Cell-Mediated Autophagy Promotes Cancer Cell Survival

William J. Buchser¹, Thomas C. Laskow¹, Philip J. Pavlik¹, Hui-Min Lin², and Michael T. Lotze¹

Abstract

Immune effector cells integrate signals that define the nature and magnitude of the subsequent response. Experimental measures for immune cell-mediated lysis of tumors or virally infected targets rely on average responses of permeability or apoptotic changes within a population of targets. Here, we examined individual target cells following interaction with lymphoid effectors. We found that human peripheral blood lymphocytes not only provide lytic signals but also promote autophagy in the remaining cells. At high effector-to-target ratios, autophagy was induced in several human tumors, as assessed by induction of LC3 puncta and diminished p62. Natural killer cells are a primary mediator of this process. In addition, target cell autophagy was enhanced by provision of interleukin (IL)-2, whereas IL-10 attenuated this effect, and cell-to-cell contact strongly enhanced lymphocyte-mediated autophagy. Although IFN-γ can induce autophagy in target cells, IFN-α acted directly on the targets or in concert with lymphocytes to diminish target autophagy in some cell types. Importantly, cell-mediated autophagy promoted resistance from treatment modalities designed to eradicate tumor cells. Our findings therefore show that the lymphocyte-induced cell-mediated autophagy promotes cancer cell survival and may represent an important target for development of novel therapies. Cancer Res; 72(12); 2970–9. ©2012 AACR.

Materials and Methods

Cell culture

Human pancreatic cancer lines Panc2.03 and Panc3.27 from American Type Culture Collection. Colon cancer HCT116s were a gift from Dr. Bert Vogelstein (John Hopkins University, Baltimore, MD), 786-0 from Dr. Jodie Maranchie and WSS1 from Lisa Butterfield (University of Pittsburgh School of Medicine, Pittsburgh, PA). Human bladder cancer cell lines (T-24) were stably transfected with GFP-LC3 were a gift from Umamaheswar Duvvuri (University of Pittsburgh Medical Center, Pittsburgh, PA). Human bladder cancer cell lines (T-24) were authenticated using antifungal mixture (PenStrep, Gibco/Invitrogen) in a C211 medium (IDEXX Radil). Cells were cultured in complete media; RPMI-1640 (Thermo/Hyclone) supplemented with 10% heat-inactivated FBS (Mediatech), and antibiotic-antifungal mixture (PenStrep, Gibco/Invitrogen) in a
humidified incubator 37°C with 5% CO₂. Cell lines were passaged, split, counted, and seeded 4,000 to 5,000 cells per well in 55 μL of complete media on 384-well collagen-I–coated plates (BD Labware 35-6832) for 24 hours.

Treatment of cells
We used recombinant human IFN-γ (R&D Systems) and recombinant IFN-α from Intron-A (Schering-Plough). In cell–cell contact and diffusible factor experiments, supernatant was removed from coculture wells at 2 and 6 hours, spun down, and the top fraction was applied to naive culture wells for the remaining 22 and 18 hours, respectively. Transwell experiments were carried out in 24-well plates using 0.4-μm polycarbonate Transwell supports (Corning/Costar).

Human peripheral blood mononuclear cell
Peripheral blood mononuclear cells were isolated as previously described (21). Briefly, peripheral blood mononuclear cells were collected from the buffy coat using Ficoll-Paque Plus (Amersham Biosciences), rinsed, and stored in 20% FBS with 10% dimethyl sulfoxide (DMSO) at −80°C. Cells were thawed and incubated for 2 hours in T-75 flasks to remove adherent monocytes. Lymphocytes (82% of cells in PBLs) were rinsed and cultured with or without recombinant human IL-2 (Proleukin; Prometheus) in RPMI + 10% FBS. These PBLs were harvested and mixed with tumor cells at various effector-to-target (E:T) ratios in complete media. NK cells were negatively selected with the EasySep Human NK cell Enrichment Kit (Stem Cell Technologies) resulting in 74% purity.

