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IXA4 INDUCES ANTIGEN CROSS-PRESENTATION IN MURINE MESENCHYMAL STROMAL CELLS AND SUPPORTS MELANOMA VACCINATION

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ABSTRACT

Background: Antigen cross-presentation is a key function for cell-based cancer vaccination. Mesenchymal stromal cells (MSCs) provide an experimentally tractable platform, but the cellular states that permit efficient presentation of exogenous proteins remain incompletely defined. We investigated whether IXA4, a pharmacological activator of IRE1α-associated signaling, induces cross-presentation and supports MSC-based melanoma vaccination.

Methods: Primary murine bone-marrow-derived MSCs were conditioned with IXA compounds and assessed using ovalbumin-dependent B3Z reporter assays, flow cytometry, secretome profiling, and RNA sequencing. Pathway inhibitors and *Xbp1* perturbations were used to examine signaling contributions. Tumor lysate-loaded, IXA4-reprogrammed MSCs (IRM-4 cells) were evaluated in B16F0-bearing mice, alone or with programmed cell death protein 1 (PD-1) blockade.

Results: IXA4 induced ovalbumin-dependent reporter activation at a tolerated working concentration of 5 μM. The response was sensitive to IRE1 RNase inhibition and accompanied by increased surface H2-K^b, altered soluble mediator composition, and transcriptional remodeling of endoplasmic reticulum proteostasis and vesicular trafficking. No clear increases were detected in the bulk antigen-handling or oxidant-sensitive assays examined. IRM-4 vaccination delayed melanoma progression, with the most sustained benefit observed alongside PD-1 blockade. Responsiveness varied among MSC preparations and was associated with baseline unfolded protein response programs. *Xbp1*-edited cells retained an IXA4-associated response, whereas enforced XBP1s expression did not reproduce the cross-presentation phenotype.

Conclusions: IXA4 conditioning supports functional antigen cross-presentation and experimental MSC-based melanoma vaccination. The dependence of this response on the

starting cellular state, rather than XBP1s abundance alone, identifies functional potency testing and mechanistic validation as priorities for further development.

Keywords: Mesenchymal stromal cells; IXA4; IRE1α; XBP1; Unfolded protein response; Antigen cross-presentation; Cell-based vaccine; Melanoma; PD-1

BACKGROUND

Therapeutic cancer vaccines aim to generate tumor-directed T-cell immunity by improving antigen delivery and presentation. Dendritic cell (DC) vaccination has broadened antigen-specific T-cell responses in patients with melanoma [1], while Sipuleucel-T has demonstrated that an autologous cellular product containing antigen-presenting cells can improve survival in metastatic castration-resistant prostate cancer [2]. Although these clinical precedents establish the feasibility of cell-based vaccination, the induced responses were modest. There is therefore a need for experimentally tractable cellular platforms in which antigen-processing and presentation states can be manipulated mechanistically to improve therapeutic responses.

Mesenchymal stromal cells (MSCs) provide a non-hematopoietic platform whose immune phenotype can be altered by conditioning or engineering. Earlier studies showed that interferon-γ can induce conditional antigen-presenting properties in MSCs, although these effects are highly dependent on the stimulation context [3, 4]. More recent genetic approaches that alter proteasome composition have generated MSC-based vaccines with MHC class I cross-presentation and antitumor activity in mice [5–7]. Pharmacological conditioning with A1 or AccuTOX has likewise been associated with the acquisition of cross-presentation properties alongside changes in intracellular antigen handling and stress-response programs [8, 9], while studies using targeted xenoantigens further link intracellular protein degradation to MSC vaccine activity [10]. Collectively, these findings identify intracellular proteostasis and antigen handling as candidate determinants of the cross-presenting MSC state, but the regulatory programs that enable this phenotype remains incompletely defined.

The unfolded protein response (UPR) is a major regulator of endoplasmic reticulum (ER) proteostasis and is coordinated through inositol-requiring enzyme 1α (IRE1α), protein kinase

R-like ER kinase (PERK), and activating transcription factor 6 (ATF6) [11, 12]. The RNase activity of IRE1α generates spliced X-box-binding protein 1 (XBP1s) from Xbp1 mRNA and can also mediate regulated IRE1-dependent decay (RIDD). These two IRE1α RNase outputs are related but not equivalent as XBP1s induces ER chaperone and quality-control programs, whereas RIDD can alter selected RNA abundance independently of XBP1s-mediated transcription [13, 14]. Studies in DCs further indicate that the effects of IRE1α/XBP1 signaling on antigen-presenting function are context dependent and can vary with cellular state and the balance among IRE1α outputs [15–17]. These observations raise the possibility that pharmacological engagement of IRE1-associated proteostasis could promote cross-presentation in MSCs, but whether such responsiveness is determined by XBP1s abundance or by the broader UPR-associated cellular state remains unclear.

Several IXA compounds were originally characterized as pharmacological activators of IRE1/XBP1s signaling that preferentially induce adaptive ER proteostasis programs in the systems examined [18]. We therefore compared three IXA lead candidates (IXA1, IXA4, and IXA6) in primary murine bone-marrow-derived MSCs to determine whether this class of compounds can induce functional cross-presentation. We then characterized the transcriptional and therapeutic consequences of IXA4 conditioning (the best activator), including its ability to support tumor lysate-based melanoma vaccination, and examined variability among independent MSC preparations. Finally, we tested under Xbp1 perturbation whether IXA4 responsiveness tracks with XBP1s abundance or with a broader UPR-associated cellular state. Together, these experiments evaluate IXA4 conditioning as a pharmacological strategy for MSC-based vaccination while defining cellular-state features that may govern functional potency.

METHODS

Animals and ethical approval.

Female and male C57BL/6 mice, 6-10 weeks of age, were obtained from Charles River (Montreal, QC, CANADA) and maintained under pathogen-free conditions at the animal facility of the Institute for Research in Immunology and Cancer, Université de Montréal. Animal procedures were approved by the Université de Montréal animal care committee (protocol #25-026). Animals were euthanatized by Carbon dioxide (CO_2) overdose followed by cervical dislocation. Our work has been reported in line with the ARRIVE guidelines 2.0

Primary MSCs and cell lines.

