Published in final edited form as:

Leukemia. 2016 January ; 30(1): 74–85. doi:10.1038/leu.2015.204.

Cytoplasmic myosin exposed apoptotic cells appear with caspase-3 activation and enhance CLL cell viability

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Abstract

The degree of chronic lymphocytic leukemia (CLL) B-cell antigen receptor (BCR) binding to myosin exposed apoptotic cells (MEACs) correlates with worse patient outcomes, suggesting a link to disease activity. Therefore, we studied MEAC formation and the effects of MEAC binding on CLL cells. In cell line studies, both intrinsic (spontaneous or camptothecin-induced) and extrinsic (FasL- or anti-Fas-induced) apoptosis created a high percent of MEACs over time in a process associated with caspase-3 activation, leading to cytoplasmic myosin cleavage and trafficking to cell membranes. The involvement of common apoptosis pathways suggests that most cells can produce MEACs and indeed CLL cells themselves form MEACs. Consistent with the idea that MEAC formation may be a signal to remove dying cells, we found that natural IgM antibodies bind to MEACs. Functionally, co-culture of MEACs with CLL cells, regardless of immunoglobulin heavy chain variable region gene mutation status, improved leukemic cell viability. Based on inhibitor studies, this improved viability involved BCR signaling molecules. These results support the hypothesis that stimulation of CLL cells with antigen, such as those on MEACs, promotes CLL cell viability, which in turn could lead to progression to worse disease.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

Supplementary information is available at Leukemia's website.

Keywords

Chronic lymphocytic leukemia; B-lymphocytes; B-cell antigen receptor; apoptosis; myosin-exposed apoptotic cells; natural IgM

INTRODUCTION

The most common Western adult leukemia is chronic lymphocytic leukemia (CLL) with an incidence of ~14 620 new cases and ~4 650 deaths in the US annually.[1] This clonal $CD5^+CD19^+$ B-lymphocyte cancer expresses a unique B-cell antigen receptor (BCR) with an immunoglobulin heavy chain variable region (IGHV) gene sequence that can be classified as unmutated (U-CLL) or mutated (M-CLL) depending on the level of somatic mutations.[2] Furthermore, the antigen binding sites of many CLL BCRs fall into virtually identical "stereotyped" amino acid sequence subgroups found in nearly one third of patients.[3] The random occurrence of these stereotyped subgroups is extraordinarily unlikely, suggesting that CLL BCRs have been selected by binding to sets of common antigenic epitopes. One possible source for these common antigens is apoptotic cells. Indeed, CLL BCRs expressed as native or recombinant monoclonal antibodies (mAbs) can recognize a variety of autoantigens,[4–10] many of which are intracellular and need to be exposed extracellularly for CLL BCRs to interact with them.

Programmed cell death, such as apoptosis, is a process whereby intracellular molecules can become surface exposed.[11–13] Indeed, we have shown that myosin appears at the cell surface during apoptosis in a subgroup of cells, termed MEACs (myosin exposed apoptotic cells).[14] Furthermore, we have found that >60% of CLL mAbs tested bind to MEACs, suggesting that MEACs could provide a source of antigens, either newly exposed intracellular antigens or neoantigens produced by oxidation of molecules, which are recognized by CLL mAbs.[4,6] MEAC binding by CLL BCRs may provide a survival and/or growth signal for the leukemic cell, consistent with our finding of MEAC binding correlating with shorter patient survival.[14] Notably, drugs that interfere with BCR signaling have shown remarkable efficacy in treatment of CLL and have recently been approved by the Federal Drug Administration (FDA).[15–18]

To further investigate the role of MEACs in CLL pathogenesis, we investigated the mechanisms of MEAC formation. Apoptosis, one of the many types of cell death mechanisms, can be divided into two major pathways termed intrinsic and extrinsic.[19] Extrinsic apoptosis is triggered by an external death receptor, such as Fas, whereas intrinsic apoptosis is triggered by intracellular stress conditions, such as DNA damage and oxidative stress. Caspase-dependent extrinsic and intrinsic apoptotic pathways require the activation of caspase-3 protease. Activation of caspase-3 leads to a number of events, including the transfer of phosphatidylserine from the inner to the outer leaflet of the cell membrane, which can be detected by AnnexinV binding, and pore formation in the outer membrane, which can be detected by cell impermeant dyes such as 7-aminoactinomycin D (7AAD). Thus, cell apoptosis can be monitored by AnnexinV and 7AAD staining.

Herein, we report that both intrinsic and extrinsic apoptotic pathways lead to formation of MEACs in a caspase-3 associated manner. In addition, because many CLL mAbs bind to MEACs, one would predict that leukemic BCRs binding MEACs would lead to growth and survival of the CLL clones from many patients.[7,14] In favor of this, we also report that co-culture of MEACs with CLL cells increases their viability, which appears to involve signaling via molecules involved in BCR signal transduction.

MATERIALS AND METHODS

Cell culture

To induce apoptosis in a human T cell line (Jurkat clone E6-1, TIB-152, American Type Culture Collection, Manassas, VA, USA), cells were cultured at high density (1×10^7 cells/ml)[14] or at 5×10^5 cells/ml in the presence of 10 μM camptothecin (MP Biomedicals, Santa Ana, CA, USA) dissolved in dimethyl sulfoxide (Thermo Fisher Scientific, Waltham, MA, USA), 100 ng/ml human Fas Ligand (FasL; R&D Systems, Minneapolis, MN, USA), or 100 ng/ml anti-Fas Ab (SY-001; MBL International, Woburn, MA, USA). Z-DEVD-FMK (DEVD; MBL International) was added at 160 μM at time of camptothecin treatment or at 40 μM one h prior to FasL treatment. 2 μM Z-YVAD-FMK (YVAD; MBL International) was added one h prior to FasL treatment. Jurkat cell cultures centrifuged in Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ, USA) density gradients resulted in cell pellets of nearly pure MEACs.

Cryopreserved CLL peripheral blood mononuclear cells,[20] collected from patients after informed consent as approved by the Institutional Review Board of North Shore University Hospital (NSUH; Manhasset, NY, USA) and Long Island Jewish Medical Center (New Hyde Park, NY, USA) and in accordance with the Helsinki Declaration, were thawed and live cells were selected by Ficoll-Paque PLUS density gradient centrifugation. CLL samples were chosen based on availability with no pre-established inclusion/exclusion criteria. Live CLL cells (2×10^6/ml) were cultured alone or with purified MEACs (3:1 to 5:1 CLL cells:MEACs) at 37°C and 5% CO_2 in complete RPMI[14] with 1.0–2.5% fetal bovine serum (FBS) with or without an inhibitor: 1 μM ibrutinib (Pharmacyclics, Sunnyvale, CA, USA), 50 μM LFM-A13 (Calbiochem, EMD Millipore, Billerica, MA, USA), 1μM idelalisib (Selleck Chemicals, Houston, TX, USA), 1 μM A66 (Selleck Chemicals), or 1 μM AG490 (Sigma, St. Louis, MO, USA). IGHV sequence of CLL leukemic clone was determined by Sanger sequencing,[21] judged to be mutated if greater than 2% nucleotide difference[2] from germline according to IMGT,[22] and attributed to a stereotype subset or not (Supplementary Tables S1–S6).[3,23]