Cancer cell lines preplated for 24 hours on 384-well plates were challenged with lymphocytes or purified NK cells. Experimental conditions were prepared in round-bottom, 96-well plates (BD Falcon 35-3077). One hundred microliters of complete media was used to setup dilution series of lymphocytes (1:1 dilution factor). IL-2 and other dilutions were executed in a similar manner. Twenty microliters from each of the setup wells was added directly to the existing media on the assay plate with the cancer cells. Experimental plates were incubated in a 37°C incubator with 5% CO₂.

Staining, microscopy, and analysis
Cells were stained for immunofluorescent imaging, and microscopy was done with Cellomics ArrayScan VTi (see Supplementary Methods). Autophagy was measured by LC3 morphology (22).

Results
Increased autophagy in surviving tumor cells after lymphocyte coculture
We have developed a coculture system where dividing human cancer cell lines adhered onto collagen substrate were overlain with primary human lymphocytes. Cytolysis was measured directly by automated adherent cell counting and was equivalent to the standard 51Cr release assay and the cell counting kit (CCK;8 cell survival assay (Supplementary Fig. S1).

With the coculture model, not only could we determine the rate of lysis in a dose-dependent manner, but also we found that many target cells remained adherent even with the highest lymphocyte number delivered for extended periods (8). Autophagy is a common cellular response to stress and we hypothesized that autophagy could sustain the surviving cells and potentially protect them from lysis. Unlike controls (Fig. 1A and B), high E:T ratio cocultures have lymphocyte clusters around the remaining tumor cells (Fig. 1C), especially in the presence of IL-2 (Fig. 1D). The high lymphocyte/IL-2 condition (Fig. 1D) has fewer adherent target cells remaining because of lysis. Importantly, the remaining cells have heightened autophagy, as shown by increases in LC3 puncta area, density and number of puncta. Not only did the fraction of highly autophagic cells increase in the high E:T conditions but also the overall number of autophagic cells increased, confirming induction of heightened autophagy, and not solely a selection of autophagic cells. We next carried out a dose–response assessment for unsorted lymphocytes and negatively selected human primary NK cells. NK cells are able to kill targets more effectively than unsorted cells (Fig. 1E, analysis of covariance (ANCOVA) comparing regressions of NK vs. PBL. P = 4.1 × 10⁻⁴ in the presence of IL-2. Both unsorted lymphocytes and NK cells promoted autophagy as assessed by LC3 puncta area (NK No IL-2, P = 1 × 10⁻⁴; IL-2, P = 0.035; PBL No IL-2, P = 1 × 10⁻⁴; IL-2, P = 4 × 10⁻⁵; regression analysis). Compared with baseline, autophagy was doubled with 2:1 E:T ratios of NKs and was increased 4-fold with 5:1 E:T NK cells. Higher E:T ratios (~40:1) of unsorted lymphocytes (Figs. 1F and 3–7) were required to produce comparable levels of autophagy, suggesting that a major role of NK cells is to promote autophagy (ANCOVA comparing regression of NK vs. PBL for LC3 puncta area, P < 0.001). In addition, CD56⁺ CD3⁻ CD19⁻ cells promoted autophagy to a greater extent than the CD56⁺ lymphocyte population (not shown). Interestingly, other immune cells including macrophages and T cells (Supplementary Fig. S2) mediate cell-mediated autophagy (C-MA) as well. Consistent with previous findings, the autophagy markers p62 (23) and caveolin-1 (24) diminish with increasing autophagy (Fig. 1G and H; regressions for p62 IL-2 6000, P = 3.6 × 10⁻⁶; No IL-2, P = 0.00063) as LC3 levels increased (Fig. 1I). Induction of target autophagy required the autophagy gene ATG5 (Supplementary Fig. S3). Lymphocytes and macrophages can promote C-MA in human epithelial cancer cell lines.