Primary bone-marrow-derived MSCs were isolated from the femurs of female C57BL/6 mice and culture-adapted as described previously [5, 6]. MSCs were maintained in α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin, reported as 50 U/mL. Cells at passages 4-6 were used for compound comparisons. Eight independently prepared MSC populations, designated S1-S8, were used to examine response heterogeneity. B16F0 melanoma cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium containing 10% FBS and penicillin-streptomycin. The B3Z reporter cells, provided by Michel Desjardins (Université de Montréal), were cultured in RPMI 1640 supplemented with 10% FBS, penicillin-streptomycin, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, and 0.5 mM of β-mercaptoethanol. Cells were maintained at 37°C in 5% CO_2. Cell-culture media and supplements were obtained from Wisent Bioproducts (Saint-Jean-Baptiste, QC, CANADA).

Compound exposure, cell counts, and immunophenotyping.

For the tolerability screen, MSCs were seeded in 24-well plates at the reported density of 2.0 × 10^4 cells per well. On the following day, IXA1, IXA4, or IXA6 was added at 1-20 μM for 48 h. Dimethyl sulfoxide (DMSO) served as the vehicle control. Cells were washed, collected, and counted using trypan blue exclusion. A working concentration of 5 μM was selected for subsequent IXA comparisons. Annexin-V staining was used to assess apoptosis-associated fluorescence after compound exposure. MSC immunophenotyping was performed by flow cytometry using antibodies against CD44-PE (clone IM7; catalog no. 103023), CD45-PE (clone I3/2.3; catalog no. 147711), CD73-PE (clone TY/11.8; catalog no. 567215), and CD105-PE (clone MJ7/1B; catalog no. 562759), with corresponding isotype controls. All flow antibodies were purchased from BD Biosciences (San Jose, CA, USA).

OVA cross-presentation and B3Z reporter assay.

MSCs were seeded at 2.0 × 10^4 cells per well in 24-well plates and incubated for 48 h in fresh medium containing 1 mg/mL ovalbumin (OVA) and the indicated IXA compound (all used at 5 μM). Cells were washed before the addition of 5 × 10^5 B3Z reporter cells per well. Reporter activity was measured as a β-galactosidase-dependent absorbance readout at 570 nm using the previously described assay approach [5, 7, 8]. SIINFEKL-pulsed MSCs served as a peptide-presentation control. The A1 molecule, supplied by Biopeptek (Malvern, PA, USA), was used at 50 μM as a positive conditioning control [8]. Titration studies used IXA4 at 5-15 μM, MK-28 at 50-150 μM, and BIX at 5-15 μM. For inhibitor-related experiments, 4μ8C (5 μM), ISRIB (10 μM), and AEBSF (6.5 μM) were added with IXA4 during antigen loading, and cells were washed before reporter co-culture. The washout experiment assessed reporter responses at 24, 48, and 72 h after IXA4 removal.

Surface MHC class I, antigen uptake, and proteolysis.

Cell surface H2-K^b-PE (purchased from BD Biosciences; clone AF6-88-5; catalog no. 562832) was measured by flow cytometry. For OVA uptake measurements, 5×10^4 MSCs were seeded per well in 12-well plates and exposed to 1 μg/mL OVA-Alexa Fluor 647 (Waltham, MA, USA) with IXA4 for 3 h at 37°C. Cells were collected and washed with phosphate-buffered saline (PBS) before analysis. For bulk OVA proteolysis measurements, cells were incubated with 10 μg/mL DQ-OVA (Waltham, MA, USA) and IXA4 for 1 h at 37°C, washed, and cultured in regular medium for a further 3 h before collection. Fluorescence was acquired on a BD FACSCanto II using FACSDiva.

Oxidant-sensitive flow-cytometric assays.

MSCs were seeded at 2.0×10^4 cells per well in 24-well plates and treated with 5 μM IXA4 for 48 h. Cells were washed with PBS, detached using trypsin, and washed with ice-cold PBS containing 2% FBS. MitoSOX (Thermo Fisher Scientific; catalog no. M36008) was applied at 5 μM in PBS for 30 min at 37°C. Dihydroethidium (DHE - Thermo Fisher Scientific; catalog no. D11347) staining was performed at 10 μM in PBS for 30 min at 37°C. After staining, cells were washed once and resuspended in PBS containing 2% FBS. Samples were maintained on ice in the dark and analyzed within 1 h using the FACSCanto II/FACSDiva system.

Cytokine and chemokine profiling.

MSCs (1×10^6 cells) were cultured in complete α-MEM for 24 h, then washed twice with PBS before adding 5 μM of IXA4 in serum-free α-MEM for another 24 h. Conditioned medium was collected and stored at 4°C. Fresh serum-free medium without IXA4 was added, and a second collection was performed 24 h later. The two collections were pooled and concentrated 80-fold using Amicon Ultra-4 centrifugal filters (MilliporeSigma, Oakville, ON, CANADA) with a

nominal 3-kDa molecular-weight cutoff, centrifuged at 4500 × g for 1 h at 4°C. Concentrates were aliquoted and stored at -80°C before multiplex analysis by Eve Technologies (Calgary, AB, CANADA) using a Luminex-based assay. Control and IXA4-conditioned samples were normalized and compared as relative cytokine and chemokine compositions.

B16F0 tumor lysate preparation.

B16F0 cells were detached using 0.05% trypsin and washed three times with PBS. The reported centrifugation setting for these washes was 400 × g for 10 min. Cell pellets underwent five cycles of freezing in liquid nitrogen and thawing using boiling water, with vortexing or shaking between cycles to homogenize the material. Lysates were clarified at 4500 × g for 10 min at 4°C. Supernatants were collected, quantified using a Bio-Rad protein assay (Mississauga, ON, CANADA) according to the manufacturer's instructions, aliquoted, and stored at −80°C.

