One siRNA pool targeting the λ constant region stops λ light chain production and causes terminal endoplasmic reticulum stress

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Running title: λ light chain constant region siRNA

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Keywords: immunoglobulin λ light chain constant region; siRNA; plasma cells; unfolded protein response; amyloidosis
Key Points:

1. Ig light chain and antibody production by plasma cells is significantly reduced by siRNA for the light chain constant region

2. In plasma cells making intact antibodies, knockdown of light chains can cause terminal ER stress due to unpaired heavy chains.

ABSTRACT

In systemic AL amyloidosis, λ light chains produced by clonal plasma cells cause organ damage and early death. In pursuit of novel therapy, we developed one pool of siRNA targeting the constant region of λ light chains (si[IGLCCR]) that substantially and promptly reduces λ light chain production and secretion by human plasma cells regardless of sequence diversity. In clones producing intact IgGλ antibodies (containing paired heavy and light chains), the secretion of intact antibodies is reduced and all three branches of the unfolded protein response (UPR) are activated by accumulation of unpaired IgG heavy chains in the endoplasmic reticulum (ER). Moreover, an ER stress response can then become terminal with effector caspase activity mediated in part by the transcription of the BH3-family member NOXA. This pool of siRNA can be used to reduce pathologic λ light chain production and cause apoptosis in human plasma cells making intact IgGλ antibodies.
INTRODUCTION

The immunoglobulin light chains in systemic light-chain amyloidosis (AL) have a κ-to-λ case rate of 1-to-4 and cause cardiac-related deaths in up to 25% of patients within months of diagnosis. Current therapies for AL aim to reduce light chain production, and include steroids, alkylating agents, immunomodulatory drugs and proteasome inhibitors. While responses are notable, incurability and early cardiac death are more the norm, and more effective therapies are needed.

Although the unfolded protein response (UPR) is constitutively active in plasma cells, and although the pairing of light and heavy chains is under strict intracellular quality control in the endoplasmic reticulum (ER), we learned in initial experiments that variable-region targeted λ light chain knockdown could trigger a reactive UPR and a terminal ER stress response, while knockdown of IgG heavy chain expression had no impact on cell growth or viability. We then hypothesized that λ light chain (IgL) production could be disrupted by siRNA targeting consensus sequences in the IgL constant region (CR) mRNA with one siRNA pool. We now report that this pool (si[IGLCCR]) significantly reduces IgL production and secretion by human plasma cells without regard for unique variable region gene sequences and that, in human plasma cells making intact antibodies, treatment with si[IGLCCR] results in the intracellular retention of unpaired Ig heavy chains (IgH), the activation of the UPR and up-regulation of genes involved in ER stress signaling that can cause NOXA-mediated mitochondrial depolarization and caspase-dependent apoptosis. In addition, si[IGLCCR] treatment can substantially reduce IgL message and intracellular IgL in AL patient plasma cells producing monoclonal IgL and can also increase caspase 3/7 activity in clones making intact antibodies.
MATERIALS AND METHODS

Cells

ALMC1 and ALMC2 cells (gift of Diane Jelinek) were cultured as described\textsuperscript{10}. MM.1S and MM.1R cells from American Type Culture Collection (Manassas, VA, USA), and EJM and OPM-2 cells from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), were cultured as directed. These cell lines were chosen because they are human myeloma cell lines that produce λ light chains (IgL). Z-VAD-FMK, L-phenylalanine mustard and arachadonic acid were from Sigma-Aldrich (St Louis, MO).

Clinical specimens obtained under an institutional review board approved protocol from patients with confirmed diagnosis of systemic AL amyloidosis and monoclonal IgL disease were used for CD138-selection as previously described\textsuperscript{11}. This study was conducted in accordance with the Declaration of Helsinki.

IgL and IgH gene identification

Monoclonal IgL and IgH genes for ALMC1, ALMC2 and EJM cells were identified and sequenced as previously described\textsuperscript{12}.

siRNA

Individual cell lines were evaluated for tolerance to streptolysin-O (SLO; Sigma-Aldrich) reversible permeabilization for transfection of siRNA, and the optimal effective concentrations of SLO and siRNA were determined. A similar approach testing tolerance was used for patient plasma cells (Supplementary Fig. 1)\textsuperscript{13}.

All siRNA agents were obtained from Dharmacon (Thermo Scientific, Lafayette, CO) using www.thermo.com/sidesign with modifications to minimize seed-region off-target effects (ON-TARGET\textsuperscript{plus} SMARTpool\textsuperscript{TM}). The siRNA pools we used are in the Supplement and custom-designed siRNA pools are defined in Supplementary Table 1.
Caspase 3/7 Assay

Luciferin-based caspase 3/7 activity assays (Promega; Madison, WI) were performed following manufacturer’s instructions on a Promega GloMax microplate luminometer in triplicate for each situation with 5x10^3 cells per well and reported as relative luminescence units (RLU).

MTT Assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation and viability assays (Invitrogen) were performed according to manufacturer’s instructions in triplicate with 2.5x10^4 cells per well and read on a microplate absorbance reader (BIO-RAD, Hercules, CA). Cell viability was also assessed by trypan blue staining where indicated.

