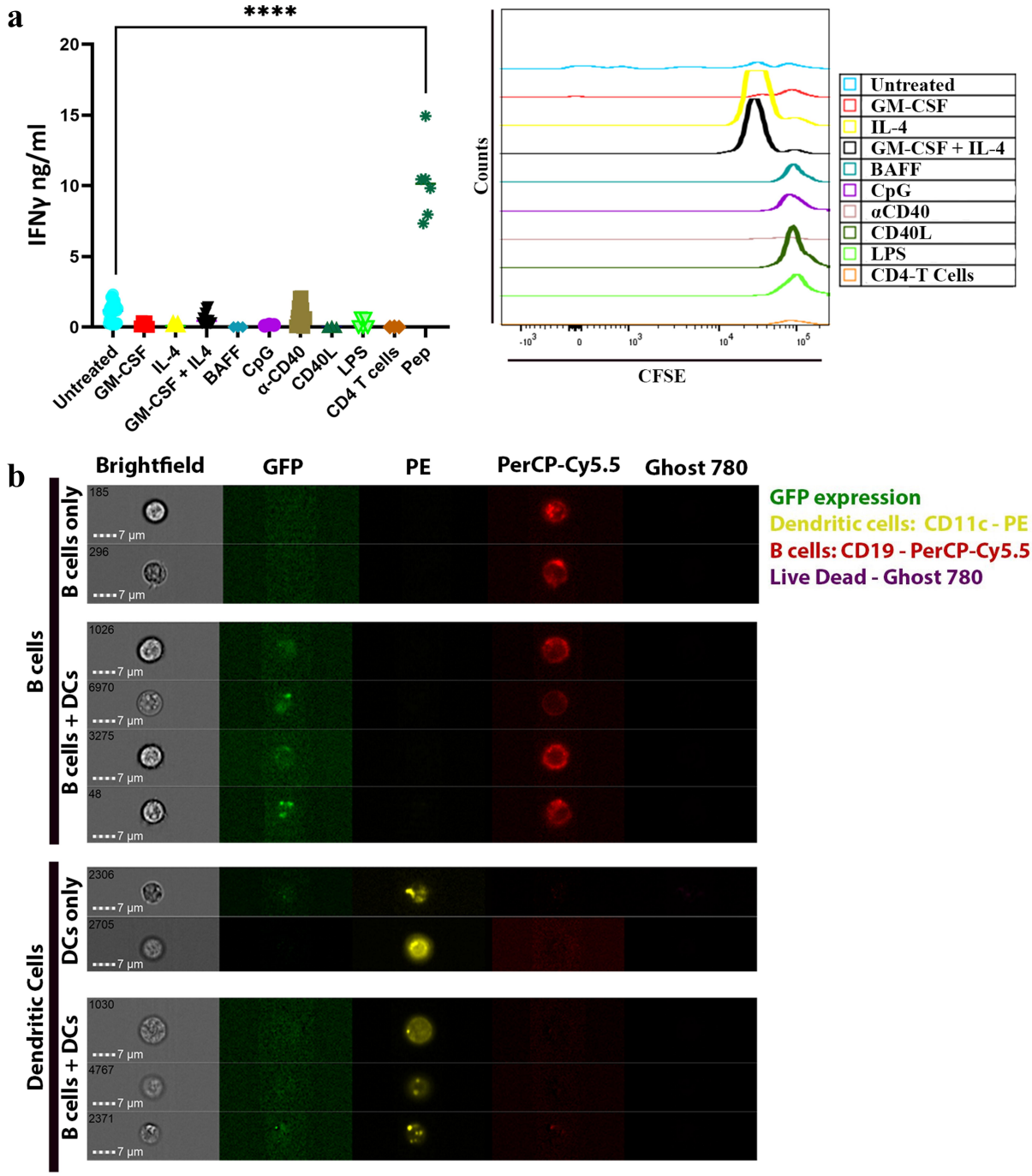

Figure 1. B cells translate antigen encoded by plasmid DNA when co-cultured with DCs. B cells from C57Bl/6 mice were isolated from spleens using negative selection and incubated with ovalbumin-expressing plasmid DNA for passive uptake. Activation agents [BAFF (400 ng/ml), CpG (5 μM), αCD40 (10 μg/ml), CD40L (1 μg/ml), LPS (10 μg/ml), IL-4 (20 ng/ml), GM-CSF (25 ng/ml), and CD4-T cells] and CFSE-labeled CD8 T cells from OT1 mice were added to B cells the following day. SIINFEKL peptide (pep) was used in place of one of the activation agents as a positive control. After five days of incubation, IFNγ was measured by ELISA and proliferation of CD8 T-cells was measured by loss of CFSE dye (panel a). Asterisks (****) indicate $p < 0.0001$. B cells and DCs isolated from C57Bl/6 spleens using negative selection were incubated with GFP plasmid DNA either individually or in combination. After three days of culture, 3500-4000 cells were analyzed using Amnis imaging flow cytometer for expression of GFP, and cells with any detectable GFP fluorescence were directly visualized (panel b). IFN-γ ELISA results are pooled from two to five independent experiments depending upon the treatment groups, with samples assessed in triplicate. Proliferation plots are representative from three similar independent experiments.

were added to the culture. As in Figure 1a, we analyzed CD8 T cells for IFNγ secretion and proliferation. Absence of secreted IFNγ (Figure 2a) and proliferation of CD8 T cells (data not shown) signified that neither individual culture nor co-culture of B cells and DCs could elicit CD8 T cell activation after passive uptake of DNA in this *in vitro* system. We next examined if inclusion of OVA-specific CD4 T cells (OT2 mice) and/or GM-CSF to this co-culture could augment antigen presentation through B cells. Both of these were added along with PKH67-labeled CD8 T cells. GM-CSF was added to maintain and promote survival of DCs *in vitro* (Supplemental Figure 2), whereas CD4 T cells were included to support antigen presentation through B cells. GM-CSF was able to promote activation of antigen-specific CD8 T cells when B cells and DC