IRM-4 vaccine preparation and tumor treatment

MSCs were incubated for 48 h with B16F0 lysate at 0.05 or 0.5 mg/mL, as specified for each cohort, with or without 5 μM of IXA4. Cells were washed with PBS, detached with Accutase (Sigma Aldrich, Oakville, ON, CANADA), and counted. Each vaccine dose contained 5×10^5 cells in 100 μL. B16F0 cells were washed three times with PBS before administration. Mice received 5×10^5 tumor cells subcutaneously (SC) on day 0. The reported vaccination protocol used 5×10^5 MSCs administered SC at a site distal to the tumor on days 3 and 10. For combination treatment, anti-PD-1 antibody (clone RMP1-14, Assay Genie), or the corresponding isotype, was administered intraperitoneally at 200 μg per dose every 2 days for a total of six doses. The anti-PD-1 treatment was initiated at day 10 (e.g. with the second vaccine dosing). The first female cohort received the vaccine prepared with 0.05 mg/mL lysate, while the second cohort received the vaccine prepared with 0.5 mg/mL lysate. The male cohort

used only the 0.5 mg/mL lysate treatment. Tumor volumes were monitored with digital calipers for up to 6 weeks or until ulceration and/or a tumor volume of 1000 mm^3. Mice were euthanized by CO_2 inhalation when endpoints were reached. Survival is described here as time to the reported humane endpoint.

RNA isolation and sequencing.

One million MSCs were treated with 5 μM of IXA4 for 48 h, detached, washed, and collected for RNA isolation using the QIAGEN RNeasy Mini Kit (Toronto, ON, CANADA). Total RNA was quantified using Qubit, and 500 ng was used for library preparation following the previously reported workflow [5]. Libraries were diluted to 10 nM and quantified using the KAPA Library Quantification Kit (KK4973). The reported sequencing platform was an Illumina HiSeq 2000 with HiSeq Reagent Kit v3 and 200-cycle paired-end chemistry, using a pooled library concentration of 1.7 nM.

Transcriptomic analysis

Gene-level counts were generated from the STAR (version 2.7) alignments to the Mus musculus GRCm38 (mm10) reference genome using the STAR ReadsPerGene output. [19] Ensembl gene identifiers were converted to gene symbols and biotypes with the biomaRt R package; only protein-coding genes were retained, and duplicated symbols were removed. Each MSC preparation was sequenced as three independent replicates per condition. Downstream analyses were performed in R using DESeq2 [20], clusterProfiler, biomaRt, pheatmap, and ggplot2. For visualization, counts were variance-stabilizing transformed (vst). Principal component analysis (PCA) was performed with prcomp (centered and scaled) on the 2000 most variable genes across control samples. Differential expression was tested with DESeq2 Wald tests and Benjamini-Hochberg adjustment: baseline differences between responders and non-

responders (S4/S5 versus S7/S8) were assessed in control samples with a ~response design after retaining genes with zero counts in fewer than 5% of responder samples, and IXA4 treatment effects were assessed separately within responders and within non-responders with a ~treatment design after filtering low-count genes. Gene set enrichment analysis (GSEA) was performed with clusterProfiler on genes ranked by the Wald statistic, using the MSigDB mouse hallmark collection (mh.all, v2024.1) and a mouse-ortholog-mapped human Reactome collection (Mouse_Human_Reactome, March 2025), with gene-set sizes of 10-500 and Benjamini-Hochberg adjustment; hallmark terms were grouped into ten broad categories, and terms with q <= 0.1 are reported. [21, 22] Gene Ontology biological-process and Reactome over-representation analysis in IXA4-treated responsive MSCs was performed with clusterProfiler using MSigDB-associated gene sets and mouse-to-human ortholog mapping; the gene ratio was defined as the number of contributing genes divided by the size of the pathway or term. The ER-stress-associated gene set shown in Fig. 5D was defined as the union of core-enrichment genes of the Reactome pathways 'PERK regulates gene expression' (R-HSA-381042) and 'ATF4 activates genes in response to endoplasmic reticulum stress' (R-HSA-380994) across the three GSEA contrasts, restricted to genes differentially expressed (adjusted $p < 0.1$) in the treatment comparisons, with Xbp1 and Manf added. Heatmaps display row-wise z-scores of vst-transformed counts.

Generation of XBP1s-overexpressing and *Xbp1*-edited MSCs

The responsive MSC population S4 was used to generate XBP1s-overexpressing and CRISPR-edited populations. The XBP1s expression construct was synthesized by GenScript (Piscataway, NJ, USA) and introduced by viral transduction as previously reported. [5, 6, 10] The engineered populations are referred to as MSC-XBP1s and MSC-XBP1^{mut}, respectively. Cas9 ribonucleoprotein (RNP) complexes were prepared with HiFi Cas9 v3 (Integrated DNA

Technologies; reported stock concentration, 62 μM) and synthetic crRNA and tracrRNA diluted to 200 μM. Equimolar crRNA and tracrRNA were incubated at 95°C for 5 min and cooled to room temperature. The guide duplex was mixed with Cas9 at a Cas9:guide molar ratio of 1:1.5 and incubated for 10 min at room temperature. For reverse transfection, mixture A contained 5 μL targeting or control RNP, 50 μL Opti-MEM, and 2 μL Plus Reagent. Mixture B contained 50 μL Opti-MEM and 4 μL Lipofectamine LTX. The mixtures were combined and incubated for 15 min at room temperature, then dispensed into culture wells before the addition of 4×10^5 MSCs per well. After 6 h, medium was replaced with α-MEM containing FBS and antibiotics. At 48 h, cells were harvested in 100 μL QuickExtract DNA Extraction Solution. Lysis was performed at 65°C for 6 min and 98°C for 2 min, followed by a 4°C hold. The target locus was amplified using forward primer 5′-GATGAATATACCCGCGCGTC-3′ and reverse primer 5′-CGCTTCCCAGGTTAGACACT-3′. A 50-μL reaction contained 5 μL lysate, 1 μL primer solution reported at 10 μM, 25 μL AmpliTaq Gold reagent, and 19 μL water. Thermal cycling comprised 94°C for 10 min; 35 cycles of 95°C for 30 s, 55.9°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 7 min. Products were Sanger sequenced by Génome Québec. The sequence-deconvolution software is described inconsistently as TIDE or "EditCo"/Synthego and requires identification. After bulk analysis, cells were plated by limiting dilution in 96-well plates at a nominal density of one cell per well. Selected clones were analyzed with the same workflow. Three clones were described as fully edited by sequence deconvolution.