Flow cytometry

For MEAC formation and apoptosis analysis, flow cytometry was performed as described previously[14] using 10 μg/ml rabbit anti-myosin IgG (BT-564; Biomedical Technologies, Alfa Aesar, Ward Hill, MA, USA) and 5 μg/ml fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (711-095-152; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) with 4 μl phycoerythrin (PE)-conjugated AnnexinV (AnnexinV-PE) and 4 μl 7AAD (BD Biosciences, San Jose, CA, USA). For analysis of IgM binding to

MEACs, cells were stained with 25 μg/ml normal human serum IgM (I8260; Sigma; 2 different lots), umbilical cord blood IgM (6 anonymous samples; Tissue Donation Program at The Feinstein Institute for Medical Research), or control clonal IgM (OBT1524; AbD Serotec, Raleigh, NC, USA or MM021; NSUH) and rabbit anti-myosin IgG followed by FITC-conjugated goat anti-human IgM $F(ab')_2$ (2022-02; Southern Biotech, Birmingham, AL, USA) and PE-conjugated goat anti-rabbit IgG (4050-09; Southern Biotech). Samples were collected using a LSRII (BD Biosciences) and analyzed with FlowJo (FlowJo LLC, Ashland, OR, USA).

For CLL cells after co-culture with MEACs, 50 μl ($2–3 \times 10^6$ cells/ml) were stained with 2 μl allophycocyanin-conjugated anti-CD19 (50 μg/ml mouse mAb SJ25C1; 340437; BD Biosciences) and 10 μl FITC-conjugated anti-CD3 (25 μg/ml mouse mAb UCHT1; IM1281U; Beckman Coulter, Brea, CA, USA) and then stained with 1–2 μl AnnexinV-PE and 2.5 μl 7AAD as before.[14] Cell samples were collected on a LSRII and gated on $CD19^+CD3^-$ cells to isolate CLL cells for apoptosis analysis with FlowJo.

Protein extraction and immunoblot

Cells were solubilized with a Potter-Elvehjem homogenizer (Corning, Corning, NY, USA) for 50 hand strokes and differentially centrifuged to produce cytosol and soluble membrane protein fractions.[24] Protein samples were separated by SDS-polyacrylamide gel electrophoresis[25] and electroblotted to nitrocellulose membranes (GE Healthcare Amersham, Piscataway, NJ, USA), which were probed by standard methods[7,25,26] using rabbit anti-myosin (BT-564), rabbit anti- GAPDH (polyclonal FL-335; sc-25778; Santa Cruz Biotechnology, Dallas, TX, USA) IgG, mouse monoclonal anti-CD3ε (clone APA1/1; 05-785; EMD Millipore) IgG1, or mouse monoclonal anti-PARP (clone C-2-10; AM30; EMD Millipore) IgG1, followed by horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG or sheep anti-mouse IgG Abs (NA934 or NA931; GE Healthcare) that were developed using the ECL Western Blotting Analysis System (GE Healthcare Amersham) and exposed to autoradiography film (Hyblot CL; Denville Scientific, South Plainfield, NJ, USA).

Microscopy

Cryopreserved CLL B cells ($1–2\times10^6$/ml) were thawed and labeled with 0.375 μM carboxy-fluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Life Technologies, Grand Island, NY, USA) in phosphate-buffered saline (PBS) for 10 min at 37°C. Jurkat cells undergoing spontaneous apoptosis ($1–2\times10^6$/ml) were labeled with CFSE as above or with 3 μg/ml Alexa Fluor 680 Dye (Invitrogen) in PBS for 30 min on ice. CLL B cells and Jurkat cells were mixed (1:1) and incubated at 37°C and 5% CO_2 for 3 h in complete RPMI with 10% FBS. Cell mixtures (500 μl) were incubated on 12 mm circular 0.15 mm thick cover glasses (Thermo Fisher Scientific) in 24-well tissue culture plates (BD Biosciences) at 37°C and 5% CO_2 for up to 40 h, fixed, stained with rabbit anti-myosin (1:100; BTI-567; Biomedical Technologies) +/− goat anti-CD23 (1:100; AF123; R&D Systems) Abs and Rhodamine Red-X-conjugated donkey anti-rabbit IgG (1:250; 711-295-152; Jackson ImmunoResearch Laboratories) +/− Dylight 649 conjugated donkey anti-goat IgG (1:200; 705-495-147; Jackson ImmunoResearch Laboratories), and then mounted on microscope slides as

previously described.[14] Alternatively, labeled CLL and Jurkat cell mixtures (1:1; 1 ml) were incubated in 12-well tissue culture plates (BD Biosciences) at 37°C and 5% CO_2 for 1 h in complete RPMI with 10% FBS, then stained with rabbit anti-myosin IgG (BTI-564; 25 μg/ml) and Rhodamine Red-X-conjugated donkey anti-rabbit IgG (1:200), resuspended in 5 μl Cell-Adherence Solution (Crystalgen, Commack, NY, USA) on cover glasses, and mounted on microscope slides as before.[14]

Statistics

Statistical significance was calculated using Prism 6 (GraphPad Software, San Diego, CA, USA) from adequately powered sample sizes for two-tailed tests; either Wilcoxon matched-pairs signed rank test, Mann-Whitney test, or one-way analysis of variance (ANOVA) with Tukey multiple comparison test where appropriate based on test assumptions and observed variances.

RESULTS

MEACs develop and increase over time as a consequence of both intrinsic and extrinsic apoptosis

Because MEAC binding to CLL mAbs correlated with shorter patient survival,[14] MEAC formation may play a role in CLL pathogenesis. Therefore, we investigated the mechanism of MEAC formation. Jurkat cells were induced to undergo intrinsic or extrinsic apoptosis and MEAC formation was measured by the surface membrane exposure of intracellular myosin as detected by flow cytometry. Camptothecin induces the intrinsic apoptosis pathway.[19,27] A representative time course experiment (Figure 1a) showed that camptothecin induced a large % of early apoptotic cells ($7AAD^-$ $AnnexinV^+$) after 4 h, which decreased over time as the % of late apoptotic cells ($7AAD^+$ $AnnexinV^+$) increased (Figure 1a top row). MEAC formation also increased with time and accumulated later at 16 h (Figure 1a bottom row). Note that even as late as 24 h only a portion of $AnnexinV^+$ cells were MEACs (49.1%), as evidenced by $myosin^+$ surface staining. Thus, only a subgroup of intrinsic apoptotic cells forms MEACS. As an internal control, the $AnnexinV^-$ live cells were $myosin^-$, indicating that viable cells do not display myosin and that myosin must be brought to the extracellular surface for detection. This experiment was repeated another 10 times and the results of all 11 experiments are summarized graphically (Figure 1b), showing that MEACs appeared and accumulated later in apoptosis, after the appearance of $AnnexinV^+$ cells. The % MEACs increased with time of camptothecin incubation and was an increasing fraction of total apoptotic ($AnnexinV^+$) cells with time. We next confirmed that intrinsic apoptosis induced by culturing Jurkat cells at high density, which leads to spontaneous apoptosis over several days, also leads to accumulation of MEACs with time. The results of 18 experiments are summarized in Figure 1c. The same phenomena seen with camptothecin-induced apoptosis were observed over time with spontaneous apoptosis: with both the % MEACs and fraction of MEACs in total $AnnexinV^+$ cells increasing and yet MEACs remaining a subgroup of total $AnnexinV^+$ cells.