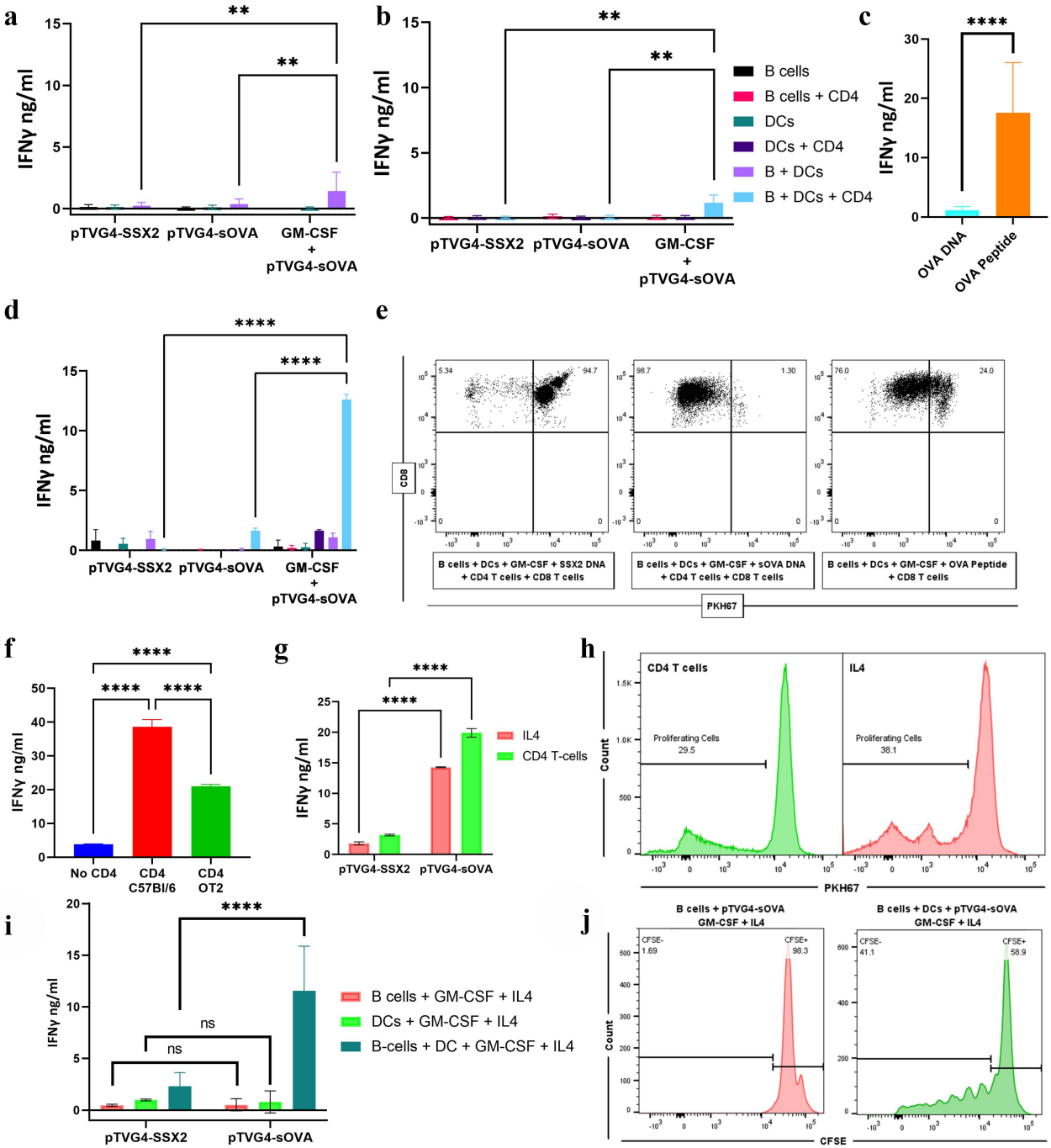

Figure 2. DNA-loaded B cells activate antigen-specific CD8 T cells when co-cultured with DCs and CD4 T cells or IL-4. B cells and DCs were isolated using negative selection from C57Bl/6 mouse splenocytes. B cells and DCs were then incubated with OVA plasmid DNA (or pTVG4-SSX2 control plasmid) either individually or in combination for 24 hours. On the next day, PKH67-labeled CD8 T-cells negatively selected from OT1 mice spleens were added, and with or without GM-CSF. After five days of culture, supernatants were analyzed for IFNγ secretion via ELISA (panel a). An identical study was performed with inclusion of CD4 T-cells that were negatively selected from OT2 mice spleens and added to the culture on the day following DNA uptake (panel b). B cells and DC were similarly co-cultured with GM-CSF and either DNA or SIINFEKL peptide (positive control) and similarly analyzed after five days (panel c). Cell culture was performed as in 2A and 2B, except that DCs were added the day following DNA loading of B cells, along with GM-CSF and CD8 T cells. After five days of culture CD8 T cells were analyzed for IFNγ secretion by ELISA (panel d) and for T-cell proliferation by PKH67 dye dilution (panel e). Asterisks (**) indicate $p < 0.01$, with comparison made between B cells and DCs co-culture groups. CD4 T cells were isolated from C57Bl/6 mice spleens or OT2 mice spleens, and cultured with DC and DNA-loaded B cells as in 2D. IFNγ secretion was measured by ELISA (panel f). CD4 T cells were replaced by the addition of 20 ng/mL IL-4 to the co-culture as in 2D, and IFNγ secretion (panel g, i) and proliferation of CD8 T cells (panel h, j) were measured. Asterisks (****) indicate $p < 0.0001$. IFN-γ ELISA results are pooled from two independent experiments for panels a, b and c, from three independent experiments for panel i, and are from one experiment for panels d, f and g; samples were assessed in triplicate. Flow cytometry proliferation plots are representative of one or more similar, independent experiments.

were co-cultured with plasmid DNA, as demonstrated by modest IFNγ release (Figure 2a,b) but not to the level observed with cognate peptide stimulation of OT1 CD8 T cells (Figure 2c).

Reasoning that DCs might outcompete B cells for DNA uptake, we next tested providing DNA to B cells prior to culture with DCs. Specifically, DCs were added to the culture 24 hours after B cells had been loaded with OVA DNA. As

shown in Figure 2d, we observed an increased amount of secreted IFNγ from the supernatant of B cell and DC co-culture, compared to individual cultures. We similarly observed proliferation of antigen-specific CD8 T cells, but only when B cells were cultured with DC (Figure 2e). Importantly, addition of CD4 T cells and GM-CSF appeared to be required in this co-culture, since we observed a loss in CD8 T-cell proliferation and IFNγ secretion in the absence of CD4 T cells and/or GM-CSF (Figure 2d). Individual cultures of B cells or DCs, each loaded with DNA, did not display proliferation of CD8 T cells or increased levels of secreted IFNγ, with or without addition of CD4 T cells and/or GM-CSF (Figure 2d).

We further analyzed DCs for their classification into DC1 and DC2 subtypes. Our data demonstrated that at day 0, the majority of DCs are either DC1a and DC2, however 2 days after the culture there was an increase in the DC1b subtype. GM-CSF was required for persistence of all DC subtypes *in vitro* (Supplemental Figures 2a–c).