Quantitative transcript measurements.

Quantitative polymerase chain reaction (qPCR) measurements assessed *Atf4*, *Atf6*, and the *Xbp1s*/*Xbp1* readout, together with *Scara3*, *Col6a*, *Bloc1s1*, *Dnajb9*, and *Manf*. RNA extraction kits were obtained from QIAGEN and used as previously reported. [5, 6, 10]

Statistical analysis

The reported analysis framework used one-way analysis of variance (ANOVA) for group comparisons, log-rank testing for survival, and mean ± standard deviation (SD) for quantitative plots. Significance symbols denote *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

Use of generative artificial intelligence

ChatGPT (OpenAI) was used to assist with language editing, manuscript organization, literature-assisted revision, and adaptation to the journal format. The tool was not used to generate experimental data or to perform statistical analyses. Author review and approval of the final wording and scientific interpretation remain required before submission.

RESULTS

IXA4 induces OVA cross-presentation at a tolerated working concentration.

To determine whether pharmacological activation of IRE1-associated signaling could establish a cross-presenting MSC phenotype, we first compared IXA1, IXA4, and IXA6, three compounds reported to activate IRE1/XBP1s signaling (**Fig. 1A**) [18]. We began with a concentration screen to identify conditions compatible with continued cell expansion. Culture-adapted murine bone-marrow-derived MSCs at passages 4-6 were exposed to 1-20 μM of each compound for 48 h. Cell numbers increased at 1-5 μM, whereas expansion was limited at 10 μM and counts declined at 20 μM (**Fig. 1B**). To complement the cell count measurements with an apoptosis-associated readout, we assessed Annexin-V staining. The representative profiles showed no evident treatment-associated increase under the chosen working concentration of 5 μM (**Fig. 1C**) and the innate MSC profile was retained (e.g. $CD44^{+}CD45^{-}CD73^{+}CD105^{+}$ - **Fig. 1D**). We thus selected that concentration for all subsequent experiments.

To test cross-presentation of an intact exogenous protein, MSCs were incubated with OVA and co-cultured with B3Z reporter cells, which respond to SIINFEKL presented by H2-K^{b}. SIINFEKL-pulsed MSCs provided a peptide-presentation control. Among the IXA compounds tested at 5 μM, IXA4 elicited the strongest OVA-dependent B3Z response (**Fig. 1E**). We next asked whether increasing IXA4 exposure would further improve this response. A concentration of 5 μM was sufficient to induce strong reporter activation while increasing IXA4 to 10 or 15 μM provided no additional benefit (**Fig. 1F**). To assess persistence after conditioning, we measured reporter activity following compound removal. The response decreased across the 24-72 h interval, indicating that the initial presentation phenotype was not maintained at the same magnitude after washout (**Fig. 1G**).

To examine whether the phenotype could also be elicited by compounds associated with other UPR branches, we next tested MK-28, a reported PERK activator, and BIX, a binding immunoglobulin protein (BiP)-inducing compound associated with ATF6-dependent transcriptional effects [23, 24]. MK-28 elicited a smaller B3Z response under the conditions tested, whereas BIX did not reproduce the IXA4-associated response (**Fig. 1H-I**). Together, these experiments identify 5 μM IXA4 as a tolerated conditioning regimen that induces functional OVA cross-presentation in murine MSCs, with activity that declines after compound withdrawal.

IXA4-associated cross-presentation is IRE1 RNase-sensitive but is independent of bulk antigen-handling, reactive oxygen species (ROS) production or soluble mediators.

To investigate the signaling contribution to IXA4-induced cross-presentation, we next combined IXA4 treatment with pharmacological inhibitors of UPR during OVA loading. The IRE1 RNase inhibitor 4μ8C substantially reduced B3Z activation (**Fig. 2A**). In contrast, ISRIB, which promotes eukaryotic initiation factor 2B (eIF2B) activity and counteracts the integrated stress response downstream of eukaryotic initiation factor 2α (eIF2α) phosphorylation, had no impact on OVA cross-presentation in response to IXA4. Likewise, treatment of IRM-4 cells with AEBSF, a serine protease inhibitor that can prevent ATF6 cleavage, did not produce a comparable reduction under the conditions examined [25–27]. This pattern supports an IRE1 RNase-sensitive component of the response.

We next assessed surface H2-K^b to determine whether the enhanced reporter activation was accompanied by greater MHC class I availability. IXA4-treated MSCs showed indeed a modest but significant increase in surface H2-K^b fluorescence relative to untreated controls (**Fig. 2B-C**). To test whether increased antigen capture or bulk degradation could account for the

functional response, we measured OVA-Alexa Fluor 647 and DQ-OVA fluorescence respectively. Neither treatment showed an IXA4-associated increase (**Fig. 2D-E**). To examine whether the phenotype was accompanied by an increase in the oxidant-sensitive signals measured here, we analyzed MitoSOX and DHE fluorescence. Neither probe showed an evident increase after IXA4 treatment (**Fig. 2F-G**).

Because conditioning could affect MSC-mediated immune communication beyond antigen display, we also profiled cytokines and chemokines in conditioned medium. IXA4 altered the relative composition of the measured soluble mediators (**Fig. 2H**). The cytokine profile shifted toward interleukin-6 (IL-6) and macrophage colony-stimulating factor (M-CSF), with smaller relative contributions from granulocyte colony-stimulating factor (G-CSF) and granulocyte–macrophage colony-stimulating factor (GM-CSF). The chemokine profile showed a larger CXCL10/IP-10 fraction and a smaller CCL5/RANTES fraction, while CCL2/MCP-1 and CXCL1/KC remained prominent. Collectively, IXA4-associated cross-presentation is sensitive to IRE1 RNase inhibition and accompanies increased surface MHC class I and altered soluble mediator composition, without a detectable increase in the bulk antigen-handling or oxidant-sensitive readouts examined.