Flow Cytometry

All antibodies used are listed in Supplementary Table 2. Antibodies were titrated for optimal use and used with appropriate isotype controls. Flow cytometry was performed in our core facility on a FACSCalibur Cytometer (Becton Dickinson; Franklin Lakes, NJ). Mean fluorescence intensity (MFI) in each case was computed minus that of isotype control. Flow cytometry for intracellular immunoglobulin was performed with fluorescein (FITC) - conjugated anti-human IgG heavy chain and phycoerythrin (PE)-conjugated anti-human Ig λ light chain antibodies titrated for optimal use with appropriate isotype controls. Cells were permeabilized by using CytoFix/CytoPerm Fixation/Permeabilization kit (BD Pharmingen, Franklin lakes, NJ), then stained with antibodies and acquired. MFI was analyzed with FlowJo (Tree Star, Ashland, OR).

The Annexin V/PI kit was from BD Pharmingen (San Jose, CA). As a control for Annexin V/PI staining melphalan (L-phenylalanine mustard; Sigma-Aldrich, St Louis, MO) was dissolved in acid alcohol and used fresh at a concentration of 25µM. For this assay, cells were harvested, washed twice and suspended in Annexin V labeling buffer with FITC-
Annexin V and propidium iodide (PI) as described in the kit and acquired. The percentage of apoptotic cells was computed with FlowJo.

**Immunoblots (IB) and immunoprecipitates (IP)**

All antibodies used are listed in Supplementary Table 2. Cells were washed with PBS, pelleted and lysed in modified RIPA assay buffer (Pierce/Thermo Scientific, Rockford, IL) with protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and sodium pervanadate (Sigma-Aldrich) and MG132 (Millipore, Billerica, MA). After protein determination with BCA kit (Pierce/Thermo Scientific), equal amounts of denatured protein were subjected to 4% to 20% gradient SDS-PAGE and electroblotted onto Immobilon-P PVDF transfer membrane (Millipore). After blocking nonspecific binding, blots were probed with appropriate primary antibodies and corresponding horseradish peroxidase–conjugated anti-rabbit or anti-mouse secondary antibodies. Signal was revealed with SuperSignal West Pico chemiluminescence reagent (Pierce/Thermo Scientific), detected by using ImageQuant LAS4000 mini and analyzed with ImageQuant TL (GE Healthcare Life Sciences, Pittsburgh, PA).

For immunoprecipitation, 200 µg of total lysate protein were pre-cleared, and then antigen-specific antibodies were added and the mix was incubated overnight at 4˚C with rotation. Then 20 µL of protein A/G agarose beads were added and the mix was further incubated for another 4 hours. After spinning down the beads and washing three times with modified RIPA/IP buffer, 25 µL of reducing sampling buffer were added to the pellets and the suspension was reduced and denatured through boiling, ice-chilling and spinning at 15,000 X g for 10 minutes at room temperature. Equal volumes of cleared supernatants were subject to gradient SDS- PAGE, further blotting and probing with antibodies.

**RT-qPCR**

RNA was extracted and cDNA generated using standard methods. RT-qPCR (qPCR) was performed in our core facility using TaqMAN Gene Expression Assays with all
primers and probes from Applied Biosystems (Foster City, CA) on an Mx 3000P platform and related software (Stratagene; La Jolla, CA). For qPCR, expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Primers and probes are listed in the Supplement.

**Enzyme-linked immunosorbent assays (ELISA)**

All antibodies used are listed in Supplementary Table 2. Cultures were inoculated at a cell density of $10^6$/ml of complete medium in all experiments for comparison, incubated for one day, and then supernatants of the suspensions were obtained from three independent repeat experiments for ELISA. For cells producing IgH and IgL, the same supernatants were used for measurements of both proteins. To measure the amounts of immunoglobulins in supernatants, quantitative ELISA was done by using anti-human IgG heavy chain and anti-human Ig\(\lambda\) light chain antibodies with sandwich enzyme-linked immunosorbent assays (Bethyl Laboratories, Montgomery, TX) according to manufacturer’s protocols. Optical density was read on a microplate reader (BIO-RAD) and protein quantity was calculated according to standard curves.

**Microarray**

Gene expression studies were performed using cDNA from 3 paired specimens of ALMC1 cells treated with si[IGLCR] or si[-] on the Illumina platform using the standard protocols for HumanHT-12 v4 Expression BeadChip at the Yale Center for Genome Analysis, New Haven CT. The BeadStudio suite of programs was used to calculate the expression values for probe sets (Illumina Inc., San Diego, CA). These studies can be accessioned with NCBI GEO number GSE54507.

**Statistics**

PRISM (GraphPad, San Diego, CA) was used for descriptive statistics and analyses. All experiments were repeated a minimum of three times in triplicate wells unless
otherwise noted. For microarray, Bioconductor packages Lumi/Limma were used to calculate the fold changes and $P$-values\textsuperscript{16,17}. TmeV was used to produce the heatmap of gene expression values for the regulated genes\textsuperscript{18} whose expression differed by $> 1.5$ fold with multiple hypothesis testing with $P < 0.10$. Web-based resource DAVID was used to calculate the enrichment of functional categories (DAVID Bioinformatics Resources 6.7, NIAID, NIH)\textsuperscript{19}. The combined heatmap and Gene Ontology (GO) enrichment categories were generated using the GeneAnswers package in Bioconductor\textsuperscript{20}. 