We next tested extrinsic apoptosis induced in Jurkat cells with either FasL (N=8) or anti-Fas Ab (N=6) (Figure 1d–e). Again, we observed the same phenomena as with intrinsic

apoptosis, increasing % MEACs and fraction of MEACs in total AnnexinV$^+$ cells over time, with MEACs remaining a subgroup of total apoptotic cells at all times.

Apoptotic cells can be divided into early and late stages based on 7AAD staining. When MEAC formation was analyzed in relation to apoptotic stage, we found that both early (7AAD$^-$ AnnexinV$^+$) and late (7AAD$^+$ AnnexinV$^+$) apoptotic cells contained the MEAC subgroup. A representative example with camptothecin-induced apoptosis (Figure 1f) showed that, after 4 h incubation, 7.8% of early apoptotic cells and 20.2% of late apoptotic cells were myosin$^+$; while after 24 h incubation, 35.8% of early and 78.6% of late apoptotic cells were myosin$^+$. Similar results were seen with spontaneous, as well as FasL or anti-Fas induced apoptosis. Thus, MEACs can be found in both early and late apoptosis, with the highest fraction of MEACs existing in late apoptosis after prolonged incubation.

MEAC formation associates with caspase-3 activation

Since early apoptotic cells become myosin$^+$ and some late apoptotic cells are myosin$^-$, myosin positivity on apoptotic cells may not simply be the result of cell membrane perforation resulting in leakage of the detecting Ab inside cells. One possible explanation may be that myosin surface membrane exposition may be an active deliberate process that occurs initially during phosphatidylserine exposure and increases during cell membrane pore formation. To investigate this possibility in both intrinsic and extrinsic apoptosis, we tested if caspase-3 activation, the biochemical feature common to both these pathways, was necessary (Figure 2a). To test the intrinsic apoptosis pathway, Jurkat cells were cultured with camptothecin for 17 h, resulting in 68% apoptotic cells and 50% MEACs. The addition of a caspase-3 inhibitor, DEVD, resulted in a substantial reduction in both apoptotic cells (25%) and MEACs (16%) (Figure 2b).

To test the extrinsic apoptosis pathway, Jurkat cells were cultured with FasL for 17 h with or without DEVD. The addition of DEVD substantially reduced apoptosis from 74% to 14%, and MEAC formation from 57% to 10% (Figure 2c).

In contrast, the addition of a caspase-1 inhibitor (YVAD) to FasL-treated Jurkat cells had no inhibitory effect on ether apoptosis or MEAC formation (Figure 2d). Thus, these data support the hypothesis that MEAC formation may be a programmed apoptotic process associated with caspase-3 activation.

Myosin cleavage and membrane localization during MEAC formation

During apoptosis, myosin is cleaved into at least two large fragments of approximately 125 and 95 kDa in size.[28] To test if myosin cleavage also occurs during MEAC formation and furthermore if myosin cleavage products are mobilized into the cell membrane, we analyzed proteins from the cytosol and membrane fractions of Jurkat cells treated with or without camptothecin were analyzed on Western blots with anti-myosin Abs (Figure 2e). In untreated cells, the full-length myosin protein (250 kDa) is largely detected in the cytosol fraction, with very little if any found in the membrane fraction. After camptothecin treatment, the amount of full-length myosin decreases in the cytosol, with the majority of myosin appearing in the membrane fraction detected as two large fragments of 149 and 94 kDa. The same result is observed with FasL-induced apoptosis (Figure 2f). Moreover, we

tested if FasL-induced cleavage of myosin and migration into the cell membrane was associated with caspase-3 activation by incubation of these cultures with DEVD (Figure 2f). The addition of DEVD prevented the appearance of myosin cleavage products and migration into the membrane. As a control, FasL-induced PARP cleavage was also inhibited by DEVD as expected. So both intrinsic and extrinsic apoptosis lead to formation of MEACs via caspase-3 associated cleavage of myosin, with migration of cleaved products into the cell membrane (Figure 2a).

CLL cells undergo apoptosis and form MEACs

Because intrinsic and extrinsic apoptosis lead to the production of MEACs, one would expect that most cell types could form MEACs and potentially provide antigens to interact with CLL BCRs. To test if CLL cells themselves form MEACs, viable CLL cells were cultured, harvested on four consecutive days, and analyzed for apoptosis and myosin exposure by flow cytometry. Results from cultures with cells from two CLL patients, CLL321 and CLL693 representative of U-CLL and M-CLL patients, respectively, showed that CLL cells underwent apoptosis (AnnexinV$^+$) in a routine culture environment, which increased over time (Figure 3a). Similarly, MEAC (AnnexinV$^+$, myosin$^+$) formation increased over time. The production of MEACs by CLL cells was confirmed by confocal microscopy by co-staining cells from CLL693 with CFSE and anti-myosin or with anti-CD23 and anti-myosin (Figure 3b–c). Thus, CLL cells, either from U-CLL or M-CLL patients, form MEACs during apoptosis, and hence can potentially provide autoantigenic material to stimulate live CLL cells. Results of further testing of this are reported below.

Natural IgM antibodies bind MEACs

Since MEAC formation occurs via major apoptotic pathways, this type of programmed cell death may serve a specific beneficial purpose, such as marking dying cells for clearance. Natural Abs, which appear early in life and without immunization, may function in the removal of apoptotic cells.[29] Because natural Abs comprise nearly the entire IgM repertoire at birth,[29,30] we examined cord blood IgM for reactivity with MEACs from Jurkat cells undergoing spontaneous apoptosis. Cord blood IgM bound MEACs at levels over that of control secondary Ab alone and the negative control clonal IgM OBT1524 (Figure 4). The level of MEAC binding (mean fluorescence intensity ratio (MFIR) = 12.46) was >2-fold higher than the positive control clonal IgM MM021 (Figure 4) and was also higher than the MFIR levels previously observed with human serum IgG Abs (MFIR = 3.38 – 7.93).[14] Normal human adult serum IgM, which still contains a substantial fraction of natural Abs despite the inclusion of IgM Abs derived from the adaptive immune response,[29,30] also bound MEACs (MFIR=4.95) at levels similar to the positive control but >2-fold lower than that observed for cord blood IgM (Figure 4). Finally, natural IgM did not bind myosin$^-$ cells, showing that these Abs are specific for the MEAC subset of apoptotic cells. Thus, the data support the idea that MEAC formation is a specific apoptotic mechanism that marks a subset of apoptosing cells, possibly for clearance, and that natural IgM binds these cells via a genetically encoded immunoreactivty.