Lymphocytes effectively lyse and induce autophagy in multiple epithelial cancer lines
We examined whether lymphocytes had the ability to induce autophagy in several human epithelial cancer cell lines including those derived from the colorectum, pancreas, kidney, and bladder. All cell types were sensitive to lysis with increasing PBL: E:T ratios (Fig. 1J, first row; χ² analysis, independent experiments with statistically decreasing slopes). Autophagy was consistently induced and the measurement of LC3 by LC3 puncta area (average size of the autophagosomes), was repeatable across cell types and experiments (Fig. 1J, second row). WSS1 did not share this phenotype, possibly because of more variable basal autophagy. Lymphocyte-induced changes in the number of LC3 puncta (Fig. 1J, third row) and the individual punctum LC3 density were more cell-type specific. These
results indicate significant and reliable autophagic changes induced by lymphocytes for pancreatic, colorectal, renal, and bladder cancer cell lines.

To verify that the autophagic increase was not simply a precursor to cell death, cultures were established for various time periods (Supplementary Fig. S4A–S4C). Autophagic cells could be identified for at least 5 days, and both the number and percentage of autophagic cells increased at high E:T conditions when compared with vehicle controls (Supplementary Fig. S4D). Additional studies using caspase inhibitors did not
prevent C-MA (Supplementary Fig. S5). Therefore, induction of autophagy by lymphocytes is not caused directly by induction of cell death pathways, and it likely relates to extended cell survival. Bafilomycin, a late autophagy inhibitor, was used to halt progression of so-called autophagic flux, which results in uncleared autophagosomes otherwise targeted for lysosomal degradation. In the presence of PBLs, bafilomycin-treated targets had both enhanced LC3 puncta and the lysosome marker lysosome-associated membrane protein, LAMP1 (Supplementary Fig. S6), consistent with a block in autophagosome fusion and degradation.

**Autophagic flux measured by imaging LC3**

Some autophagic characteristics of epithelial cancer cells are shown in Fig. 2. These patterns were context-dependent and associated only with particular cell lines or treatments. Cells in control conditions (Fig. 2A) had diffuse LC3 with difficult-to-discern puncta. In the pancreatic, renal, and bladder cancer cell lines, there was a pool of nuclear LC3 and less cytoplasmic LC3. Several patterns emerged that were mirrored across most cell lines tested. The addition of lymphocytes produced autophagy with more puncta appearing in the cytoplasm. These puncta were quite large and possessed indistinct borders (Fig. 2B). The overall amount of LC3 in the cytoplasm and the number of puncta were somewhat higher than the control, but the largest difference was in the area (size) of the puncta. Other treatments, such as IFN-γ, produced a different pattern of autophagy. Although there was an increase in the size and number of LC3 puncta, the largest increase was the LC3 density within individual puncta (i.e., the intensity of LC3; Fig. 2C). These bright puncta were also easier to identify because of more distinct borders. The localization of the puncta within the cell was also distinct, depending on the context (cell type and treatment). The renal cancer cell line 786-0 (Fig. 2D) had a high level of juxta-nuclear LC3 at basal conditions, which was often observed as a diffuse perinuclear signal with defined borders and 1 or 2 bright perinuclear puncta. Occasionally, a small mist of cytoplasmic LC3 was also observed. The classic autophagy inducer, serum starvation, and chloroquine inhibition also produced distinct LC3 patterns (Supplementary Fig. S7). Staining with the lysosomal marker LAMP1 indicated puncta of both LC3 and LAMP1 in...
Cytokines regulate induction of autophagy

Unsorted PBLs were used henceforth because they can readily be induced to express lymphokine-activated killer (LAK) activity (25) and to establish the response for the mixture of cells that would exist in a tumor microenvironment in individuals treated with IL-2. We sought to determine the dose response for IL-2 in regard to lymphocyte-induced target autophagy. With no lymphocytes, cancer cells maintained the same level of autophagy through increasing IL-2 treatment (as expected, no significant effect of IL-2). With peripheral lymphocytes present, IL-2 enhanced autophagy in the target cells (Fig. 3A, right; linear regression, \( P = 0.035 \)). Denatured (boiled) IL-2 was unable to increase autophagy, indicating that the mannnitol and SDS excipient of IL-2 did not contribute to induction (data not shown). IL-10 was expected to act on the lymphocytes to regulate autophagy in the targets (26). MHC-I expression was used as a positive control. In Fig. 3B (top right), MHC-I was increased in cancer cells by lymphocyte coculture, especially with the addition of IL-2. MHC-I was unchanged by direct treatment of cancer targets with dose-escalating IL-10. IL-10 steadily decreased the expression of MHC-I otherwise induced by high E:T ratio of lymphocytes both with \( (P < 0.001) \) and without IL-2 \( (P < 0.001) \). The effect on autophagy was similar (Fig. 3B, right). LC3 puncta area was diminished with IL-10 treatment in coculture, particularly with concurrent IL-2 treatment (No IL-2, \( P < 0.005 \); with IL-2, \( P < 0.0005 \)). Therefore, IL-2 potentiated C-MA whereas IL-10 attenuated it.