RESULTS

Knockdown of IgL not IgH causes effector caspase activation

With siRNA targeted to the IgL or IgH variable region mRNA of ALMC1 (and ALMC2) or EJM cells, at 24 hours expression of IgL was reduced as appreciated by flow cytometry dot plot (Fig.1a), and expression of either IgL or IgH was reduced as shown by flow cytometry histograms (Fig.1b) and by differences in MFI (Fig.1c). With this approach to knockdown of IgL, IgH or IgL-and-IgH, reductions in cell viability and proliferation and increases in caspase 3/7 activity were observed in cells with IgL but not IgH or IgL-and-IgH knockdown (Figs.1d, 1e). With IgL knockdown increased intracellular staining for IgH was observed (Figs. 1a, 1f).

Knockdown of IgL activates the unfolded protein response and causes an ER stress response

After knockdown of IgL in clonal cells making IgGλ, IgH production was maintained while intracellular IgL was diminished (Fig. 2a). From lysates of control si[-] and si[IGLC] treated cells, IgH was pulled down to assess immunoprecipitates (IP) for IgL and for the chaperone GRP78. The results (Fig. 2b) show that IgH is associated with GRP78. Moreover, IP of GRP78 shows association with IgH as well although we note that in the immunoblots from both cell lines there may be contamination in the IgH lanes by the IP antibody for GRP78. Nevertheless, the relevant features of IgL knockdown, GRP78 induction and IgH/GRP78 association can be appreciated.

The UPR is triggered by dissociation of GRP78 from the activators IRE1α, PERK and ATF6. In Figure 2c, prompt activation of the UPR is seen with expression of CHOP, GRP78 and XBP1s. In Figure 2d increased production of IRE1α, GRP78 and CHOP is seen within hours of UPR activation. Given the evidence for likely caspase-dependent apoptosis with IgL knockdown, the timeline of changes in expression of pro-apoptotic BH3 family members was studied and four-fold up-regulated expression of NOXA and up-regulated
expression of *PUMA* but not *BIM* in ALMC1 cells, and to a lesser degree of *PUMA* and *NOXA* in EJM cells, was seen (Fig. 2e). Moreover, by 20 hours after IgL knockdown, 25% of ALMC1 cells showed evidence of mitochondrial depolarization reversible by pan-caspase inhibition, consistent with the results in Figures 1d and 1e, and therefore caspase dependent (Fig. 2f). EJM cells treated in the same way did not display increased levels of mitochondrial depolarization. By AnnexinV/PI staining, in ALMC1 cells there was an average of 29.8 +/- 6.8% specific apoptosis associated with IgL knockdown (n=3) and none with IgH or combined IgL-and-IgH knockdown. Pan-caspase inhibition reversed this effect (Figure 2g).

In EJM cells, despite the modest increase in caspase 3/7 activity appreciated by bioluminescence (Fig. 1e) and the transient up-regulation of *NOXA* (Fig. 2e), evidence of mitochondrial depolarization by JC-1 staining (Fig. 2f) and of early or late apoptosis by AnnexinV/PI staining was not found (data not shown).

The differences between ALMC1 and EJM cells were then examined further. ALMC1 cells produce an intact IgGλ with excess IgL while EJM cells have two populations by flow cytometry, including a minor one that makes IgL only (Fig. 1a). The efficiency of IgL knockdown with variable region siRNA was lower in EJM than in ALMC1 cells (Fig. 1c) and the relative decrease in cell viability and proliferation (Fig. 1d) and increase in caspase 3/7 activity with IgL knockdown were also smaller in EJM than ALMC1 cells (Fig. 1e). (Of note, the efficiency of *GAPDH* knockdown was best in ALMC1 cells (Supplementary Fig. 1)). Both cell lines, however, have similar timelines of IgL knockdown (Fig. 2a) and both show the co-association of IgH and GRP78 (Fig. 2b) and activation of the UPR (Fig. 2c, 2d). Unlike the *CHOP* preeminent pattern of UPR activation in ALMC1 cells, a *GRP78* preeminent pattern occurs in EJM cells (Fig. 2c).

In ALMC1 cells, CHOP is significantly increased compared to EJM cells (Fig. 2h) and there was more NOXA, an MCL-1 antagonist, pulled down with MCL-1 from ALMC1 than from EJM cells (Fig. 2i). CHOP is viewed as a mediator of a terminal UPR, and MCL-1, an anti-apoptotic Bcl-2 family member, as a critical co-mediator of apoptosis in human myeloma
These results are consistent with a terminal ER stress response in ALMC1 but not EJM cells.

Multiple factors may contribute to this difference, including the presence of an IgL only sub-population of EJM cells and the lower efficiency of IgL knockdown in EJM cells, as well as the differences between ALMC1 and EJM cells with respect to the ratios of IgL and IgH production (shown below) and the production by EJM cells of IgL of two different sizes (Supplementary Fig. 2). Although the process of intact antibody formation is disrupted by IgL knockdown, the amount of IgL available in EJM cells may be adequate to match the amount of intracellular IgH waiting to be paired.