MEACs may associate with CLL cells and prevent their apoptosis

Because MEACs that derive from any cell type, including CLL cells themselves, are commonly formed during apoptosis, they could interact with CLL cells in vivo, especially in solid lymphoid tissues where these cells reside in large numbers. To test if CLL cells interact with MEACs, we examined the proximity of CLL cells and MEACs in co-culture by confocal microscopy. Using two different labeling methods, CLL cells could be found to contact MEACs (Figure 5a–b). To test if CLL cells were functionally affected by MEACs, the level of spontaneous CLL cell apoptosis was examined by flow cytometry after co-culture with MEACs. CLL cell apoptosis (AnnexinV^{+}) decreased after MEAC co-culture in most experiments. Results with 3 CLL patient cells are shown in Figure 5c illustrating the decrease in overall apoptosis from 53.3, 38.6 and 12.63% to 11.81, 22.25 and 7.71% for CLL1239, CLL851 and CLL827, respectively. This MEAC-induced decrease in CLL cell apoptosis and the reciprocal increase in CLL cell viability could was prevented by fixation (Supplemental Figure S1), suggesting that fixation-sensitive antigens may be involved and this MEAC-induced effect is not simply due to non-specific cell density interactions. Next, cells from a large CLL patient cohort (N=76) were tested for the effect of MEAC co-culture on spontaneous CLL cell apoptosis (Figure 6a, Supplementary Table S1). This led to a statistically significant increase in CLL cell viability after co-culture with MEACs (P<0.0001). Furthermore, this was the case regardless of the IGHV mutation status of the clone and the presence or absence of stereotopy. U-CLL and M-CLL cells had similar increases in viability after co-culture with MEACs (median % increase 10.19 and 7.11, respectively, P = 0.4856, Mann-Whitney test). The 15 CLL patient samples exhibiting stereotyped CLL BCR showed variable MEACs co-culture responsiveness, with increases in viability ranging from 2.32 to 85.53 % (Supplementary Table S1).

MEACs effect on CLL cells is reversed by BCR signaling inhibitors

To test if the effect of MEACs on CLL cell viability was dependent on cell signaling, Bruton tyrosine kinase (BTK) inhibitors (ibrutinib or LFM-A13; N=23 and 18, respectively) were added to these co-cultures. Ibrutinib is an irreversible inhibitor that covalently binds to Cys481 in the ATP-binding domain of human BTK, a key molecule in BCR signaling,[31] which was recently approved by the FDA for treatments of relapsed refractory CLL and 17p^{-} CLL.[15–17] LFM-A13 is a reversible inhibitor that competitively binds to the ATP-binding domain of BTK at a ~20-fold lower binding affinity than ibrutinib and is currently not used in a clinical setting.[32] Due to its lower binding affinity, a 50-fold higher concentration of LFM-A13 was needed to achieve results comparable to ibrutinib at 1 μM. At these concentrations, both ibrutinib (Figure 6b) and LFM-A13 (Figure 6c) significantly inhibited the MEAC-related co-culture increase in CLL cell viability (P<0.0001). This inhibition was similar for both U-CLL and M-CLL cells treated with ibrutinib (median % inhibition 217.0 and 133.8, respectively) or LFM-A13 (median % inhibition 54.2 and 71.8, respectively). The CLL patient samples exhibiting stereotyped CLL BCRs showed wide variation in inhibition by ibrutinib (88.24 to 577.42 %; Supplementary Table S2) or LFM-A13 (−37.75 to 301.62 %; Supplementary Table S3). LFM-A13 and ibrutinib affected these cultures similarly, because patient cells treated with both inhibitors (CLL1019, CLL1159, CLL1161, CLL1200, CLL1282, CLL1312, CLL1325, CLL1326, CLL1496, and CLL1649)

tended to have similar % decreases in viable cells (Supplementary Tables S2 and S3). Thus, BTK inhibition prevents MEAC promotion of CLL cell viability.

To further confirm that molecules involved in BCR signaling may be important for this effect, we tested idelalisib, a reversible inhibitor of the p110δ catalytic subunit isoform of phosphotidylinositol 3-kinase (PI3K).[33] PI3Kδ is the major expressed PI3K catalytic subunit in CLL and is the dominant isoform involved in BCR signaling.[34] Idelalisib was recently approved by the FDA to treat relapsed refractory CLL in combination with rituximab.[18] In our co-culture experiments, idelalisib significantly inhibited the MEAC-induced increase in CLL cell viability (Figure 6d, $P = 0.0138$, Supplementary Table S4). As controls, we tested A66, an inhibitor of the alpha isoform of the p110 subunit of PI3K,[35] and AG490, an inhibitor of Janus kinases (JAKs).[36] Although PI3Kα is ubiquitously expressed, its effects on BCR signaling are much less than that of PI3Kδ, which is expressed predominantly in lymphocytes.[37] JAKs are intracellular tyrosine kinases required for cytokine receptors signaling and are not directly involved in BCR signaling.[38] A66 and AG490 did not significantly inhibit MEAC-induced CLL cell viability (Figure 6e–f, Supplementary Table S5–S6). Consistent with this result, A66 or AG490 inhibitors did not prevent MEACs from increasing CLL cell viability (Figure 6e–f, $P = 0.0006$ and $P = 0.0002$, respectively). Thus, inhibitors of BTK or PI3Kδ, but not PI3Kα or JAKs, block MEAC-induced increase in CLL cell viability, supporting the hypothesis that BCR signaling molecules are involved in this effect.

DISCUSSION

MEAC binding to recombinant CLL mAbs in vitro correlated with shorter patient survival, consistent with autoantigen stimulation being involved in the growth and evolution of the leukemic clone.[14] MEACs may provide an abundant source of such antigens, which are not likely limiting in vivo for several reasons. First, because both intrinsic and extrinsic pathways of apoptosis lead to cleavage of intracellular myosin, exposure on the cell surface (Figure 2) and production of MEACs (Figure 1) with caspase-3 activation, in principle, any cell type can form MEACs, including CLL cells themselves (Figure 3). Second, there is an abundance of MEAC antigens in vivo because of normal cell turnover (~10^{11} per day),[39] CLL cell turnover (0.5%–2.3% deaths per day),[40,41] or induction of damage in vivo (e.g. ischemia, infection, inflammation). In this regard, it is essential to recognize that MEACs do not simply provide myosin fragments for BCR interactions. Rather, they supply a large number of other autoantigens that the apoptotic process makes available to immune receptors.[4,6,42] Surface membrane exposed myosin is primarily serving as an indicator of the type of cell involved in the process.