IXA4-responsive MSCs induce ER quality-control and vesicular-trafficking transcriptional programs

To identify cellular programs that could connect IXA4 conditioning to enhanced antigen cross-presentation, we profiled transcriptional changes after 48 h of treatment in responsive MSCs. Enrichment analysis identified biological processes associated with Golgi vesicular transport, ER-to-Golgi transport, ER stress responses, protein folding, ER-associated degradation (ERAD), and antigen processing and presentation (**Fig. 3A**). These findings directed attention

399 to intracellular handling and transport rather than increased bulk antigen uptake alone. To
400 determine whether the enriched categories reflected distinct gene sets or shared components,
401 we next examined the corresponding gene-pathway map. Substantial overlap was evident
402 among the proteostasis, trafficking, and antigen-presentation categories (**Fig. 3B**), indicating
403 that the enrichments represent interconnected functions rather than wholly independent
404 pathways. We then examined individual transcript patterns to identify candidate components
405 underlying this coordinated response. The expression heatmap showed increased expression of
406 ER chaperones and quality-control factors, including *Calr*, *Canx*, *Pdia3*, *Derl1*, *Edem1*,
407 *Edem2*, *Ube2g2*, *Ube2j2*, and *Vcp* (**Fig. 3C**). Trafficking-associated genes included *Sec23b* and
408 the *Sec24* paralogs, together with *Rab1a*, *Rab6a*, *Rab6b*, *Rab32*, *Bet1*, *Stx6*, and multiple *Tmed*
409 family members. Endocytic and sorting components, including *Ap2a2*, *Ap2m1*, *Epn2*, *M6pr*,
410 *Chmp6*, and *Tsg101*, extended the signature beyond ER protein folding alone. These patterns
411 link IXA4 responsiveness to coordinated proteostasis and vesicular-trafficking programs,
412 identifying candidate processes for enhanced peptide-MHC class I display without establishing
413 a specific antigen-processing route [13, 28].
414
415 **Tumor lysate-pulsed IRM-4 cells delay melanoma progression, with the most sustained**
416 **benefit alongside PD-1 blockade**
417 To determine whether the *in vitro* conditioning approach supported therapeutic activity with
418 tumor-derived antigens, we loaded MSCs with B16F0 lysate in the presence or absence of
419 IXA4 and administered the cells after SC tumor implantation (**Fig. 4A**). The treatment groups
420 included lysate-pulsed control MSCs, lysate-pulsed IRM-4 cells, anti-PD-1 alone, and
421 combinations of either cellular product with anti-PD-1, together with a tumor-only control.
422 This design allowed assessment of IXA4 conditioning within the cellular vaccine and
423 comparison of cellular treatment alone with treatment alongside checkpoint blockade. We first

evaluated vaccines prepared with 0.05 mg/mL tumor lysate in female mice. IRM-4 treatment (blue line) delayed tumor growth and prolonged time to the reported survival endpoint relative to the tumor-only group. The IRM-4 plus anti-PD-1 group (red line) showed the most sustained response in this cohort (**Fig. 4B-C**). To examine the effect of antigen-loading concentration, a second female cohort received cells prepared with a tenfold higher lysate concentration of 0.5 mg/mL. The combination group again showed the most durable tumor-control and endpoint-survival pattern among the displayed treatments (**Fig. 4D-E**). Increasing lysate concentration did not produce a clear improvement in the IRM-4 monotherapy across these separate cohorts but had a substantial effect on the combinatory therapy. To assess whether the treatment pattern extended beyond the female cohorts, we evaluated the 0.5 mg/mL formulation in male mice. Combination treatment again delayed tumor progression and produced a favorable endpoint-survival pattern (**Fig. 4F-G**). Overall, tumor lysate-pulsed IRM-4 cells showed therapeutic activity in the B16F0 model, with the most sustained benefit observed when vaccination was administered alongside PD-1 blockade.

MSC preparations differ in IXA4 responsiveness and baseline UPR-associated expression.

To assess the reproducibility of IXA4 conditioning across independently prepared MSC populations, we compared OVA-dependent B3Z responses in eight different MSC preparations where responses varied substantially. In this experiment, both S4 and S5 triggered efficient B3Z activation whereas populations S1, S2, S3, and S6 generated a partial or moderate response followed by an inexistant response using the S7 and S8 populations (**Fig. 5A**). This variation prompted us to examine whether functional responsiveness was associated with broader differences in cellular state. PCA of the 1000 most variable genes was used to determine whether transcriptional differences aligned with the functional response classes. The

449 preparations separated in a manner associated with these classes, with PC1 and PC2 accounting
450 for 48.8% and 27.0% of the variance, respectively (**Fig. 5B**). To distinguish differences present
451 before treatment from those induced by IXA4, we compared baseline and treated
452 transcriptomes in responder preparations S4/S5 and non-responder preparations S7/S8 using
453 GSEA and the MSigDB hallmark collection (**Fig. 5C**) [21, 22]. IXA4 treatment in responders
454 was associated with positive enrichment of cell-cycle, metabolic, and stress-response
455 programs, including MYC/E2F targets, glycolysis, oxidative phosphorylation, and the UPR.
456 Several programs, including MYC/E2F targets and the UPR, showed the opposite treatment-
457 associated direction in non-responders. The baseline comparison was also consistent with
458 greater expression of selected stress- and UPR-associated programs in non-responsive
459 preparations. To inspect these differences at the level of individual transcripts, we next
460 examined an ER-stress-associated gene set that included *Xbp1*, *Hspa5*, and *Manf* (**Fig. 5D**).
461 *Manf* expression was relatively high at baseline in non-responders and decreased after IXA4
462 treatment, whereas responders showed the converse treatment-associated pattern. Previous
463 studies show that XBP1 can regulate transcription of mesencephalic astrocyte-derived
464 neurotrophic factor (MANF) and that MANF can interact with IRE1α and modulate its
465 signaling in other cell types [29, 30]. Thus, our observations suggest MANF as a possible
466 candidate component of feedback regulation. Together, these data identify the baseline UPR-
467 associated state and its response to conditioning as correlates of MSC cross-presentation,
468 demonstrating that IXA4 responsiveness is not uniform across preparations.
469
470 **Xbp1 perturbation uncouples IXA4-induced cross-presentation from XBP1s abundance.**
471 To determine how UPR state relates to IXA4-induced cross-presentation, we first measured
472 Atf4, Xbp1s/Xbp1, and Atf6 expression in control MSCs after OVA loading, IXA4 treatment,
473 or combined IXA4/OVA exposure (**Fig. 6A**). The Xbp1s/Xbp1 readout increased after IXA4