Treatment of plasma cells with si[IgLCR] reduces immunoglobulin secretion and cell viability

In order to design si[IgLCR], one pool of siRNA specific for IgL CR, four consensus region targets in the IgL constant region were identified. Each target is listed below the relevant CR consensus sequence in Table 1 (the pool is in Supplementary Table 1). The availability of one pool would in theory allow reduction of IgL production and secretion in multiple specimens without regard for variable region sequences; it would also allow an evaluation of the differences in secretion between ALMC1 and EJM cells using the same reagent. (Subsequently the IgL CR of both cell lines was determined to be concordant with the siRNA targets (data not shown)).

Five human myeloma cell lines that produce IgL were treated with si[IgLCR], demonstrating a 45% average reduction in intracellular IgL MFI after less than a day (Fig. 3a) and by ELISA an overall reduction of IgL secretion of 45% (Figs. 3b, 3c). IgH secretion was markedly reduced as well at 1 day after IgL knockdown (Fig. 3d). EJM cells secreted the most IgL (Fig. 3b). When treated with si[IgLCR] EJM cells secreted about 35% less IgL in 1 day than control cells (Fig. 3c). In comparison, ALMC1 cells treated with si[IgLCR] secreted about 60% less (Figs. 3b, 3c). EJM cells also secreted about half the amount of IgH produced by ALMC1 cells and when treated with si[IgLCR] secreted only 14% less IgH; in
contrast, ALMC1 cells treated with si[IGLCCR] secreted almost 50% less IgH (Fig. 4d). This difference led to the concept that the ratio of post-knockdown IgL and basal IgH secretion might be related to the triggering of the terminal UPR. This ratio was greater in EJM cells at 3 (30μg/ml ÷ 10μg/ml per day per million cells) versus 0.5 (10μg/ml ÷ 20μg/ml) in ALMC1 cells. There likely were lower levels of unpaired IgH in EJM than in ALMC1 cells with IgL knockdown, a difference that may contribute to the lack of a terminal ER stress response in EJM cells.

To examine further the activation of the UPR and changes in NOXA expression with CR knockdown, ALMC1 cells that secrete both intact IgGλ and IgL were compared to MM.1S cells that secrete only IgL. As shown in Figure 3e, CHOP, GRP78, XBP1S and NOXA were all substantially up-regulated in ALMC1 but not in MM.1S cells, while the efficiency of IgL knockdown was the same for both. Caspase 3/7 activity and cell viability and proliferation were then studied after si[IGLCCR] treatment, and the former was increased and the latter decreased significantly in cells making intact IgGλ (Figs. 3f, 3g).

The terminal ER stress response with si[IGLCCR] treatment is NOXA dependent

The heatmap of genes significantly regulated in ALMC1 cells by si[IGLCCR] treatment is shown in Supplementary Figure 3. CHOP (also known as DDIT3) is the most highly up-regulated gene. The detailed depiction of the most up-regulated genes (Fig. 4a) and the integrated view provided by a Gene Ontology heatmap (Fig. 4b) of the functional classes of activated genes demonstrate that a high level of ER stress is induced by the load of unpaired IgH and that genes involved in the UPR and ERAD are up-regulated in concert.

The circuitry of intrinsic terminal ER stress responses in clonal plasma cells remains unclear. The IRE1α pathway and the downstream activation of JNK by phosphorylated ASK1 can lead to apoptosis of plasma cells. Arachadonic acid can inhibit ASK1 phosphorylation in plasma cells by activating protein phosphatase 5. In cells treated with si[IGLCCR], however, there was no increase in phosphorylated JNK at 24 hours by IB and no effect of arachadonic acid at 72 hours by MTT assay, suggesting that IRE1α-mediated
activation of ASK1 did not play a significant role in si[IGLCr]-related apoptosis (data not shown).

A simultaneous double knockdown technique was then employed, asking whether simultaneous knockdown of IgL expression along with CHOP or NOXA would change the level of caspase 3/7 activity. There was no difference in the levels of caspase 3/7 activity between si[IGLCr] and si[IGLCr+CHOP] treated cells at 169+/− 31% and 170+/−34% of controls respectively (P=0.87, n=5), while si[CHOP] treated cells had levels that were 102+/−43% of controls. Also, cells treated with si[IGLCr+CHOP] showed increased levels of NOXA as did si[IGLCr] treated cells (Fig. 4c). In contrast, si[IGLCr+NOXA] treatment significantly reduced caspase 3/7 activity (Fig. 4d, 4e). Although NOXA up-regulation had been noted in EJM cells (Fig. 2e), increased expression began earlier, was greater and was observed for a longer time in ALMC1 than in EJM cells (Fig. 2e), consistent with the comparative increase of NOXA in ALMC1 cells in the IP of MCL-1 (Fig. 2i).