CD4 T cells and IL-4 each support in vitro antigen processing and presentation

We next tested whether the CD4 T cells needed to be antigen-specific in this *in vitro* system. B cells were loaded with DNA as before, and cultured with DCs and antigen-specific (from OT2 mice), or antigen nonspecific (from C57Bl/6 mice), CD4 T cells. We observed that both types of CD4 T cells led to secretion of IFNγ when used in the *in vitro* antigen presentation system (Figure 2f). This suggested that CD4 T cells were playing a helper cell role in this co-culture, potentially by release of a cytokine(s). Others have demonstrated that IL-4 can substitute for the helper function of CD4 T cells[17–19], and hence we specifically evaluated IL-4. As shown in Figure 2(g,h) we found that the requirement for CD4 T cells with DNA-loaded B cells and DCs to activate CD8 T cells, and lead to IFNγ release or T-cell proliferation, could be replaced by co-culture with IL-4. Moreover, IL-4 could not replace the requirement for both B cells and DC (Figure 2i,j). We further analyzed the activation status of B cells following the *in vitro* culture. We observed increased expression of CD83, CD86, MHCI, and MHCII (Supplemental Figure 1C) in the presence of DCs and IL-4, and a greater number of live B cells in the *in vitro* culture, after culture with DCs (Supplemental Figure 1C). This demonstrated that these culture conditions with DCs and IL-4 promoted the survival of B cells. Given this finding, all subsequent studies included GM-CSF and IL-4 and did not include CD4 T cells.

B cells licensed by DCs are the primary antigen presenting cells for plasmid DNA

As co-culture of DNA-loaded B cells and DCs was required for activation and proliferation of antigen-specific CD8 T cells, there was a possibility that DCs were either cross dressing or cross presenting the antigen expressed by the B cells. To address this, we first re-purified DNA-loaded B cells and DCs after three days of co-culture using magnetic bead selection. These re-purified B cells and DCs were then individually cultured with PKH67-labeled CD8 T cells in the presence of GM-CSF and IL-4. As shown in Figure 3a,b only re-purified B cells, and not re-purified DC, were able to activate CD8 T cells, leading to their proliferation and release of IFNγ. To further address the APC cell type directly activating CD8 T cells in this system, we performed similar studies using B cells and DCs from MHC class I knockout (MHCI-KO) mice. We found that MHCI-KO DCs did not affect the activation and proliferation of CD8 T cells, or the levels of secreted IFNγ. On the contrary, use of MHCI-KO B cells negatively impacted activation and proliferation of CD8 T cells and also resulted in significantly lower levels of secreted IFNγ (Figure 3c,d). Collectively, these data demonstrate that B cells were the primary antigen presenting cells in this co-culture and interacted directly with CD8 T cells. DCs, on the other hand, acted as helper cells that enabled and licensed B cells to process the antigen encoded in plasmid DNA for presentation through MHC I.

Cell-cell interaction between B cells and live DCs is essential for licensing of B cells by DCs

We next sought to understand the nature of the interaction between B cells and DCs. Our first approach was to test if protein(s) expressed on the surface of DCs or factor(s) secreted by DCs were essential in licensing of B cells. For this, we prepared lysates from DCs and supernatant from cultured DCs. We utilized these fractions in lieu of whole live DCs either alone or in combination with DNA-loaded B cells. Neither of these DC fractions were able to satisfy the requirement of whole live DCs by DNA-loaded B cells, as demonstrated by the loss in CD8 T cell proliferation and secreted IFNγ (Figures 4a,b). Similarly, paraformaldehyde-fixed DCs and heat-killed DCs were not able to replace live DCs in the *in vitro* system (data not shown). We further tested if there was requirement of physical interaction between B cells and DCs using trans-well plates. Each one of the physical separations of DNA-loaded B cells, DCs, or CD8 T cells resulted in loss of CD8 T cell proliferation and loss in secreted IFNγ. Only when all the three cell types were allowed to interact physically, we observed CD8 T cell proliferation and IFNγ secretion (Figures 4c,d). These results suggested that a membrane-bound factor on live DC was required to license B cells to present a DNA-encoded antigen.

Cell-cell interaction between B cells, CD8 T cells and live DCs results in release of pro-inflammatory cytokines and chemokines

We next wished to determine functional changes that occurred in DNA-loaded B cells and DC following co-culture. We analyzed the supernatants from DNA-loaded B cells cultured with DCs and CD8 T cells for changes in secreted cytokines and chemokines. This was evaluated in the presence or absence of GM-CSF and IL-4. In line with our previous results, increased expression of cytokines such as IFNγ, TNF-α, IL-1Ra, TIMP1 and RANTES were observed in the co-culture group (Figure 5a,b, Supplemental Figure 3). ICAM1 (soluble CD54) was also found at increased concentrations in the co-culture