474 treatment and was highest in the combined IXA4/OVA condition, as opposed to a modest
475 change for Atf4 and Atf6. Thus, under the conditions used for the cross-presentation assay,
476 IXA4 preferentially increased the XBP1-splicing-associated readout rather than producing
477 equivalent changes across all measured UPR markers. We next asked whether basal UPR-
478 associated expression distinguished cells that were functionally responsive to IXA4. The
479 responsive S4 preparation showed lower baseline Atf4, Xbp1s/Xbp1, and Atf6 readouts than
480 the non-responsive S8 preparation and the S4-derived XBP1s-overexpressing population (**Fig.**
481 **6B**). Functional testing of the engineered populations further separated cross-presentation from
482 XBP1s abundance. More specifically, Xbp1-edited MSCs (MSC-sXBP1^{mut}) retained OVA-
483 dependent B3Z activation and displayed a larger IXA4-associated reporter response than
484 parental control MSCs (**Fig. 6C**). In contrast, enforced XBP1s expression did not reproduce
485 the IXA4-induced phenotype and was associated with a reduced OVA/IXA4 reporter response
486 relative to parental MSCs (**Fig. 6D**). SIINFEKL-pulsed cells remained strongly stimulatory in
487 both engineered populations, indicating that the divergent responses to intact OVA were not
488 accompanied by a general loss of peptide presentation to the B3Z reporter. These differences
489 in cross-presentation could not however be explained by changes in the total surface H2-K^{b} as
490 flow-cytometric profiles showed comparable H2-K^{b} in control, MSC-sXBP1^{mut}, and XBP1s-
491 overexpressing MSCs, with IXA4-associated increases in the indicated populations (**Fig. 6E-**
492 **F**). Importantly, XBP1s-overexpressing MSCs retained substantial surface H2-K^{b} despite their
493 reduced OVA-dependent B3Z response. These findings argue that total surface MHC class I
494 abundance is insufficient to account for the functional differences and instead implicate events
495 upstream of, or more specific than, bulk H2-K^{b} display, such as antigen processing, peptide
496 loading, or trafficking.
497

We then characterized the transcriptional states produced by MSC-sXBP1^{mut} and XBP1s overexpression (**Fig. 7A-B**). XBP1s-overexpressing MSCs showed a high-output UPR-associated profile, including increased Atf4, Atf6, Dnajb9, and Manf. In contrast, MSC-sXBP1^{mut} cells showed relatively low Dnajb9 and Manf expression despite an elevated Xbp1s/Xbp1 qPCR readout. This discordance indicates that the increase expression of the edited non-functional form of XBP1s is uncapable of transcriptional activity. Finally, we examined expression of *Scara3*, *Col6a*, and *Bloc1s1*, three transcripts selected as candidate RIDD-associated readouts (**Fig. 7C**). Their expression was lower in the MSC-sXBP1^{mut} population, with additional IXA4-associated decreases in several comparisons, whereas control and XBP1s-overexpressing cells showed different response patterns. These results are consistent with altered IRE1-associated RNA regulation in the edited state. Overall, these perturbation experiments show that the IXA4-associated cross-presentation phenotype does not increase monotonically with XBP1s abundance. In other words, MSC-sXBP1^{mut} cells retained a strong functional response, whereas XBP1s overexpression generated a high-UPR transcriptional state without reproducing efficient OVA cross-presentation. Together with preserved SIINFEKL responses and surface H2-K^{b}, the data support a model in which the configuration of IRE1/UPR signaling, rather than XBP1s abundance alone, constrains the cross-presenting state.

DISCUSSION

This study identifies IXA4 conditioning as an approach to induce functional presentation of exogenous OVA by murine MSCs and to generate a tumor lysate-loaded cellular product with antitumor activity in the B16F0 model. The central finding is not that MSCs become equivalent to conventional DCs, but that a defined pharmacological intervention changes their antigen-presentation phenotype in association with ER proteostasis and trafficking programs. Variation among MSC preparations and the divergent outcomes of *Xbp1* perturbations further show that the response cannot be reduced to a model in which more XBP1s necessarily produces more cross-presentation (**graphical abstract**).

The functional and transcriptional observations support a connection between intracellular proteostasis and antigen display. IXA4 increased surface H2-K^{b} and OVA-dependent B3Z activation without an obvious increase in OVA uptake or processing. This pattern is compatible with altered antigen routing, peptide loading, or peptide-MHC trafficking rather than increased bulk antigen capture. The enrichment of ER chaperones, ERAD-associated factors, and vesicular-transport components provides plausible molecular candidates, consistent with established XBP1s-dependent proteostasis programs [13, 28]. The redox findings likewise require a compartment-specific interpretation. MitoSOX and DHE showed no detectable treatment-associated increase in the assays presented, but these probes do not exclude ER-localized redox changes. A recent report showed that IXA4 can inhibit the ER transporter solute carrier family 33 member 1 (SLC33A1), which promotes ER hyperoxidation and activation of adaptive IRE1/XBP1s signaling [31]. It thus reinforces the distinction between pharmacological activation of an IRE1-associated pathway and direct binding to IRE1α and may not suggest the complete absence of ROS-independent mechanism.