TGF-\( \beta \) can act on NK cells to diminish target lysis and IFN-\( \gamma \) production (8). We have found that blocking TGF-\( \beta \) in the coculture diminishes target cell autophagy in a dose-dependent manner (Supplementary Fig. S8), indicating that TGF-\( \beta \) may be one of the molecular signals responsible for autophagy induction.

Cell–cell contact enhances lymphocyte-mediated autophagy

To evaluate mechanisms promoting C-MA, we asked whether soluble factors released from lymphocytes were sufficient for induction. Supernatants from lymphocyte/cancer cocultures were applied to adherent cancer cell cultures (Fig. 4A). With supernatant treatment, autophagy was slightly increased when compared with untreated cancer cells (in HCT116 and Panc2.03) but less than with cell–cell contact (50:1 E:T ratio coculture, \( P < 0.035 \); Panc2.03, not significant). With peripheral lymphocytes present, IL-2 enhanced autophagy in the target cancer cells (Fig. 2F). In a parallel experiment, autophagy was promoted by direct lymphocyte coculture, with only minimal increases observed when cells were separated by a permeable Transwell (Fig. 4B, ANCOVAs comparing regressions between Transwell and cell–cell contract for HCT116, \( P = 0.0014 \); Panc2.03, \( P = 0.0013 \); T-24, \( P = 0.012 \); Dunnett’s posttests displayed in figure). In both instances, overall LC3 intensity in the cancer cell lines was increased by both direct coculture and Transwell conditions (data not shown), consistent with the presence of a diffusible factor that is sufficient to upregulate LC3 expression, but not to markedly increase autophagic flux. Cell–cell contact was required for full increases of autophagy in the cancer cell lines tested.

NK cells express specific receptors, which either activate or inhibit effector function. We have found that the activating receptor NKGD2 is not responsible for triggering C-MA (Supplementary Fig. S9). On the other hand, the family of killer cell Ig-like receptors (KIR) do seem to play a role, at least in some cell types. KIR inhibition both by a pan-KIR blocking antibody and by a SHP2 phosphatase (downstream of the ITIM domain in KIR-L forms) inhibition produces increases in effector IFN-\( \gamma \).
HCT116, \( P < 0.001 \); using the lowest 2 concentrations), IFN-\( \gamma \) had the largest effect on LC3 density (Figs. 5C and 2C) but also led to increases in the number and area of the puncta (not shown). Lymphocytes induced increases in L33 puncta area (Fig. 5F, linear regressions \( P < 0.0001 \) for all cell types). STAT1/2 are downstream of IFN signaling, and we observed increases in the nuclear localization of STAT1 and STAT2 after lymphocyte coculture (Supplementary Fig. S11A–S11B), indicating STAT activation. Although both lymphocytes and IFN-\( \gamma \) induced autophagy, the phenotype of the target cells was different, suggesting that molecular pathways driven by lymphocyte coculture other than IFN-\( \gamma \) may be involved. In addition, soluble IFN-\( \gamma \) receptor (IFNgR1) reduced upregulation of MHC-I but was not able to block C-MA (Supplementary Fig. S11C–S11F), confirming that additional factors or surface molecules can trigger the process.