Treatment of patient plasma cells with si[IGLCr] reduces IgL production and increases caspase 3/7 activity

With CD138-selected specimens from patients with AL highly enriched suspensions of plasma cells were secured11. The patient characteristics, number and uses of specimens are detailed in Supplementary Table 3. In Figure 5a, an example of prompt and effective treatment with si[IGLCr] causing an 85% reduction in intracellular IgL immunofluorescence is shown. In Supplementary Figure 4, immunoblots of 2 specimens are shown, demonstrating significant reductions in IgL with si[IGLCr] treatment. As shown in Figure 5b, in 16 specimens evaluated by qPCR, the average reduction in IgL message exceeded 70%, and as shown in Figure 5c in 13 specimens evaluated by flow cytometry the average reduction in MFI was 51%. In 10 instances for which there are both qPCR and flow cytometry data, as shown in Figure 5d, the reductions in MFI were significantly correlated with the reductions in message by linear regression analysis (r²=0.56, P < 0.01). In 5 instances, the plasma cells made both an intact immunoglobulin and IgL, and in those cases
a pattern of increased intracellular IgH immunofluorescence related to the degree of
reduction of IgL immunofluorescence could be seen (Fig. 5e).

Caspase 3/7 activity was evaluated with si[IGLCR] or si[-] in 6 specimens making IgL
only, giving RLU of 24186+/−19114 and 22999+/−15772 in si[IGLCR] and si[-] treated
 aliquots respectively (P=0.57, two-tailed paired t-test). In contrast, when caspase 3/7 activity
was evaluated in 5 specimens making intact antibodies, as shown in Figure 5f, there was a
significant increase in caspase 3/7 activity with si[IGLCR] treatment (P=0.04, two tailed
paired t test). These results support the conclusion that one siRNA pool targeting consensus
sequences in the IgL constant region message can effectively reduce both IgL message and
IgL protein production without regard for the diversity of IgL variable region sequences, and
can also trigger a terminal ER stress response in cells making intact antibodies.
DISCUSSION

We report that the targeting of consensus sequences in the constant region of λ light chain genes with one pool of siRNA (si[IGLC\text{CR}]) can rapidly and substantially reduce λ light chain (IgL) production in numerous clones of λ plasma cells without regard for the diversity of the variable regions. Moreover, in λ plasma cells making intact Ig, si[IGLC\text{CR}] also can activate both UPR and ERAD with terminal potential because of the stress associated with unpaired IgH in the ER. Preliminary data indicate that the terminal potential is inversely related to the ratio of residual IgL to basal IgH, a hypothesis amenable to further testing. The ratio depicts the relative availability of a light chain to pair with a heavy: with a ratio < 1 both the excess of unpaired IgH and the ER stress-related terminal potential may be higher.

Activation of the UPR is a complex interplay of pathways that can be associated with either restored homeostasis or apoptosis\textsuperscript{27}. The UPR is constitutively activated in plasma cells as they differentiate from B cells with increased demand for protein folding and ER trafficking capacity; it is particularly the activity of XBP1s that escorts the metamorphosis of B cells into morphologically distinct antibody-producing cells\textsuperscript{6}. In myeloma, the UPR has been a major theme in the study of the mechanisms of action of proteasome inhibitors\textsuperscript{28-31}. The importance of the PERK-ATF4-CHOP arm of the UPR as a potential indicator of caspase-dependent apoptosis highlights a paradoxical aspect of the role of the UPR in plasma cell biology\textsuperscript{32}.

In this report, knockdown of IgL production and accumulation of unpaired intracellular IgH activate the UPR and ERAD in plasma cells making intact immunoglobulins but not in plasma cells making only IgL. Of note, the loss of IgH production with manufacture of only IgL, and the possible toxicity of IgH to plasma cells, are fundamental themes in plasma cell biology\textsuperscript{7,33-36}. After passage in culture, hybridomas often lose IgH production and make IgL only, and the majority of human myeloma cell lines produce IgL without IgH partners\textsuperscript{33}. IgL-deficient mice attain a complete block in B cell development at the stage when light-chain rearrangement should occur, resulting in surface IgM deficiency, retention of unpaired IgH in the cytoplasm, and lack of plasma cells\textsuperscript{37}. Normal human plasma cells make more IgL than
IgH, possibly to minimize intracellular accumulation of unpaired IgH. In 20% of cases of multiple myeloma, only IgL are made. Moreover, when myeloma relapses after plateau phase, we often observe “light-chain escape”, the emergence of a modified phenotype in which IgL replace, or are produced far in excess of, IgH.

The model of terminal UPR and ERAD activation that we report is caspase and NOXA dependent. Regulation of NOXA has been related to DNA damage as well as ERAD and epigenetic changes that enhance the activity of specific transcription factors; moreover, in vitro, bortezomib-induced apoptosis has been shown to depend on NOXA. The model we report provides the opportunity to investigate in greater detail how plasma cells may overcome, adapt or succumb to intrinsic terminal ER stress signals.

siRNA therapeutics are in clinical trials for transthyretin-related amyloidosis, delivered via lipid nanoparticles targeting hepatic cells producing transthyretin; moreover, investigators interested in AL have recently demonstrated that RNA interference with IgL production is feasible and merits further study. Targeting specific types of cells, such as clonal marrow plasma cells, for RNA interference poses additional major challenges. The significance of the results with si[IGLCCR] with respect to turning off IgL production and secretion may prove relevant to light-chain mediated diseases. Some of the AL patient samples treated with si[IGLCCR] were obtained from patients whose plasma cell disease had not responded to conventional therapies including bortezomib; the reduction of IgL message in si[IGLCCR] treated cells may reflect the importance of RNA degradation to the malignant plasma cell phenotype and, although the reductions were in some cases substantial, they were also notably variable, accenting the need for skepticism as this work continues. Further study of si[IGLCCR] must seek to optimize siRNA design, packaging and delivery for in vivo testing, and this report encourages those on-going efforts.
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AUTHOR CONTRIBUTIONS