MSC heterogeneity is another important biological and practical feature of the study. Non-responsive preparations had a different baseline transcriptional state and responded differently to IXA4. Rather than viewing this variation solely as a technical obstacle, the results suggest that the starting state constrains pharmacological conditioning. Enforced XBP1s expression was associated with poor OVA-dependent cross-presentation despite retained SIINFEKL responses and surface H2-K^{b}, whereas the edited population retained a substantial IXA4-associated response. Although the higher *Xbp1s*/*Xbp1* transcript alongside low *Dnajb9* and *Manf* expression in MSC-XBP1^{mut} cells may result from the expression of a non-functional XBP1 gene product, it also indicates that activation of XBP1 within a specific threshold may help coordinate the IRE1α response to avoid RIDD as observed in the MSC-XBP1s population. Consistent with this idea, XBP1 loss in DCs can alter IRE1 RNase output and antigen-presenting function, whereas tumor-associated ER stress can impair immunity through a different cellular program [15, 17]. MANF is relevant here because previous work links its expression to XBP1 activity and shows that it can regulate IRE1α signaling [29, 30]. The *Manf* patterns observed in our studies are compatible with feedback-associated states, but a causal chain from MANF to altered IRE1 output and loss of MSC cross-presentation may require additional testing.

CONCLUSIONS

IXA4 conditioning induces functional cross-presentation of exogenous antigen in responsive murine MSCs and supports tumor lysate-based vaccination in the B16F0 melanoma model, with the most sustained therapeutic benefit observed alongside PD-1 blockade. The response is associated with ER proteostasis and trafficking programs and is constrained by the starting UPR-related cellular state. These findings support further development of IRM-4 as an experimental cellular vaccine while emphasizing the need for functional potency testing,

validated genetic models, and direct resolution of the mechanisms linking conditioning to antigen presentation and antitumor immunity.

LIST OF ABBREVIATIONS

α-MEM: α-minimum essential medium

AEBSF: 4-(2-aminoethyl)benzenesulfonyl fluoride

ANOVA: Analysis of variance

ATF4/ATF6: Activating transcription factor 4/6

BiP: Binding immunoglobulin protein

cDC1: Type 1 conventional dendritic cell

CRISPR: Clustered regularly interspaced short palindromic repeats

DC: Dendritic cell

DHE: Dihydroethidium

DMSO: Dimethyl sulfoxide

eIF2α/eIF2B: Eukaryotic initiation factor 2α/2B

ER: Endoplasmic reticulum

ERAD: ER-associated degradation

FBS: Fetal bovine serum

G-CSF: Granulocyte colony-stimulating factor

GM-CSF: Granulocyte–macrophage colony-stimulating factor

GSEA: Gene set enrichment analysis

IL-6: Interleukin-6

IRE1α: Inositol-requiring enzyme 1α

IRM-4: IXA4-reprogrammed mesenchymal stromal cells

ISRIB: Integrated stress response inhibitor

M-CSF: Macrophage colony-stimulating factor

MANF: Mesencephalic astrocyte-derived neurotrophic factor

MFI: Mean fluorescence intensity; exact summary statistic to confirm

MHC: Major histocompatibility complex

MSC: Mesenchymal stromal cell

MSigDB: Molecular Signatures Database

NES: Normalized enrichment score

OVA: Ovalbumin

PBS: Phosphate-buffered saline

PCA: Principal component analysis

PD-1: Programmed cell death protein 1

PERK: Protein kinase R-like ER kinase

qPCR: Quantitative polymerase chain reaction

RIDD: Regulated IRE1-dependent decay

RNP: Ribonucleoprotein

ROS: Reactive oxygen species

RRID: Research Resource Identifier

SC: Subcutaneously

SD: Standard deviation

SLC33A1: Solute carrier family 33 member 1

UPR: Unfolded protein response

XBP1s: Spliced X-box-binding protein

Declarations

Ethics approval and consent to participate

Animal procedures were approved in September 2026 by the Université de Montréal animal care committee for the project *Development of new therapies for modulation of the immune system* (Protocol #25-026). No human participants, human material, or human data were included. Thus, consent to participate is not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Materials and reagents are available from the corresponding author, Moutih Rafei, upon reasonable request. The RNA-seq datasets generated in this study will be deposited in the Gene Expression Omnibus (GEO Submission (GSE350162) - NCBI tracking system #26102930).

AI usage disclosure

ChatGPT (OpenAI) was used to assist with manuscript organization, literature synthesis, and preparation of preliminary draft language. All scientific interpretations, descriptions of experimental methods and results, source data, statistical analyses, references, and final manuscript wording were independently reviewed and verified by the authors, who take full responsibility for the submitted work. No experimental data were generated or altered by the AI system.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JPB. performed most *in vitro* and *in vivo* experiments. YA-B, ML, JA, RF, PM, and AC contributed to *in vitro* experiments, data analysis, and schematic preparation. ST contributed to study design. NE-H performed transcriptomic analyses and contributed to data interpretation. MR conceived and supervised the study, analyzed the data, and prepared the original manuscript draft. All authors contributed to manuscript editing.

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FIGURE LEGENDS

Fig. 1. IXA4 induces OVA cross-presentation at a tolerated working concentration. A) Schematic of IRE1α-associated *Xbp1* splicing and downstream ER programs, with the IXA compounds tested. The schematic does not establish direct binding to IRE1α. **B**) MSC counts at baseline and after 48 h of exposure to 1-20 μM of IXA1, IXA4, or IXA6. DMSO was the vehicle control. **C**) Representative Annexin-V profiles after compound exposure. **D**) Representative CD44, CD45, CD73, and CD105 profiles in control and IXA4-treated MSCs, with isotype controls. **E**) B3Z responses after co-culture with MSCs exposed to OVA and the indicated IXA compound at 5 μM. SIINFEKL was a peptide-presentation control. **F**) IXA4 titration (5-15 μM), with A1 (50 μM) as a positive conditioning control. **G**) B3Z responses at the indicated 24-, 48-, and 72-h intervals after IXA4 removal. **H-I**) Responses to MK-28 (50-150 μM) and BIX (5-15 μM), respectively. Reporter absorbance was measured at 570 nm. The reported summary represents the mean ± SD with *p<0.05, **p<0.01, or ***p<0.001. Figure 1A was created in BioRender. Farah, R. (2026) https://BioRender.com/clgodp4

Fig. 2. IRE1 RNase-sensitive cross-presentation accompanies altered MHC class I display and soluble mediator composition. **A**) B3Z responses to OVA-loaded MSCs treated with IXA4 alone or with 4μ8C, ISRIB, or AEBSF. Reporter-only, MSC, OVA, and SIINFEKL conditions provide the indicated controls. **B**) Representative surface H2-K^b profiles in untreated (NT) and IXA4-treated MSCs. **C**) H2-K^b fluorescence quantification. Four measurements are displayed per condition. **D-E**) Representative cell-associated OVA-Alexa Fluor 647 and DQ-OVA fluorescence, respectively. **F-G**) Representative MitoSOX and DHE profiles. **H**) Relative cytokine and chemokine composition of concentrated conditioned medium. The statistics for the reported summary are *p<0.05, **p<0.01, or ***p<0.001.