MEAC formation appears to be an active program, since antigens like (but not limited to) myosin may be purposefully modified and exposed on the cell surface (Figure 2). One reason for such translocation is to indicate that this apoptotic cell is to be removed and recycled. Since natural IgM Abs can recognize MEACs (Figure 4), this is consistent with the rationale of apoptotic cell removal.[39] It is also consistent with at least some CLL clones deriving from B cells that produce natural autoreactive Abs that are used as their surface receptors and potentially as their secreted effector molecules. Possible sources of these Abs

could be B-1 cells, which express CD5 in mice and in ~75% of human B-1 cells ($CD20^+$, $CD27^+$, $CD43^+$), marginal zone B cells, transitional B cells, or antigen-experienced mature $CD5^+$ B cells, as have been proposed.[30,43–45]

Just as apoptotic cell infusions into mice stimulate natural Ab producing B cells,[46] MEACs may stimulate CLL cells in a similar manner. Indeed, MEAC co-culture with CLL cells consistently improved CLL cell viability (Figures 5–6, Supplementary Table S1). A role for the BCR in this process is implied by blocking this effect with inhibitors of BTK and PI3Kδ, key molecules in the BCR signaling pathway, but not with inhibitors of PI3Kα and JAKs (Figure 6, Supplementary Tables S2–S6). However, MEACs stimulated CLL cells from all types of patients, not distinguishing between U-CLL and M-CLL and not showing an IGHV stereotype preference. Similarly, BTK and PI3Kδ inhibitor treatments did not show any preferential effects on CLL subtypes, as they inhibited MEAC-induced CLL cell viability regardless of IGHV mutation status or stereotypy. In contrast, recombinant CLL mAbs showed a bias toward U-CLL and stereotype IGHV mAbs binding MEACs well.[14] One explanation for these non-overlapping data may be due to the complex nature of MEACs, which have numerous surface molecules that may interact with multiple different receptors and signaling pathways of the CLL cell, in addition to the antigen:BCR interaction. For instance, CLL cells may receive an additional second non-BCR signal from MEACs that boosts the overall survival signal. There are a number of candidates for this second signal within the CLL microenvironment.[47] For example, BTK and PI3Kδ inhibitors may also block a second receptor pathway that is involved in cell survival. Associated with the clinical success of ibrutinib and idelalisib treatments of CLL is an initial marked lymphocytosis, which may be explained by inhibition of chemokine receptor signaling and loss of integrin-mediated adhesion.[16,18] Thus, chemokine and adhesion mediated signaling are possible candidates for the potential second signal between MEACs and CLL cells.

Finally, inhibition of BTK and PI3Kδ would not only block antigen-induced BCR signaling, but also possibly inhibit “tonic” BCR signaling, which is required for normal B-cell survival.[48,49] CLL cells generally exhibit a higher than normal level of constitutive BCR signaling.[50] Indeed, this idea is supported by the observation that BTK and PI3Kδ inhibitors generally inhibited viability of CLL cells beyond the increase induced by MEACs (>100%, Supplementary Tables S2–S4), whereas PI3Kα and JAK inhibitors did not (Supplementary Tables S5–S6). This tonic BCR signaling could be due to CLL MEACs arising in culture that stimulate the BCR of surviving CLL cells. Another possibility is the tonic signal is due to autonomous CLL BCR signaling via recognition of an autoepitope within the BCR itself (e.g., the second framework region).[9,51] Such low-level tonic, possibly anergic signaling might be overcome when a stronger antigen signal is available to provide a stimulatory growth signal. One could thereby distinguish antigen-BCR reactivities that lead to basic CLL cell survival from those that lead to growth and expansion. In sum, our results are consistent with the theory that antigenic stimulation of CLL cells via the BCR and possibly other receptors promotes CLL cell viability and may influence the level of disease activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Piers Patten, Barbara Sherry, Patricia Mongini, Sophia Yancopoulos, and Rajendra N. Damle (The Feinstein Institute for Medical Research) for helpful discussions; and Marlin Lee “Buzzer” Hefti for advice, support, and encouragement. Ibrutinib was generously provided by Dr. Joseph Buggy (Pharmacyclics). This study was supported by grants from NIH (R01 CA81554, M01 RR01853), The Karches Foundation, The Muriel and Frank Feinberg Foundation, The Marks Foundation, The Jerome Levy Foundation, The Leon Levy Foundation, and the Tebil Foundation, Inc.

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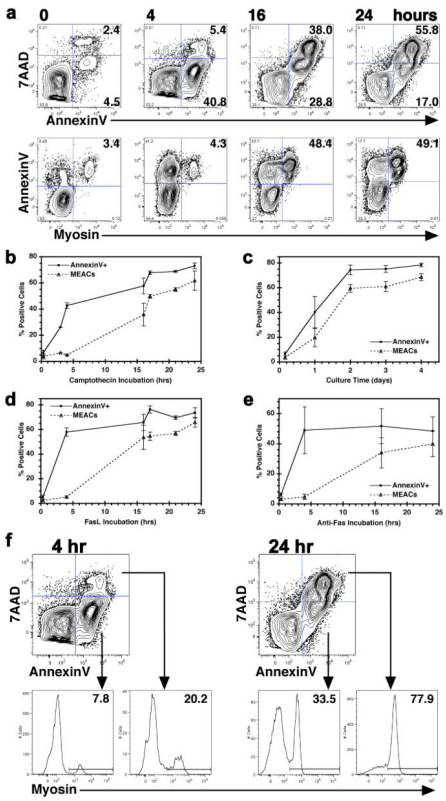

Figure 1.

MEACs accumulate over time during both early and late apoptosis. (**a**) Flow cytometric analyses of Jurkat cells are displayed as contour plots of fluorescence intensity shown on five-log scales with BiExponential transformation. Cells were treated with camptothecin for 0, 4, 16, or 24 h to induce apoptosis and stained with rabbit anti-human myosin, FITC-conjugated anti-rabbit IgG, 7AAD and AnnexinV. Representatives of 11 experiments are shown. The top row shows plots of 7AAD and AnnexinV illustrating changes in live ($7AAD^-$, $AnnexinV^-$), early apoptotic ($7AAD^-$, $AnnexinV^+$), and late apoptotic ($7AAD^+$, $AnnexinV^+$) cell populations over time. The bottom row shows AnnexinV and anti-myosin plots for the same experiment. (**b**–**e**) % of total apoptotic cells ($AnnexinV^+$) and MEACs were determined by flow cytometry as in (**a**) and plotted over time of incubation with means ± SD shown. (**b**) Summary of camptothecin induced apoptosis and MEAC formation (N=11). (**c**) Summary of spontaneous apoptosis and MEAC formation (N=18). (**d**) Summary of FasL-induced apoptosis and MEAC formation (N=8). (**e**) Summary of anti-Fas induced apoptosis and MEAC formation (N=6). (**f**) Representative example of camptothecin treated Jurkat cells analyzed by flow cytometry for apoptosis stage by AnnexinV and 7AAD staining after 4 h (left) or 24 h (right) of incubation. Early ($7AAD^-$, $AnnexinV^+$) and late ($7AAD^+$, $AnnexinV^+$) apoptotic cells were gated and analyzed for MEAC % by level of myosin staining (bottom).

