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COMMENT

Characterization of a novel human BFL-1-specific monoclonal antibody

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BFL-1 is the least studied pro-survival BCL-2 family member, partly due to the lack of high-quality tools, such as antibodies that can detect this protein at endogenous levels. *BFL-1* mRNA expression may not mirror BFL-1 levels due to post-translational regulation of this protein [1, 2]. Hence, there is a requirement for a reliable antibody to detect endogenous BFL-1 in cells.

To generate BFL-1-specific monoclonal antibodies, we utilized mice lacking A1 (the mouse homologue of BFL-1) [3, 4]. We hypothesized that $A1^{-/-}$ mice would generate a better immune response against BFL-1 as their immune cells have not been tolerized to the highly homologous mouse A1 protein. Hence, we immunised the mice with a truncated recombinant BFL-1 protein (aa1-151, lacking the C terminal region) and two KLH-conjugated peptides corresponding to the BFL-1 protein regions aa71–84 and aa129–154. One BFL-1 reactive monoclonal antibody identified in an ELISA screen (data not shown) was validated by Western blot analysis of lysates from HEK293T cells overexpressing FLAG-tagged BFL-1 protein [5] (Supplementary Fig. 1). To verify the specificity of the BFL-1 antibody, we examined BFL-1 protein stability in M14 (previously shown to express BFL-1 protein [6]) and SK-MEL30 melanoma cells. As expected, the protein synthesis inhibitor cyclohexamide (CHX) decreased and the proteasome inhibitor (MG132) increased the intensity of the BFL-1 protein band (Supplementary Fig. 2). To test the cross-reactivity of the BFL-1-specific antibody for its murine counterpart A1, lysates from LPS (known to upregulate *A1* mRNA [7]) stimulated lung tissues were analysed by Western blotting. Lysates from BFL-1 CRISPR deleted [8] and parental M14 cells served as controls. This demonstrated the reactivity of the monoclonal antibody to human BFL-1 but not mouse A1 (Supplementary Fig. 3).

Importantly, the BFL-1 antibody could robustly be used for BFL-1 immunoprecipitation (Fig. 1a) and for immunofluorescence staining in M14 cells (Supplementary Fig. 4 and Fig. 1b). To validate the results in the immunofluorescence setting, 2 h treatment of M14 cells with CHX or MG132 decreased or slightly increased, respectively, the APC signal corresponding to the BFL-1 protein (Fig. 1b). Mitotracker Orange was added to the serum-free growth medium for 1 h before fixation, permeabilization, and antibody staining. Notably, the BFL-1 protein was predominantly present in the cytosol and did not co-localize with the mitochondrial staining, consistent with the reported sub-cellular localization of A1, its mouse homologue [1]. Unfortunately, the BFL-1 antibody did not yield reproducible results in flow cytometry experiments (data not shown).

To validate the applicability of the monoclonal BFL-1 antibody in primary human cells, peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors, stimulated in culture for 3 days with antibodies against CD3 and CD28 (known to increase the A1 protein expression in mouse T cells [4, 9]) and cell lysates subjected to Western