**IFN-\( \alpha \) inhibits C-MA in HCT116 and 786-0 cell lines**

As expected, IFN-\( \alpha \) was sufficient to upregulate MHC-I on target cells (Fig. 6A). Additive effects on MHC-I expression were observed with the combinations of lymphocytes and IFN-\( \alpha \) (Fig. 6A, second panel monotonic; third panel additive, but no synergy). Increasing both cells and IFN-\( \alpha \) led to MHC increases with a 2-fold greater rate of change (Fig. 6A, fifth panel). Although IFN-\( \alpha \) produced the greatest MHC-I expression increase from 0 to 10,000 IU/mL and then plateaued, the combination of lymphocytes and IFN-\( \alpha \) produced greater MHC-I increases, suggesting that another cytokine (such as IFN-\( \gamma \)) was also operative. All IFN-\( \alpha \)-effects on MHC were significant across the concentration range tested (linear regression HCT116, \( P < 0.0001 \); Panc2.03, \( P = 0.0073 \); T-24, \( P = 0.014 \), and for high E:T ratio or high doses of IFN-\( \alpha \) (t tests, \( P < 0.0001 \)).

Although both IFN-\( \gamma \) and IFN-\( \alpha \) produced similar target cell changes in MHC-I expression, the effect on target cell autophagy was distinct. Depending on the cell type, IFN-\( \alpha \) either produced no significant changes in autophagy (Panc2.03, T-24) or reliably inhibited autophagy (HCT116, \( P = 2 \times 10^{-16} \)). When IFN-\( \alpha \) was added to cells, autophagic LC3 puncta dropped in size and number in a dose-dependent manner (Fig. 6B, first panel; \( P = 0.0068 \)). HCT116 autophagy was induced by increasing lymphocyte E:T ratios (Fig. 6B, second panel; \( P = 0.0015 \)). IFN-\( \alpha \) specifically inhibited autophagy induced by lymphocyte coculture (Fig. 6B, panels 4 and 5), except at the highest E:T ratio tested (panel 3). In all cases, C-MA was limited in the presence of IFN-\( \alpha \) (multiple regression’s interaction term in HCT116 and 786-0 \( P < 0.001 \)). Representative images are shown in Fig. 6C and D. When combined with a late autophagy inhibitor, there is little expansion of autophagosome number with higher doses of IFN-\( \alpha \), and when combined with an early autophagy inhibitor, there is little change in the effect of IFN-\( \alpha \) (Supplementary Fig. S12). These results confirm that IFN-\( \alpha \) does not increase autophagy in the target cells we evaluated.

**C-MA protects cells from subsequent \( \gamma \)-radiation**

We examined the remaining tumor cells after lymphocyte coculture to determine whether the cancer cells were...
protected from radiation-induced cell death. The cancer cell lines were cocultured with human lymphocytes for 24 hours at individual E:T ratios, then harvested and exposed to γ-irradiation. The cancer cells were replated for an additional 24 hours before they were analyzed. Increasing doses of γ-irradiation eradicated almost half of the cancer cells in all 3 cell lines (Fig. 7A, regressions for HCT116, P = 0.0015; 786-0, P = 2.8 × 10⁻⁶; Panc2.03, P = 6.7 × 10⁻⁶). Interestingly, HCT116 and 786-0 cells that were previously cocultured with PBLs had a significantly higher fraction of cells remaining after γ-irradiation than those cells that were cultured alone (ANOVA P < 0.0001, Dunnet’s posttests HCT116 500 rads, P = 1.2 × 10⁻⁵; 786-0 125 rads, P = 0.055; Panc2.03, not significant). γ-Irradiation itself induces a stress response in cells, increasing autophagy (Fig. 7B). These results suggest that C-MA protects cells from subsequent stressors including radiation. Chemotherapeutic agents’ efficacy was only modestly diminished following previous coculture with lymphocytes (Supplementary Fig. S13).

Discussion

Cell-mediated lysis has been well documented since the pioneering studies of the Hellstrom (31). Multiple reasons for the failure of immune effectors to fully eradicate tumor have been advanced, including immune exhaustion, factors released within the tumor microenvironment to limit effector survival or efficacy (21) in vitro and in vivo (8, 9), and so on. Relatively little attention has been given to the state of the remaining tumor cells. Here, we suggest that C-MA may play a role in inducing resistance to immune effectors and also limit the ability of tumors to respond to subsequent chemotherapy or radiation therapy (32).