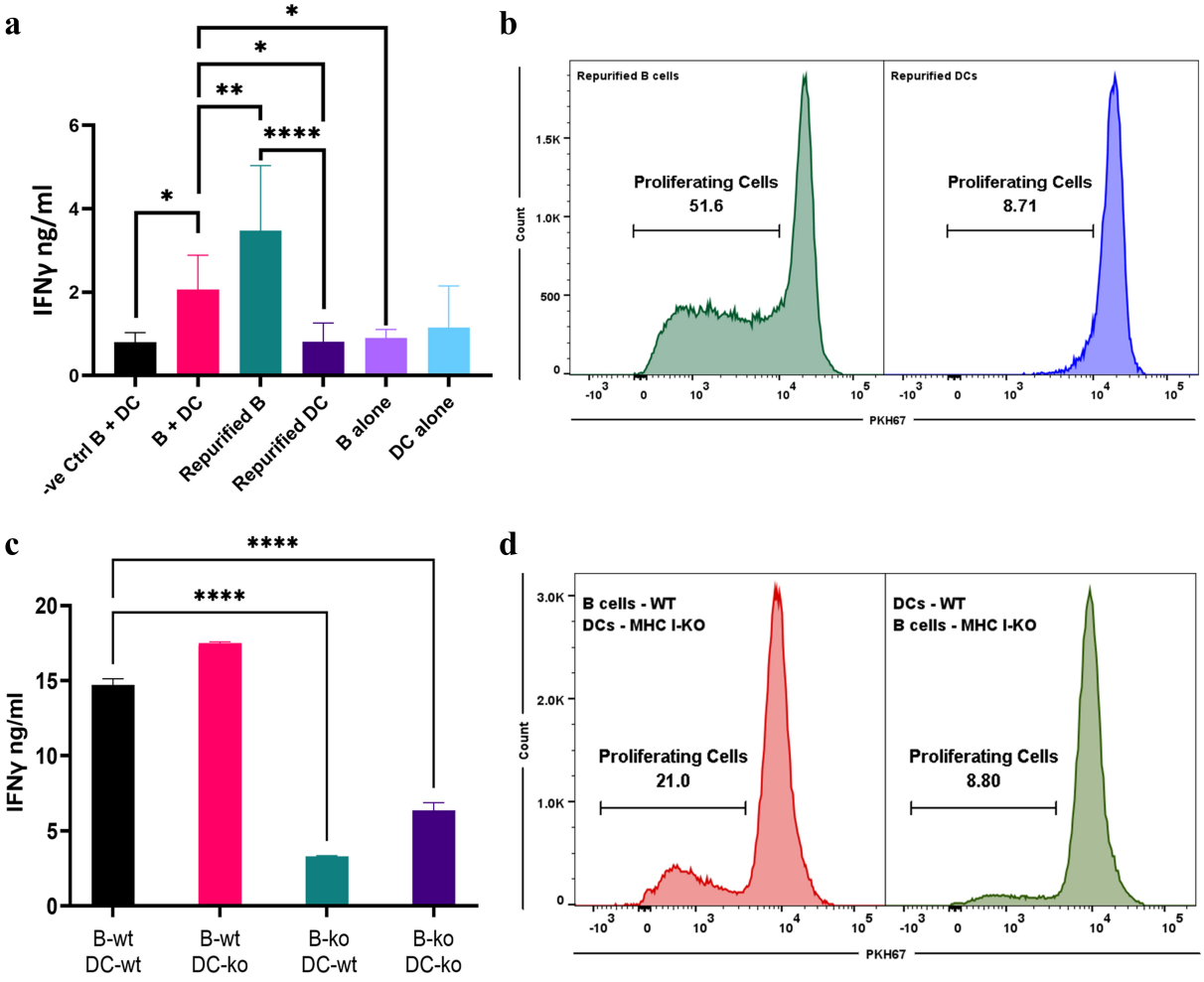

Figure 3. B cells licensed by DCs are the primary antigen presenting cells for plasmid DNA. B cells and DCs were isolated from C57Bl/6 spleens using negative and PE-positive selection respectively. OVA plasmid DNA-loaded B cells were co-cultured with DCs as in Figure 2g, but without CD8 T cells. B cells were re-purified after three days of co-culture by positively selecting DCs. PKH67-labeled CD8 T cells isolated from OT I spleen were then added to either re-purified B cells or repurified DCs. After four days of culture, CD8 T-cells were analyzed for IFNγ secretion (panel a) and proliferation (panel b). The *in vitro* assay was set up as in Figure 2g, however B cells and DCs were isolated from either C57Bl/6 mice or MHCI-KO mice spleens using negative selection. After five days of incubation with PKH67-labeled CD8-T cells, IFNγ secretion (panel c) and proliferation (panel d) were assessed. Asterisks * = $p < 0.05$, ** = $p < 0.01$, and **** indicate $p < 0.0001$. IFN-γ ELISA results are pooled from two independent experiments, with samples assessed in triplicate. Proliferation plots are representative from two similar independent experiments.

group (Figure 5b). In terms of chemokines, we observed increased concentrations of MIP1-α, and MIP1-β in the co-culture group, which was dependent on the presence of GM-CSF and/or IL-4 (Figure 5b). Furthermore, we observed increased expression of other chemotactic proteins, CCL2, CXCL2, CCL17, CCL12 and IL16 in the co-culture group (Figure 5b). These findings suggested that the interaction of DNA-loaded B cells with DC results in the production of several cytokines and chemokines that (1) promote antigen presentation, (2) promote inflammatory responses, and (3) promote chemotaxis of immune populations.

Distinct gene expression patterns are observed in DNA-loaded B cells that are licensed by DCs

Next, we wished to understand the changes occurring in B cells at the gene expression level, following their interaction with DCs. DNA-loaded B cells were cultured for 3 days with DCs and CD8 T cells and then separated into individual populations by flow cytometry. B cells were then analyzed by RNAseq. Upon principal component analysis, the biological replicates demonstrated minimal variance, however large variation was observed between B cells cultured with DCs and those not cultured with DC (Figure 6a). This was indicative of vastly different gene expression signatures. This was confirmed by MA plot showing log fold change (M) of each gene plotted against its mean average intensity/expression (A) (Figure 6b). Similar analysis was also performed to analyze the gene expression variation in DCs before and after co-culture with DCs. PC plots and MA plots of the analysis showed that like B cells, DCs also demonstrated significant gene expression changes (6A-B). There were 6845 genes that were significantly ($p < 0.05$, adjusted for multiple comparisons) differentially regulated in B cells between the two groups. The top upregulated genes in B cells after co-culture with DCs were classified under the category of cytokine and chemokine related to immune system responses, more specifically related to inflammation type responses (Figures 6c and Supplemental Figure 4). We then performed gene set enrichment analysis (GSEA)[20,21] to match this gene data set against prior defined B-cell related gene sets.