Fig. 3. IXA4-responsive MSCs induce proteostasis and vesicular-trafficking transcriptional programs. A) Enrichment of selected Gene Ontology biological processes and Reactome-associated pathways in IXA4-responsive MSCs relative to controls, grouped by functional category. The gene ratio is described as the number of contributing genes divided by the size of the term. Point size denotes gene counts and color denotes $-\log_{10}$(adjusted p). **B**) Gene-pathway associations for selected upregulated genes. Rows represent pathways and columns represent genes; the displayed size scale is labeled $-\log_{10}$(adjusted p). **C**) Relative expression of selected genes associated with antigen presentation, trafficking, protein degradation, ER stress/protein folding, and protein transport. Columns show control and IXA4-treated samples.

Fig. 4. Tumor lysate-pulsed IRM-4 vaccination delays B16F0 progression alongside PD-1 blockade. **A**) Treatment schematic for B16F0 implantation. Two MSC vaccine administrations, and six antibody doses were administered. The doses are 5×10^5 for tumor cells, and 5×10^5 for IRM-4 cells per administration, and 200 μg for the anti-PD-1 per dose. **B-C**) Tumor volume trajectories and Kaplan-Meier curves for time to the reported humane endpoint in female mice receiving MSCs loaded with 0.05 mg/mL tumor lysate. **D-E**) Corresponding results in female mice using 0.5 mg/mL lysate. **F-G**) Corresponding results in male mice using 0.5 mg/mL lysate. Groups included tumor only, anti-PD-1 alone, lysate-pulsed control MSCs, IRM-4 cells, and each cellular product with anti-PD-1. Five mice per group were used for each vaccination study with *$p<0.05$, **$p<0.01$, or ***$p<0.001$. Figure 4A was created in BioRender. Farah, R. (2026) https://BioRender.com/achwpsn

Fig. 5. MSC response heterogeneity is associated with baseline and treatment-dependent transcriptional states. **A**) B3Z responses for eight MSC preparations (S1-S8) under the indicated control, SIINFEKL, OVA, or OVA/IXA4 conditions. Based on these results, the S4 and S5 populations were designated as the responders, S1/S2/S3/S6 as partial responders, and S7/S8 as non-responders. **B**) PCA using the 1000 most variable genes. PC1 and PC2 account for 48.8% and 27.0% of the variance, respectively. **C**) Hallmark enrichment across three contrasts, from left to right: responders versus non-responders at baseline; IXA4-treated versus control non-responders; and IXA4-treated versus control responders. Point size denotes $-\log_{10}$(adjusted p) and color denotes normalized enrichment score (NES). **D**) Heatmap of the gene set designated PERK_ATF4_REGULATES_ER_STRESS_RESPONSE, which includes genes associated with other UPR outputs. Columns identify treatment and response class. The reported reporter-assay summary is represented as mean ± SD with *p<0.05, **p<0.01, or ***p<0.001.

Fig. 6. XBP1 perturbation uncouples cross-presentation from XBP1s. A) Relative *Atf4*, *Xbp1s/Xbp1*, and *Atf6* transcript expression measured by qPCR in untreated MSCs (Ctl), OVA, IXA4, or combined IXA4/OVA conditions. **B**) Baseline qPCR comparison of the same UPR-associated readouts in the S4 responsive MSC preparation, the S8 non-responsive MSC preparation, and XBP1s-overexpressing MSCs (generated using the S4 population). **C**) B3Z reporter responses following co-culture with Ctl MSCs or MSC-XBP1^{mut} under the indicated B3Z, MSC, SIINFEKL, OVA, and IXA4 conditions. **D**) Corresponding B3Z responses from Ctl MSCs or MSC-XBP1s. **E**) Representative flow-cytometric profiles of surface H2-K^{b} in Ctl MSCs, MSC-XBP1^{mut}, and MSC-XBP1s with or without IXA4. The histograms denote isotype controls. **F**) Quantification of cell surface H2-K^{b} for the indicated populations and treatments. Individual points represent individual measurements. Panels A and B have an n=5

measurements per group. For panels C and D, we have n=3 per condition, and for panel F we have n=4 per group. Quantitative data are presented as mean ± SD. Group comparisons were analyzed by one-way ANOVA as specified in the manuscript with *p<0.05, **p<0.01, or ***p<0.001.

Fig. 7. XBP1 state alters UPR and RIDD-associated transcripts. A) Relative *Atf4*, *Xbp1s/Xbp1*, and *Atf6* transcript expression measured by qPCR in Ctl, MSC-XBP1mut, and MSC-XBP1s MSCs with or without IXA4. **B**) Relative expression of the UPR/XBP1-associated transcripts *Dnajb9* and *Manf* in the same populations and treatment conditions. **C**) Relative expression of the RIDD-associated transcripts *Scara3*, *Col6a*, and *Bloc1s1*. Individual points represent the plotted measurements (n=4 per group in the displayed data), and bars represent the mean ± SD. Group comparisons were analyzed by one-way ANOVA as specified in the manuscript with *p<0.05, **p<0.01, or ***p<0.001.

Proposed state dependence of IXA4-induced MSC cross-presentation.

IXA4 conditioning promotes antigen cross-presentation in responsive MSCs through an IRE1α-associated program whose functional outcome is determined by the underlying UPR state rather than by XBP1s abundance alone. XBP1s overexpression fails to reproduce the cross-presenting phenotype, whereas Xbp1-edited MSCs retain enhanced IXA4-associated activity, supporting a state-dependent activation window in which MANF feedback and alternative IRE1 RNase outputs remain candidate regulatory mechanisms.

Supplementary Files

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