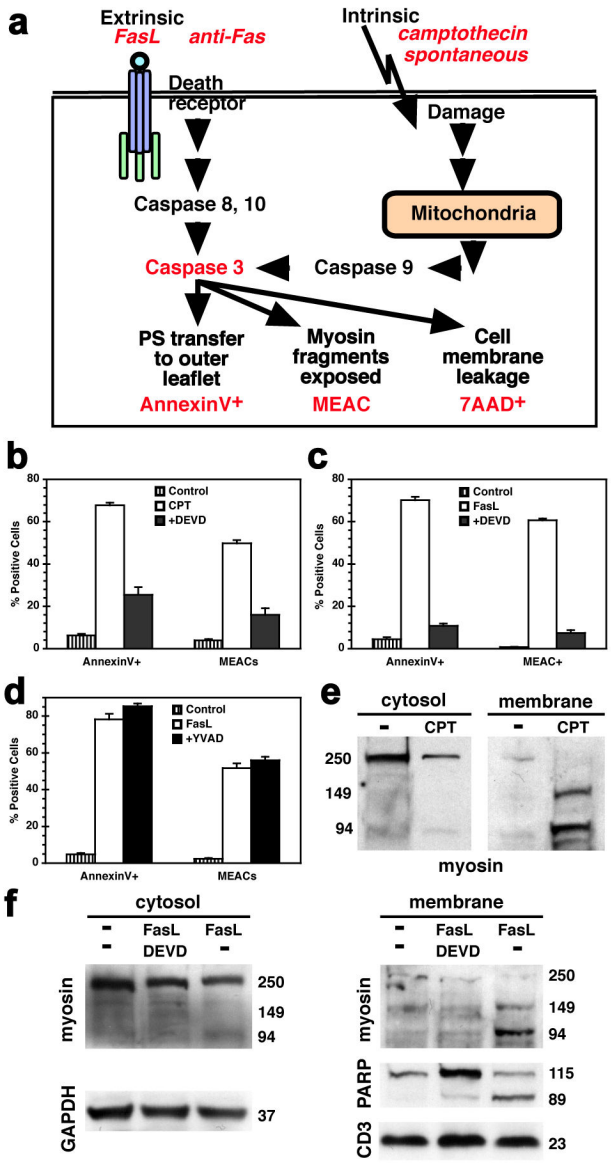

Figure 2.

Extrinsic and intrinsic apoptotic pathways form MEACs via caspase-3 associated myosin cleavage and membrane localization. (**a**) The model of extrinsic apoptotic pathway involves death receptor engagement, which we tested using FasL or anti-Fas Ab, resulting in a caspase activation cascade leading to caspase-3 activation. Three of the multiple functional consequences of caspase-3 activation are shown: phosphatidylserine (PS) exposure to the outer cell membrane (assessed by AnnexinV staining), exposure of myosin fragments to the outer cell membrane (defining MEACs), and pore formation resulting in cell membrane leakage (assessed by 7AAD staining). The model of intrinsic apoptotic pathway is initiated by intracellular damage, which we induced by camptothecin or spontaneously after prolonged culture at high density. This damage leads to mitochondrial outer membrane permeabilization and consequent caspase activation leading to caspase-3 activation and its subsequent consequences. (**b**–**d**) Apoptotic (AnnexinV$^+$) and MEAC (AnnexinV$^+$, Myosin$^+$)

populations of Jurkat cells were analyzed by flow cytometry. (**b**) Cells were incubated for 17 h in media alone or with 160 μM DEVD, a caspase-3 inhibitor, and/or camptothecin (CPT, 10 μM). (**c**) Cells were incubated in medium alone or with DEVD (40 μM, 18 h), and/or human FasL (100 ng/ml, 17 h). (**d**) Cells were incubated in medium alone or with YVAD (2 μM, 18 h), a caspase-1 inhibitor, and/or human FasL (100 ng/ml, 17 h). (**b**–**d**) Means ± SD (N=4) are shown. (**e**) Jurkat cells were incubated in media alone or with CPT (20 μM, 48 h). Cytosol and membrane fractions of cells were separated and their proteins analyzed by Western blot with rabbit anti-myosin IgG (1:500) followed by HRP-conjugated donkey anti-rabbit IgG (1:10,000). Molecular weights (kDa) are indicated. (**f**) Jurkat cells were incubated in media alone or with FasL (40 ng/ml, 20 h) with or without DEVD (40 μM, 21 h). Cytosol and membrane proteins were analyzed by Western blot using anti-myosin (1:100–400 dilution) and anti-PARP (1:100) Abs. In addition, cytosol protein blot was probed with anti-GAPDH Ab (1:200), while membrane protein blot was probed with anti-CD3ε Ab (1:200) as controls for equivalent protein loading. Ab binding detected by HRP-conjugated donkey anti-rabbit (1:1,000–5,000) or sheep anti-mouse (1:2,000–5,000) IgG. (**e**–**f**) Representatives of thirteen experiments are shown.