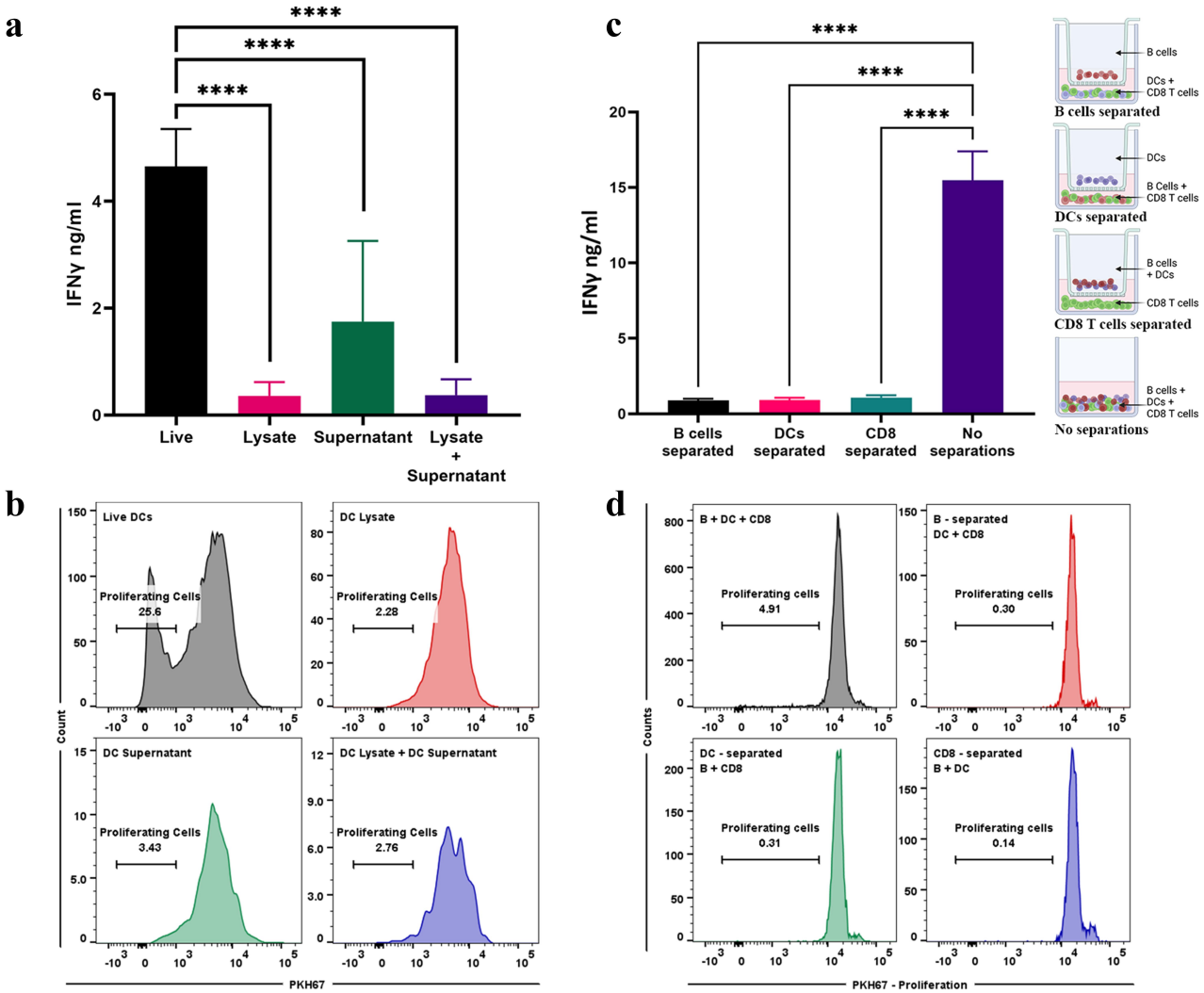

Figure 4. Cell-cell interaction between B cells and live DCs is required for licensing of B cells by DCs. OVA plasmid DNA-loaded B cells were set up in culture as in Figure 2G, however live DCs were replaced with DC lysates (prepared from DCs by repeated freeze thaw followed by sonication) or by supernatant collected from live DC cultures. Secreted IFNγ was measured by ELISA (panel a) and proliferation of PKH67-labeled CD8 T-cells was measured using flow cytometry (panel b). OVA DNA-loaded B cells and DCs were co-cultured as in Figure 2G but were separated by using trans-well culture plates. After five days of co-culture PKH67-labled CD8 T-cells were analyzed for IFNγ secretion (panel c) and proliferation (panel d). Asterisks * indicate $p < 0.05$ ** indicate $p < 0.01$ and **** indicate $p < 0.0001$. IFN-γ ELISA results are pooled from three independent experiments, with samples assessed in triplicate. Proliferation plots are representative of three similar, independent experiments.

Based on the enrichment scores and the relevance to APC function of B cells, we identified two prior defined gene sets most associated with DC-licensed B cells: B cells cultured with TLR7 agonist (imiquimod) versus TLR4 agonist (monophosphoryl lipid A) (Figure 6d,f), and B cells simulated through IgG (Figure 6E,g). Together, these gene sets suggested that the B cells licensed by DC had a gene expression profile consistent with an activated phenotype, similar to B cells activated by TLR and/or the B-cell receptor.

Based on the findings from the GSEA analysis, we next investigated the effect of a TLR7 agonist on the APC function of B cells directly. This was performed with TLR7 agonist alone or in combination with other B-cell activation agents such as CD40, CD40L and TLR9 agonist CpG. None of these treatments induced DNA-loaded B cells to activate CD8 T cells in the absence of DCs (Supplemental Figure 5). We also surveyed the literature for known cell surface interactions between B cells and DCs. We tested the possible role of the most prominent interactions associated with antigen presentation by using blocking antibodies targeting CD23-IgE or CD70-CD27. In addition, we evaluated the CD40-CD40L interaction by using APC from CD40 KO mice. Blockade of the CD70-CD27 interaction did not affect CD8 T cell proliferation or levels of secreted IFNγ (Figure 7b). On the other hand, blockade of CD23 by use of a blocking antibody (Figure 7a), or blockade of CD40-CD40L by use of B cells and DCs from CD40-KO mice (Figure 7c), impeded but did not entirely abrogate CD8 T-cell proliferation and secretion of IFNγ.

Taken together, our data suggest that possibly multiple cellular interactions between B cells and DC, including CD40-CD40L and CD23-IgE, lead to activation of B cells that have increased antigen presentation function. Our future studies will be focused on understanding these specific interactions between B cells and DCs and how this leads to changes in B cell antigen presentation function.