Target cells with high autophagy persisted in culture for at least 5 days after interaction with immune effectors. The induction of autophagy was shown for 6 individual epithelial cancer cell lines and is ATG5-dependent. Measuring autophagic flux was achieved by quantifying LC3 puncta (33–35). IL-2 further activates lymphocytes to induce LAK activity (25), enhancing lysis but also enhancing autophagy induction in the nonlysed targets. Direct cell–cell contact produced the strongest C-MA. IFN-γ was sufficient to induce autophagy in the target cells. However, the morphology of the autophagosomes was different than that observed with PBL-induced autophagy, with especially high-density LC3 puncta. The effect of IFN-α depended on the specific target cell type used. IFN-α was able to inhibit autophagy when combined with lymphocyte coculture. Both KIR and TGF-β inhibition diminished (but did not abolish) C-MA, making them candidates for the molecular mechanism.

Autophagy is a basal repair and stress response mechanism, and in many ways mediates programmed cell survival. Autophagy is important oncologically because it can both suppress tumorigenesis at early stages (36, 37) and enable cancer adaptation and recurrence after therapy in late stages.
Thus suggesting an autophagic switch arising during carcinogenesis. Although IL-2 administration can be potentially curative, it creates a systemic autophagic syndrome, limiting vital processes within tissues at the expense of cell survival (39, 40). Combination with the autophagy inhibitor chloroquine significantly enhances the effects of IL-2 (41). The balance between activating and inhibitory signals regulating NK-mediated lysis is now more nuanced, given that lymphocytes can also induce C-MA in targets. Classic activating signals for cytolysis (42) may also promote repair and survival in some circumstances.

We showed that IFN-γ, but not IFN-α, enhanced autophagy in cancer cell lines. IFN-γ induces autophagy (43) both to enhance antigen processing and presentation through MHC-I (44, 45). Increased autophagy also enhances the process of viral/bacterial digestion through xenophagy (46). In the liver for example, IFN-γ is likely responsible (along with IL-12/IL-18) for the clearance of hepatitis virus (47, 48). IFN-α primarily enhances NK cell cytolotoxicity (49). The differential response produced from these type-I and type-II IFNs provides a potential mechanism for lymphocytes to deliver differential repair/cytolytic instructions to target cells. Additional cytokines such as IL-10, which diminishes C-MA as well as IL-4 may play roles in negatively regulating C-MA.

Figure 6. C-MA is inhibited by −IFN-α. Tumor cell lines were grown alone for 24 hours and then treated with combinations of IFN-α and lymphocytes for 24 hours before measuring cytolysis, MHC-I expression, and autophagic puncta. Line charts showing MHC-I expression (A) and # LC3 puncta (B) with the 5 columns on each graph representing a dose response to IFN-α, increasing E:T ratios of PBLs with no IFN-α, a gradient of PBLs with a constant IFN-α 70,000 IU/mL, constant 50:1 E:T ratio (PBLs) with increasing concentrations of IFN-α, and a dual increase in both E:T ratio and IFN-α. All x-axes are on a log scale. Micrographs of T-24 bladder cancer (C) and Panc2.03 (D) representing the various patterns of autophagy and MHC-I expression. C, control growth of cells in Hoechst channel, LC3, MHC-I, and merge (blue, red, green, respectively). On the second row, 50:1 E:T ratio of lymphocyte coculture after 24 hours (arrowhead indicating lymphocyte nuclei). Bottom row showing IFN-γ treatment for comparison. D, Panc2.03 control (top), 70,000 IU/mL IFN-α (middle), and IFN-γ (bottom) for comparison. Scale bar, 50 μm.
Using a new image-based cytolysis assay, we show that lymphocytes can promote cell survival. The induction of autophagy, the precise signals and means for communication to induce this function, needs to be more fully evaluated to understand the complex multistep interactions between lymphoid cells and their targets. The potentially reparative or protective signal provided by lymphocytes to induce autophagy could promote wound healing or response to pathogens but could also promote the development and persistence of cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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