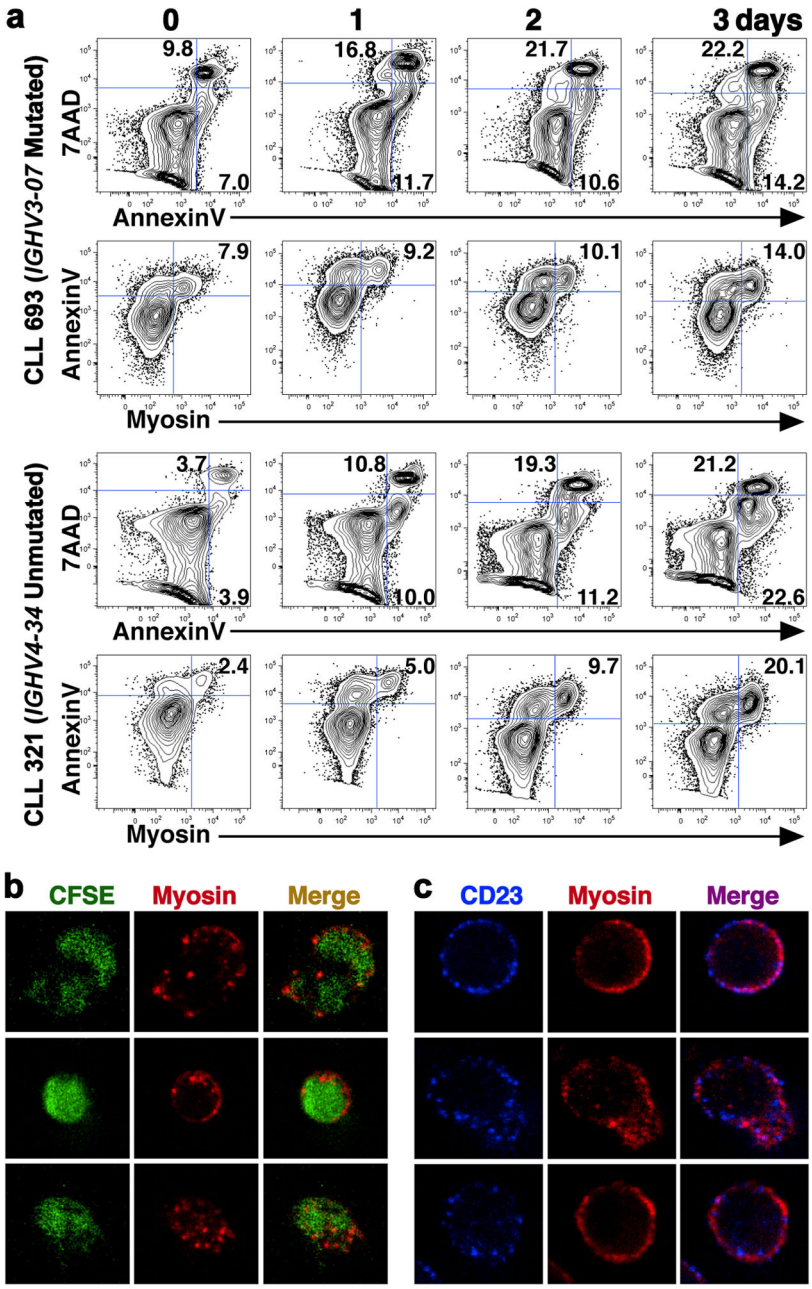

Figure 3.

CLL cells form MEACs during apoptosis. (**a**) Flow cytometric analyses of $CD19^+$ $CD3^-$ CLL cells are displayed as contour plots of fluorescence intensity shown on five-log scales with BiExponential transformation. Cells were cultured for 0, 1, 2, or 3 days and stained with rabbit anti-human myosin, FITC-conjugated anti-rabbit IgG, 7AAD and AnnexinV. Representative results from four experiments with seven CLL patient samples are illustrated with two CLL patients, CLL321 and CLL693. IGHV gene and mutation status are labeled. The top row for each patient shows plots of 7AAD and AnnexinV with % of live ($7AAD^-$, $AnnexinV^-$), early apoptotic ($7AAD^-$, $AnnexinV^+$), and late apoptotic ($7AAD^+$,

AnnexinV$^+$) cell populations over time. The bottom row for each patient shows AnnexinV and anti-myosin plots with MEAC % from the same experiment. (**b**) Apoptotic CLL693 cells stained with CFSE (green) and anti-myosin (red) were visualized separately by a confocal laser-scanning microscope system (FluoView 300-1X; Olympus) using a PLAN APO 60X/1.4 oil-objective lens with a 1.5X digital zoom and then the images were merged. Three representative CLL MEAC images are shown from multiple images obtained in 8 independent experiments. (**c**) Apoptotic CLL693 cells stained with anti-CD23 (blue) and anti-myosin (red) were visualized by confocal microscopy as in (**b**) except with a 3X digital zoom. (**b–c**) Three representative CLL MEAC images are shown from multiple images obtained in 7 independent experiments.

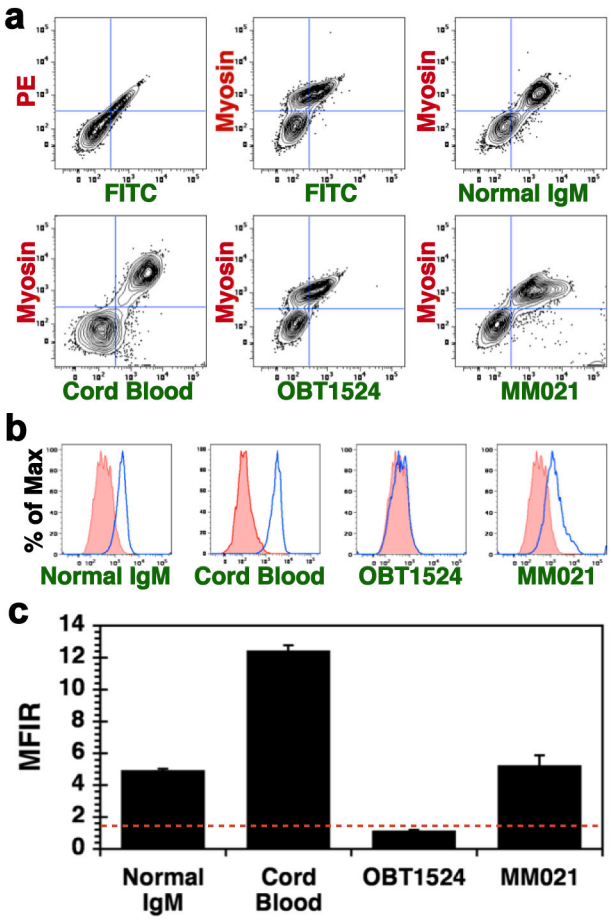

Figure 4.

Natural IgM antibodies recognize MEACs. (**a**) Flow cytometric analyses of Jurkat cells undergoing spontaneous apoptosis are displayed as contour plots of fluorescence intensity shown on five-log scales with BiExponential transformation. Cells were stained with rabbit anti-myosin and normal human adult serum IgM (Normal IgM), cord blood, or control clonal IgM (OBT1524 or MM021), followed by secondary Abs: PE-conjugated anti-rabbit IgG and FITC-conjugated anti-human IgM. Representative plots of unstained (top left panel), anti-myosin alone (top middle panel), or anti-myosin plus normal human serum (two different lots, 16 experiments), cord blood (6 different samples, 8 experiments), or control clonal IgM stained cells (remaining panels, 8 experiments) are shown. (**b**) After gating on myosin^{+} cells in above plots, histograms of the % of maximum (% of Max) fluorescent intensity for indicated staining (thick blue line) are shown relative to staining with anti-myosin alone (red shaded plot, negative control). (**c**) The mean MFIR was calculated from the geometric mean fluorescence intensity determined in panel B as a ratio over the negative control. MFIR ≥ 1.5 considered positive MEAC binding (dashed red line).[14] Average MFIR with standard error is shown for normal IgM (N=23), cord blood (N=17), OBT1524 (N=3), and MM021 (N=13).