PZ and XM conceived, designed and conducted experiments and wrote the paper; CC conducted clinical research, obtained patient marrows, and wrote the paper; LI oversaw microarrays, analyzed and presented gene expression studies, and wrote the paper; and RLC conceived the design of the research and wrote the paper.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.
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Table 1. Potential target consensus sequences in \(\lambda\) light chain constant region genes

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1IMGT = ImMunoGeneTics information system, www.imgt.org.
2Numeration is sense strand 5’ to 3’.
**Figure 1. Plasma cell light and heavy chains can be knocked down with siRNA.**

a. The flow cytometry plots in the upper row (si[-]) show that ALMC1 and EJM cells both produce intact IgG\(\lambda\) immunoglobulins but that EJM cells contain a subpopulation producing IgL without IgH. In the plots in the lower row, cells at 24 hours after si[IGLC] treatment showed a leftward shift when compared to si[-] controls (top). This shift represents a marked decrease in IgL MFI. MFI values for IgL and IgH are shown for each plot, demonstrating that MFI for IgL decreases and for IgH increases with IgL knockdown.

b. In these flow cytometry histograms, the IgL and IgH in ALMC1 cells are shown at 24 hours after treatment with si[IGLC] or si[IGH]. The MFI is markedly reduced (arrows) in both instances consistent with a marked reduction in intracellular protein.

c. ALMC1 and EJM cells produce intact IgG\(\lambda\) and IgL. Specific Interference with IgL or IgH mRNA leaves residual intracellular IgL or IgH staining as a percent of si[-] control. Plots reflect three independent repeats (median, range). Intracellular IgL MFI was reduced by a median of 72% in ALMC1 and 40% in EJM cells.

d. Reduction of IgL but not IgH or combined IgL-and-IgH production was associated with significantly reduced cell proliferation and viability by MTT assay at 24 hours (mean +/- SD).  
* \(P < 0.01\) by two-tailed paired t test in both cases (ALMC1, \(n=14\); EJM, \(n=9\)).

e. In ALMC1, ALMC2 (a sister cell line) and EJM cells, reduction of IgL but not IgH or IgL-and-IgH production was associated with significantly increased levels of caspase 3/7 activity at 24 hours (mean +/- SD).  
* \(P < 0.01\) by two-tailed paired t test, \(n=4\) for each cell line.

f. In ALMC1 cells producing intact IgG\(\lambda\), specific interference with IgL mRNA is associated with increased intracellular IgH staining as this histogram demonstrates. In this typical example, MFI for IgH is increased by 41%.

**Figure 2. Intracellular unpaired IgH trigger the UPR and can cause apoptosis.**
a. Immunoblots (IB) from ALMC1 cells (top) treated with si[IGLC] or si[-] control over a 30 hour period show reduction in IgL and persistence of IgH intracellular proteins as do IB of EJM cells (bottom) treated in the same way.

b. IB of GRP78 and IgH immunoprecipitates (IP) from ALMC1 and EJM cells treated with si[IGLC] or si[-] control show the co-immunoprecipitation of IgH with GRP78, and the marked decrease in IgL and increase in GRP78 that occur at 30 hours after si[IGLC] treatment.

c. Real-time PCR (qPCR) for markers of the unfolded protein response (UPR) was performed with cDNA from ALMC1 and EJM cells treated with si[IGLC] or si[-] control for the indicated times. In si[IGLC] treated cells there were significant increases in all three UPR markers at 8 and 16 hours. Mean +/- SD are shown with \( P < 0.01 \) compared to control by two-tailed paired t test, \( n=3 \). Three independent repeat qPCR were performed in duplicate wells.

d. Immunoblots for three markers of the UPR were performed with whole cell lysates from cells treated with si[IGLC] or si[-] control for the indicated times. As this typical IB shows, in si[IGLC] treated but not control cells there were significant increases in all three UPR markers.

e. Real-time PCR (qPCR) for pro-apoptotic BH3 family members was performed with cDNA from cells treated with si[IGLC] or si[-] control for the indicated times. In si[IGLC] treated ALMC1 cells there was a 3-to-4-fold increase in NOXA expression maintained at 8, 16 and 24 hours. The increase in NOXA expression in EJM cells, however, was lower than in ALMC1 cells and also lower than the increase in PUMA expression. Two independent repeat qPCR were performed in duplicate.

f. ALMC1 but not EJM cells treated with si[IGLC] displayed significantly increased levels of mitochondrial depolarization at 24 hours as represented by a significant decrease in the percentage of si[IGLC] relative fluorescence units (RFU) of green JC-1 monomer compared to si[-] by flow cytometry. Increased levels of mitochondrial depolarization were not present when si[IGLC] treated cells were cultured with 50μM Z-VAD-FMK. Mean +/- SD are shown with \( * P = 0.01 \) by two-tailed paired t test, \( n=3 \).
g. ALMC1 cells treated with si[IgL] displayed increased levels of apoptosis manifest as Annexin V-positivity at 24 hours. Viable cells were reduced to 64% with si[IgL] treatment. Apoptosis did not occur when cells treated with si[IgL] were cultured with a pan-caspase inhibitor (Z-VAD-FMK, 50 µM).

h. IB comparison of ALMC1 and EJM cells undergoing IgL knockdown shows that CHOP is markedly increased in ALMC1 compared to EJM cells. This IB is representative of three performed.

i. IP of MCL-1 in ALMC1 and EJM cells treated with si[IgL] indicate by IB that more NOXA is pulled down with MCL-1 from ALMC1 than from EJM cells. Normalized levels of MCL-1 and NOXA in si[IgL] lanes are shown as percentages of si[-] levels. This IP/IB is a representative of two performed.