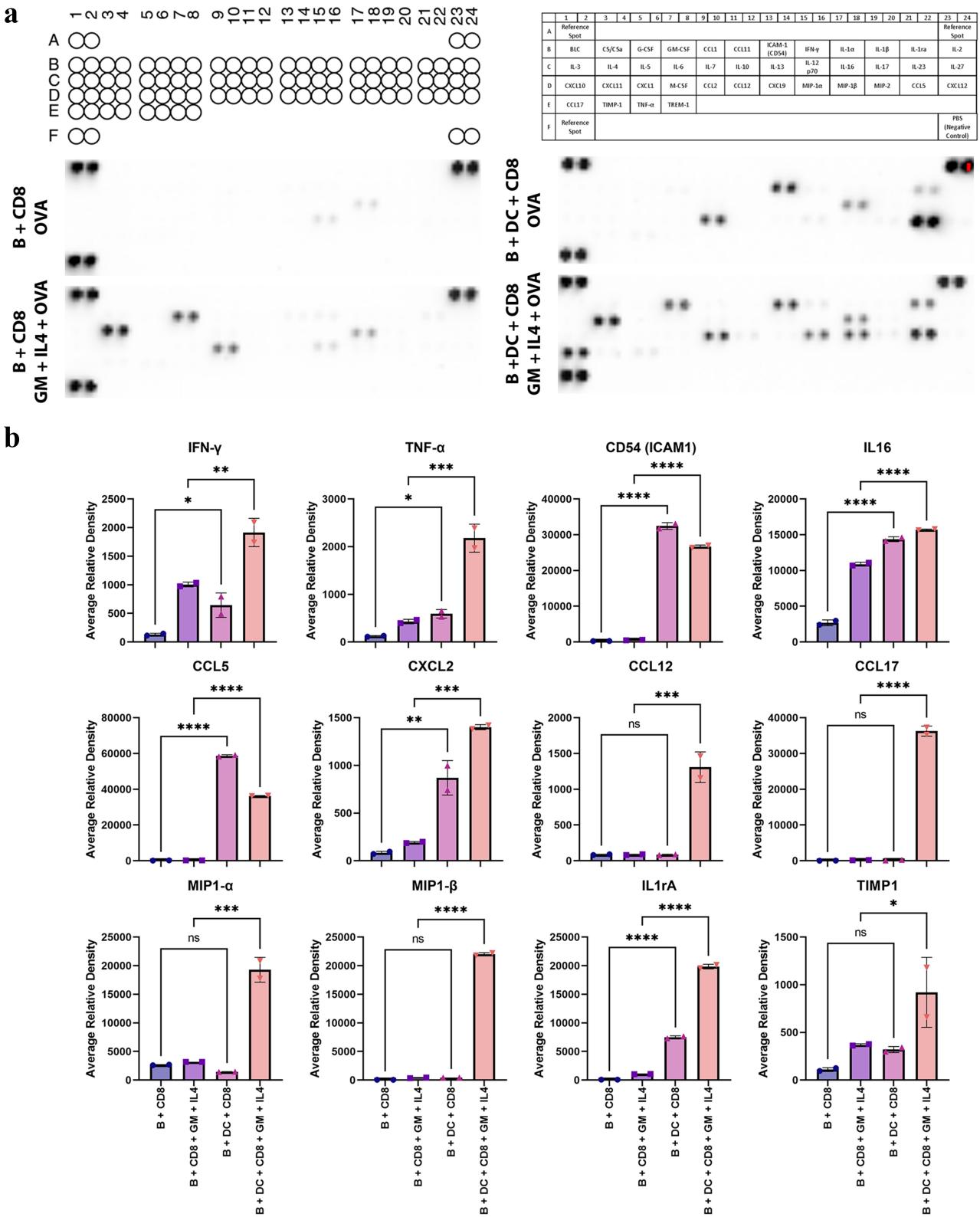

Figure 5. Cell-cell interaction between B cells, CD8 T cells and live DCs results in release of pro-inflammatory cytokines and chemokines. The *in vitro* antigen presentation assay was set up as in Figure 2G, and supernatants were collected after five days of culture. The supernatants were used for detection of immune-response related cytokines and chemokines using blot-based cytokine array. Representative blots are shown after exposure using BioRad Chemi-Doc imaging system (panel a). Relative expression was quantified by measuring the intensity of each band using NIH ImageJ software (panel b). Asterisks * indicate $p < 0.05$, ** indicate $p < 0.01$, *** indicate $p < 0.001$ and **** indicate $p < 0.0001$. Results are from one experiment, with samples assessed in duplicates, and are representative of three similar, independent experiments (Supplemental Figure 3).

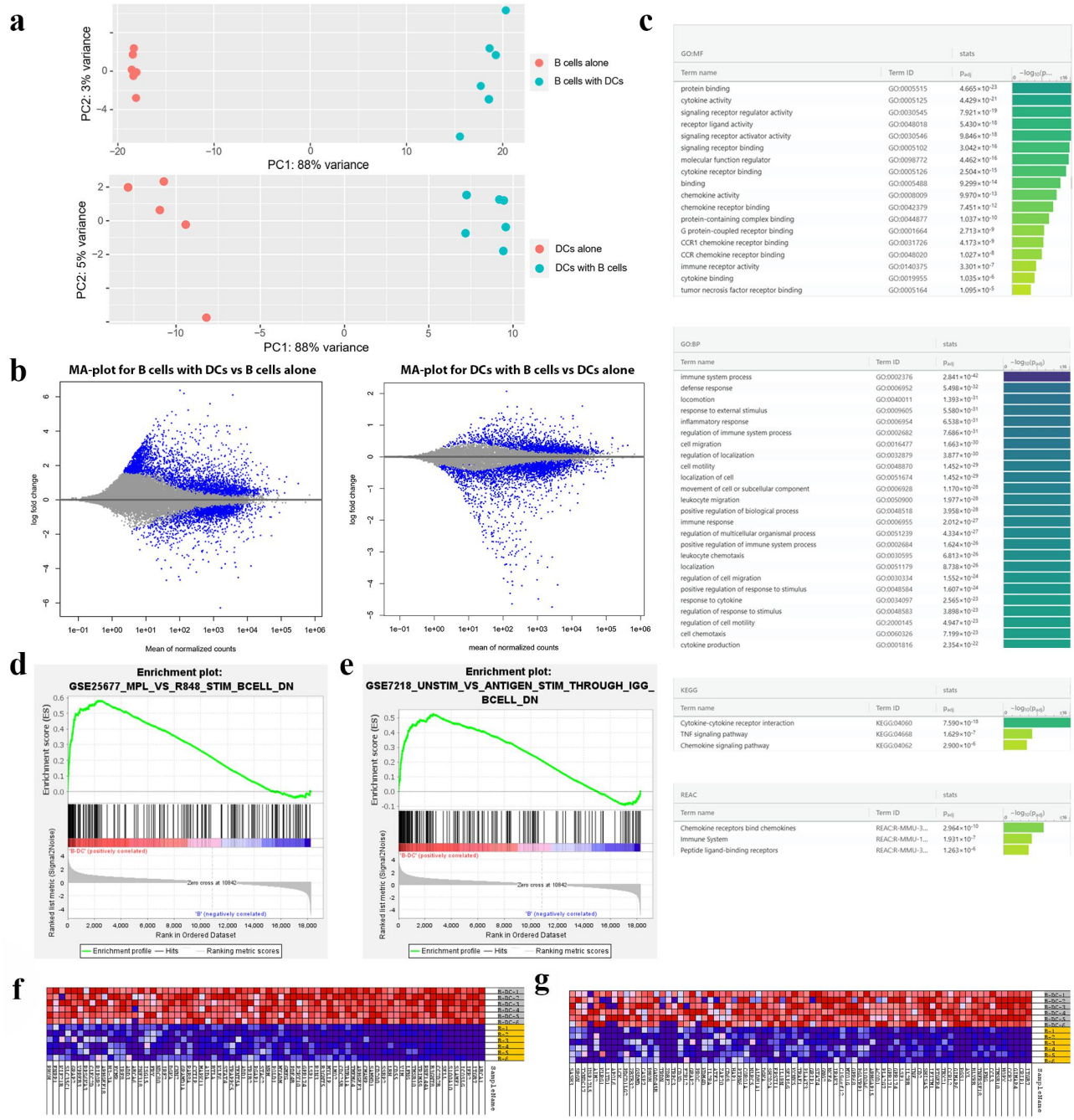

Figure 6. Distinct gene expression patterns are observed in DNA-loaded B cells that are licensed by DCs. DNA-loaded B cells were cultured, either alone or with DCs, with CD8 T-cells for three days. B cells were then sorted using CD19 cell surface marker, and DCs sorted by CD11c cell surface marker, and processed for total RNA isolation. cDNA libraries were synthesized and indexed for sequencing. After analysis by using Project Galaxy, log2-fold changes in gene expression were calculated from both groups. Shown are a PCA plot (panel a) and MA plot (panel b), representative of the whole data for each cell type. gProfiler, an online gene ontology tool, was used to categorize 400 most upregulated genes in B cells after co-culture with DCs (panel c). The two most significant enrichment plots from GSEA analysis on the resulting gene list are shown (panels d and e), and their corresponding heatmaps of most overexpressed genes in the co-culture groups are shown (panels f and g). Results are from one experiment, with samples assessed in six biological replicates.