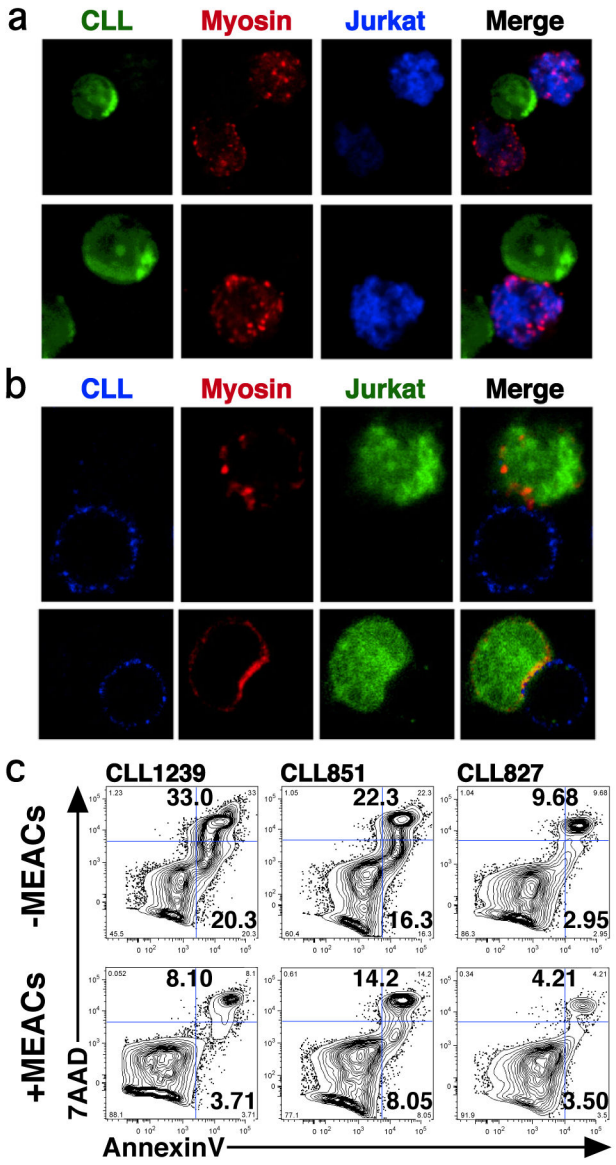

Figure 5.

MEACs associate with CLL cells and enhance CLL cell viability. (**a**–**b**) Co-culture of CLL cells with Jurkat cells previously induced to become MEACs were examined by confocal microscopy and representative images of associated cells were acquired as in Figure 3b with varying digital zoom conditions using (**a**) CLL693 cells stained with CFSE (green), dead Jurkat cells with Alexa680 (blue), and MEACs with anti-myosin (red); or (**b**) CLL1181 cells stained with anti-CD23 (blue), Jurkat cells with CFSE (green), and MEACs with anti-myosin (red). Fluorochrome results are shown individually or merged together. In (**a**), each image shows three associated cells, representatives of multiple images obtained from two independent experiments. Top row and bottom row of images were acquired with 4X or 6X digital zoom, respectively. In (**b**), each image shows two associated cells, representatives of multiple images obtained from 7 independent experiments. Top row and bottom row of images were acquired with 3X or 2X digital zoom, respectively. (**c**) Apoptotic CLL cells

after co-culture (2–4 days) with or without MEACs (top and bottom rows, respectively) are shown for three representative CLL patient cells out of 76 patient samples tested in 35 experiments. % of early apoptotic ($7AAD^{-}$, $AnnexinV^{+}$) and late apoptotic ($7AAD^{+}$, $AnnexinV^{+}$) cell populations are indicated.

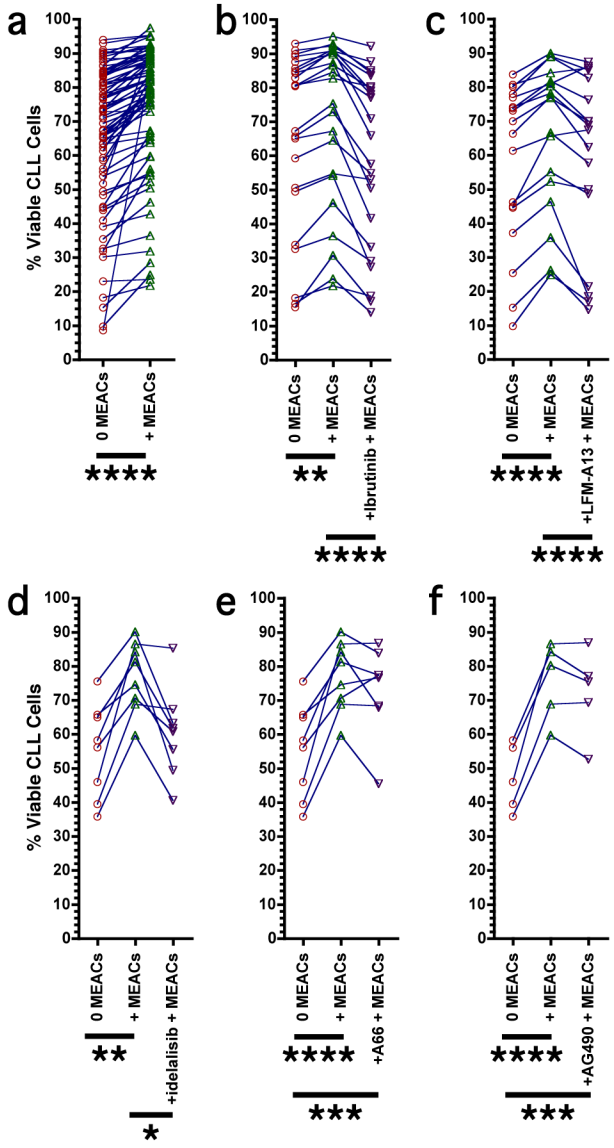

Figure 6.

MEACs may enhance CLL cell viability via BCR signaling. Co-cultures of CLL cells with Jurkat cells previously induced to become MEACs were examined as in Figure 5c. (**a**) Summary of AnnexinV⁻ viable CLL cell % after co-culture (2–4 days) with (black) or without MEACs (red), where lines connect CLL cells from the same patient (N=76). The increase in viability of patient CLL cells after co-culture is highly significant by Wilcoxon matched-pairs signed rank test (****, $P < 0.0001$). (**b**–**f**) CLL cells were co-cultured with (+ MEACs) or without MEACs (0 MEACs) plus the addition of BTK inhibitor (**b**) ibrutinib (N=23) or (**c**) LFM-A13 (N=18), PI3Kδ inhibitor (**d**) idelalisib (N=8), PI3Kα inhibitor (**e**) A66 (N=8), or JAK2/3 inhibitor (**f**) AG490 (N=5) and the % of AnnexinV⁻ viable cells was measured. Addition of MEACs significantly increased viability of CLL cells in inhibitor experiments (**, $P = 0.0013$ (**b**); ****, $P < 0.0001$ (**c,e,f**); **, P = 0.0018 (**d**); repeated measures one-way ANOVA and Tukey test). BTK or PI3Kδ inhibitors significantly reduced

MEAC-induced CLL cell increase in viability (****, $P < 0.0001$ (**b,c**); *, $P = 0.0138$ (**d**); repeated measures one-way ANOVA and Tukey test), but not with PI3Kα or JAK2/3 inhibitors. Consistently, PI3Kα or JAK2/3 inhibitors +MEACs showed a significant increase in viability compared to without MEACs (0 MEACs) (***, $P = 0.0006$ (**e**), $P = 0.0002$ (**f**); repeated measures one-way ANOVA and Tukey test), whereas BTK or PI3Kδ inhibitors did not.