**Figure 3. Knockdown of the IgL CR reduces immunoglobulin secretion and cell viability.**

a. Five human MM cell lines that secrete IgL were treated with si[IgLCR] targeting the constant region of the IgL mRNA or si[-] control, and the residual MFI at 20 hours after knockdown are shown. Mean +/- SD, n = 3 observations per cell line. In all (n=15), the mean MFI of intracellular IgL after si[IgLCR] treatment was 55% +/- 23% of control indicating a 45% average reduction in less than a day.

b. The concentrations of secreted IgL in culture supernatants from these five human MM cell lines after treatment with si[IgLCR] or si[-] control were measured by ELISA. Cultures were charged with 10⁶ cells per ml and supernatant was obtained 24 hours later for measurement. All cells treated with si[IgLCR] secreted less IgL. Mean +/- SD are shown for three independent repeats per cell line with P-values obtained with two-tailed paired t test.

c. This graph shows the percent reductions in secreted IgL with si[IgLCR] treatment. Mean +/- SD are shown for three independent repeats per cell line. The mean percent reduction for all fifteen observations was 45% +/- 23%.
d. The concentrations of secreted IgH in culture supernatants from three human MM cell lines that secrete intact IgGλ after treatment with si[IGLCCR] or si[-] control were measured by ELISA. Mean +/- SD are shown for three independent repeats per cell line with P-values obtained with two-tailed paired t test. The mean reductions in secreted IgH were 45% +/- 9%, 43% +/- 22%, and 14% +/- 8% for ALMC1, ALMC2 and EJM cells respectively.

e. ALMC1 as IgGλ producing cells were compared to MM.1S as IgL only producing cells for evidence of activation of the UPR and change in the expression of NOXA by qPCR. At 20 hours after treatment the relative fold-changes in expression of the UPR markers GRP78, CHOP and XBP1S, and of NOXA, with si[IGLCCR] are substantial in IgGλ but not in IgL only producing cells. Assays were performed three times with cDNA from three independent repeat experiments. Mean +/- SD are shown with P-values obtained with two-tailed unpaired t test. Dotted line is qPCR control.

f. Caspase 3/7 activity was measured with the GloMax bioluminescence assay in two myeloma cell lines that produce intact IgGλ (ALMC1, EJM) and two that produce only IgL (MM.1S, OPM-2) after treatment with si[IGLCCR] or si[-]. Reductions in IgL in cells producing intact IgGλ were associated with significantly increased caspase 3/7 activity while in cells producing only IgL there was no increase in caspase 3/7 activity associated with IgL reductions. (In OPM-2 cells there was an apparent decrease in caspase 3/7 activity with IgL knockdown). Mean +/-SD of the changes in caspase 3/7 activity and P-values are shown, the latter obtained with two-tailed paired t test, n=3 for each cell line.

g. Cell viability and proliferation was measured by MTT assay in two myeloma cell lines that produce intact IgGλ (ALMC1, EJM) and in two that produce only IgL (MM.1S, OPM-2) after treatment with si[IGLCCR] or si[-]. Reductions in IgL in cells producing intact IgGλ were associated with significantly decreased cell viability and proliferation while in cells producing only IgL there was no decrease compared to si[-] controls. Mean +/-SD of the changes in MTT read-outs and P-values are shown, the latter obtained with two-tailed paired t test, n=3 for each cell line.
Figure 4. Treatment with si[GLC<sub>CR</sub>] triggers ER stress and NOXA-related apoptosis.

a. Gene expression studies were performed using cDNA from 3 paired specimens of ALMC1 cells treated with si[GLC<sub>CR</sub>] or si[-] on the Illumina platform using the standard protocols for HumanHT-12 v4. Expression BeadChip and the Beadstudio suite of programs were used to calculate the expression values for probe sets (Illumina Inc., San Diego, CA). A heatmap of the 99 genes whose expression differed > 1.5 fold between si[-] and si[GLC<sub>CR</sub>] treated samples by multiple hypothesis testing at \( P < 0.10 \) is shown in Supplementary Figure 3, increasing in expression from top to bottom. *PMAIP1 (NOXA)* is indicated in the Supplementary figure with 1.54-fold increased expression in cells treated with si[GLC<sub>CR</sub]]. The 24 genes that were most up-regulated in cells treated with si[GLC<sub>CR</sub>] are shown in this figure. *HSPA5 (GRP78)* is up-regulated 2.4-fold. *CHOP* (also known as *DDIT3*) is the most up-regulated gene consistent with activation of a terminal ER stress response.