Discussion

In this article, we demonstrated that B cells that have taken up DNA by passive transfer can translate the encoded antigen, but require co-culture with DCs to present the encoded antigen to activate CD8 T cells. This presentation to CD8 T cells is by B cells, and not via cross-presentation by DCs. Further, we demonstrated that this is due to a cell-cell interaction between B cells and DCs that requires either CD4 T cells or IL-4, and this encounter results in an inflammatory response with release of multiple cytokines and cell attractant chemokines. In addition, culture of DNA-loaded B cells with DCs results in a dramatic change in B cell phenotype as evidenced by changes in gene expression profiles. As such, we provide the first evidence, to our knowledge, of DCs providing a licensing function to B cells, facilitating their function as APCs. The role of B cells as APC has been largely understudied relative to their role in humoral immunity. Our findings may be relevant to the anti-tumor role of B cells in tumors, and are certainly of

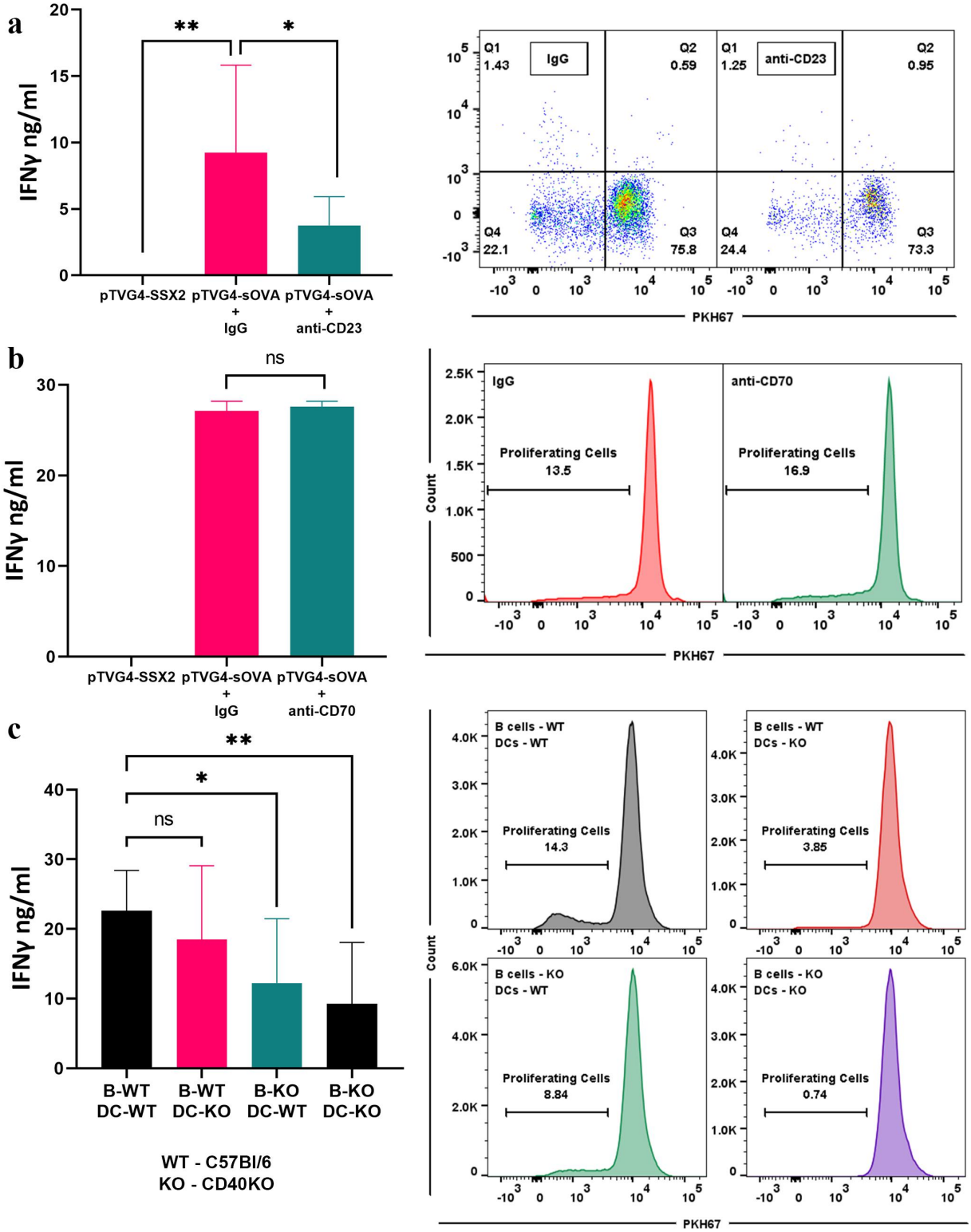

Figure 7. Blocking CD23-IgE and CD40-CD40L, but not CD70-CD27 interaction, negatively impacts B cell licensing. The *in vitro* antigen presentation assay was set up as in Figure 2g, however, blocking antibodies for CD23 (2.5 µg/ml), CD70 (10 µg/ml), or IgG (10 µg/ml) were added to the culture on the second day along with DCs and PKH67-labeled CD8 T-cells. For testing involvement of CD40-CD40L interaction, B cells and DCs were used from either wild-type C57Bl/6 or CD40-KO mice spleens. After five days of culture, proliferation of CD8 T-cells was evaluated and IFNγ secretion was measured following anti-CD23 blockade treatment (panel a), following anti-CD70 blockade treatment (panel b), and using B cells and/or DCs from CD40-KO mice spleens (panel c). Asterisks * indicate $p < 0.05$ and ** indicate $p < 0.01$. IFN-γ ELISA results are pooled from three independent experiments, with samples assessed in triplicate. Proliferation plots are representative of three similar, independent experiments.

relevance to studies to enable the use of B cells as APCs for anti-tumor vaccines.