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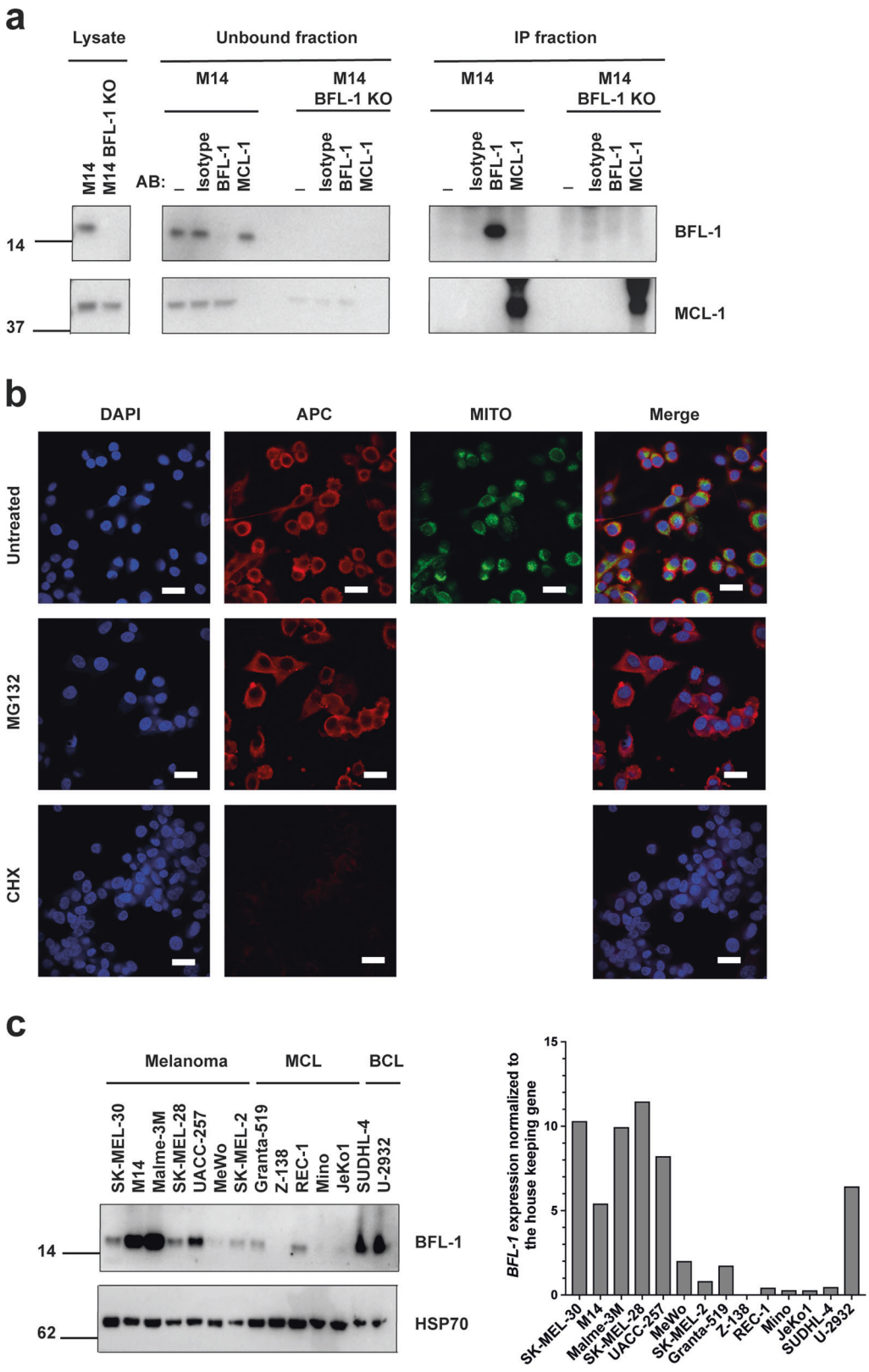

blot analysis. Importantly, we could detect a band corresponding to the correct molecular weight of BFL-1 in stimulated samples but not in the unstimulated cells (Supplementary Fig. 5). To examine whether BFL-1 protein corresponds to *BFL-1* mRNA expression, Western blot analysis and quantitative PCR analysis were performed

◀ **Fig. 1** A novel BFL-1 specific monoclonal antibody reliably detects this pro-survival BCL-2 family member, even at endogenous levels. **a** For immunoprecipitation experiments, pre-cleared melanoma cell (M14 and M14 BFL-1 KO) lysates were incubated overnight with BFL-1 antibody or an Ig isotype-matched control antibody (IgG2a/κ) and protein G-sepharose beads. Immunoprecipitation of MCL-1 was performed with a previously characterized MCL-1-specific monoclonal antibody (19C4-15 clone [10]) as a positive control for the immunoprecipitation protocol. The immunoprecipitated proteins were then subjected to electrophoresis and Western blotting. **b** M14 melanoma cells grown overnight on coverslips were treated for 2 h with cycloheximide or MG132 and stained with the primary antibody (anti-BFL-1 or Ig isotype matched control antibody at 25 μg/mL) and subsequently with anti-mouse IgG specific secondary antibodies conjugated to APC (red). Nuclei were stained with DAPI (blue) and slides mounted for confocal imaging. Mitotracker Orange was added to cells prior to fixation to stain for mitochondria. Scale bars indicate 20 μm. **c** The monoclonal antibody against BFL-1 detects BFL-1 at endogenous levels in a panel of human melanoma, mantle cell (MCL) and other B cell lymphoma (BCL) derived cell lines by Western blotting. Probing for HSP70 served as a protein loading control (left side). Total RNA was extracted from the same cell lines and converted to cDNA. RT-PCR analysis was performed using Taqman probe sets for the housekeeping gene *HMBS* and *BFL-1* with relative quantification applied using the ΔCt method (right side)

side-by-side on a human cell line panel (Fig. 1c). Since BFL-1 protein is regulated by ubiquitin-dependent proteasomal degradation [1], *BFL-1* mRNA expression data failed to fully reflect BFL-1 protein expression. The discrepancy between transcription and protein levels of BFL-1 emphasizes the importance of having a reliable BFL-1 specific antibody. Collectively, our studies describe a robust human BFL-1-specific monoclonal antibody capable of reliably detecting endogenous BFL-1 protein in cell lines and primary human cells. This antibody will enable further studies into the regulation of BFL-1 protein in homeostasis and disease.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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