Our results, at first, seemed to conflict with what we had previously reported, that B cells alone could present plasmid DNA-encoded antigens to T cells.[13] In those studies, less pure populations of cells were used, and we had used a different method for enriching B cells using magnetic bead isolations. These resulted in B cell populations that likely had small numbers of contaminating DC. In context with our current findings, this suggests that even small numbers of DC may potentially be required to enable antigen expression by B cells, as well as antigen presentation, as we observed in Figure 2a. Certainly larger numbers of DC were disadvantageous by outcompeting B cells for DNA uptake (Figure 2a–d).

To our knowledge, this is the first evidence of B cell licensing through DCs. The precise signaling between these cell types for this licensing remains unknown. One known natural interaction between B cells and DCs is when germinal center B cells capture antigen complexes from the surface of follicular DCs[22]. Apart from BCR stimulation by the antigen complex on DCs, other cell surface molecules like ICAM-1, VCAM-1 and BAFF are expressed on DCs that interact with their ligands LFA-1, VLA-1 and BAFF-R respectively on B cells; hence, these are potential ligand-receptor interactions[22]. Interactions involving integrins ICAM-1 and VCAM-1 have been shown to facilitate B cell survival[23]. Furthermore, Carrasco et al., have demonstrated that ICAM-1/LFA-1 interaction promotes B cell adhesion and synapse formation by lowering the antigen threshold for B cell activation[24]. On the other hand, stimulation through BAFF has been known to generally promote B cell survival, activation and maturation[25]. More specifically BAFF signaling has been demonstrated to promote maintenance of germinal centers[26]. In our cytokine array, we found upregulation of soluble ICAM-1 in the co-culture groups. This suggests that ICAM-1 expressed by DCs in this co-culture, and the ICAM-1/LFA-1 interaction, could play a role in promoting antigen processing and presentation by B cells. However, we currently do not know the exact interactions that are occurring during this co-culture. It is also conceivable that B cells require both a cell surface interaction(s) and a secreted cytokine/chemokine during the co-culture. We could not identify any specific interaction from the RNAseq data analysis. However, from our blocking and knockout studies, it appears that CD40/CD40L interactions are at least partially required. Understanding how each of these interactions is important for APC function of B cells is one of our future directions.

In response to co-culture with DCs, we found that B cells, as well as the DCs, changed their transcriptional phenotype dramatically. From the RNAseq analysis, we showed that this new phenotype is similar to B cells that have been stimulated through BCR or TLR7/8. It has been known that signaling through BCR is critical for B cell activation and differentiation upon interaction with antigen[27]. Internalization of antigen by BCR is the primary mode of antigen processing and presentation by B cells. This leads to BCR oligomerization and subsequently presentation of peptide through MHC[28]. It is unknown whether activation of the BCR occurs following DC co-culture, or whether the gene expression profile of DC-licensed B cells is just similar to those of BCR-activated B cells. Similarly, it is currently not known if the DC-licensed B cells are activated through TLR7/8 signaling or if their profile resembles that of TLR7/8 activated B cells. In any case, our findings demonstrate that TLR7/8 activation alone could not replace the licensing function of DC (Supplemental Figure 5). TLR7/8 activation has been shown to promote B cell proliferation, induce expression of co-stimulation molecules and augment antigen-specific immunoglobulin production[29]. These findings are consistent with the observation of a change in phenotype to cells with antigen-presentation capacity.

The role of B cells as APCs has been largely understudied. This has now become of more relevance, as recent reports have demonstrated that the presence of tumor-infiltrating B cells correlates with better prognosis for many cancer types[30]. In particular, increased numbers of tertiary lymphoid structures, where these B cells reside, have been associated with increased survival of cancer patients.[31] Although their specific role in the tumor microenvironment is not clear, there are indications that these B cells are capable of presenting antigen, as demonstrated by increased expression of antigen presentation-related surface markers. This makes understanding the role of B cells as APCs of paramount importance, and whether they are functionally different from antibody-producing B cells or regulatory B cells requires further investigation. Moreover, we have previously shown that upon priming of CD8 T cells through peptide-loaded B cells or DCs, there were differences in the resulting checkpoint marker expression on CD8 T cells[32]. Therefore, understanding the differences between B cells and other professional APC subsets in the context of their capacity to activate antigen-specific T cells is also of importance.

Overall, our findings demonstrate that B cells are the primary professional APC that can directly process and present plasmid DNA to activate CD8 T cells following passive uptake. While our intent has been to focus on passive delivery of DNA to APC, relevant to most immunization methods, a limitation of our studies is that these findings may not be relevant for other methods of DNA delivery, including the use of nanoparticles, other transfection reagents, or electroporation. In fact, these other delivery methods may be desirable to bypass DNA degradation and promote direct presentation by DC. This will be a focus of future studies, as will the evaluation of other antigen systems to validate these results. Another limitation of our study is that we have specifically focused on the activation of CD8 T cells. It remains unknown whether B cells can activate antigen-specific CD4 T cells following passive DNA uptake. This will also be a goal of future studies. Another limitation of our study is that most of our co-culture studies were performed with CD8 T cells. This was necessary to be able to evaluate the antigen-presentation function of B cells, but we cannot rule out the possibility that CD8 T cells also contributed to the activation and antigen presentation by B cells. In particular, both B cells and T cells can express CD40 and CD40L, and it is conceivable that CD8 T cells provided CD40 to partially rescue activation in the presence of CD40-KO DC or B cells. Future studies will evaluate the

activation of B cells by DC in the absence of CD8 T cells. Future studies will also be aimed at determining the precise signaling provided by DC to license B cells, as this will be important to develop novel methods of vaccination. In particular, we expect these studies could target nucleic acids specifically to B cells, or use DNA-loaded B cells for delivery as a cell-based therapeutic vaccine.

Acknowledgments

We thank the University of Wisconsin Flow Cytometry Core Facility, UW Biotechnology Center, UW Carbone Cancer Center Microscopy Center, and the NIH Tetramer Facility (Emory University, Atlanta, GA) for their support.

Disclosure statement

Douglas G. McNeel has ownership interest, has received research support, and serves as consultant to Madison Vaccines, Inc. which has licensed intellectual property related to this content. IR has no relevant potential conflicts of interest.

Funding

This work was supported by the National Institutes of Health (P30 CA014520) and the Department of Defense Prostate Cancer Research Program (W81XWH-17-1-0247).

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Authors' contributions

IR wrote the manuscript, performed all experiments, and carried out data analysis. DGM oversaw the experimental design and is responsible for the overall content as the guarantor. Both authors contributed to the writing and approval of the final manuscript.

Availability of data and material

The data generated and/or analyzed during this study are available from the corresponding author on reasonable request.

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