b. Gene Ontology (GO) heat map was generated with GeneAnswers package in Bioconductor and shows gene expression values and pathways involved for the regulated genes, indicating that the UPR, ERAD and apoptotic pathways are notably involved.\(^{18}\)

c. In this IB of si[-], si*[CHOP], si*[GLC<sub>CR</sub>], and si*[GLC<sub>CR</sub>+CHOP] treated ALMC1 cells, there were reductions in CHOP in the si*[CHOP] and double knockdown cells, while in the si*[GLC<sub>CR</sub>] and double knockdown cells there were increases in GRP78, IRE1\(\alpha\), NOXA, MCL-1 and cleaved PARP.

d. In ALMC 1 cells at 20 hours after treatment, reduction of *IgL* expression was associated with increased caspase 3/7 activity while simultaneous reduction of *IgL* and NOXA was associated with substantially reduced caspase 3/7 activity. Cells in which NOXA alone was knocked down had on average 17% less caspase 3/7 activity than controls. Mean +/− SD with \( P\)-value obtained with two-tailed unpaired t test, n=9.

e. In this IB of si[-], si*[NOXA], si*[GLC<sub>CR</sub>], and si*[GLC<sub>CR</sub> + NOXA] treated ALMC1 cells, there were increases in GRP78, IRE-1\(\alpha\) and phosphorylated PERK in si*[GLC<sub>CR</sub>] treated cells, reductions in NOXA in the si*[NOXA] and double knockdown cells but no differences in the levels of MCL-1 with NOXA knockdown.
Figure 5. Treatment with si[IGLCCR] in patient cells reduces IgL.

a. A flow cytometry plot of CD138+ marrow plasma cells from a patient with a λ light chain monoclonal gammopathy causing systemic AL amyloidosis shows the results of treatment with si[IGLCCR] or si[-]. The upper panel shows the marked reduction in IgL at 20 hours, and the lower panel shows histograms indicating a reduction of MFI in si[IGLCCR] treated cells of 85%.

b. Real-time PCR (qPCR) was performed on CD138+ plasma cells (from patients with AL) treated with si[IGLCCR] or si[-] control. Overall the average residual λ light chain message by qPCR was 0.28 +/-0.2 of control (mean +/- SD; mean is shown in figure, n=16).

c. Flow cytometry for intracellular IgL was performed on CD138+ plasma cells (from patients with AL) treated with si[IGLCCR] or si[-] control, showing the average residual MFI was 49% (n=13).

d. In 10 cases we had sufficient CD138+ plasma cells to perform both qPCR (> 4x10^5) and flow cytometry (> 10^6) after treatment with si[IGLCCR] or si[-] control. The correlation is shown between residual MFI and residual message by linear regression (line of best fit) with 95% confidence intervals (r^2 = 0.56, P < 0.01).

d. In 5 specimens both the intracellular IgL and IgH in CD138+ cells were evaluated simultaneously by flow cytometry after si[IGLCCR] treatment. This figure suggests that the lower the IgL MFI became with si[IGLCCR] treatment, the higher the residual intracellular IgH MFI became (P > 0.05).

e. Five specimens of CD138+ plasma cells from patients with AL and abnormal λ light chain levels but with immunoglobulin heavy chains (IgGλ=4, IgAλ=1; open circles are two specimens from the same patient three months apart) were treated with si[IGLCCR] or si[-]. Caspase 3/7 activity was evaluated by bioluminescence 20 hours later and significantly
higher levels of caspase 3/7 activity were seen with si[IGLC_Ce] treatment. ($P=0.04$ with two-tailed paired t test).
si[\text{\textit{IGLC}}]

\text{si[Target]}
\text{si[-]}
\text{Isotype control}

si[\text{\textit{IGH}}]
For personal use only.
Isotype control

si[IGLC]

si[-]
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**ALMC1**
- IgL
- IgH
- β-Actin

**EJM**
- IgL
- IgH
- β-actin
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ALMC1

EJM

Fold-change (s/[G.L.C.] / s[−1])

Time (hours)

P < 0.01

P < 0.01

GRP78

CHOP

XBP1s
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**ALMC1**

**EJM**


For personal use only. on November 16, 2017. For personal use only.
si[IGLC] / si[-]

ALMC1

EJM

%RFU

Z-VAD-FMK (μM)

0  50  0  50
Annexin V

PI

si[−]

si[IGLC]

si[IGLC]+Z-VAD-FMK

85.7

64

87.5
2h

**Western Blot Analysis**

**ALMC1**

**EBM:**
- si[–]
- si[IGLC]
- si[–]
- si[IGLC]

**GRP78**

**IgH**

**IgL**

**CHOP**

**β-actin**
IP: MCL-1
IB:

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*si[IGLCCR]*

*si[-]*

IgL (μg/mL)

![Graph showing IgL levels with *si[IGLCCR]* and *si[-]* contrasts for different cell lines.](image)
3c
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Caspase 3/7 Activity (RLU)

Mean Δ %  +149  +11  +11  -29

P-value  0.002  0.03  0.06  0.22

si[−]  si[IGLCCR]
Mean $\Delta$ %

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- si[−] and si[IGLCCR]

- Genes: HSPA5, SYVN1, VIMP, FAM129a, CRELD1, SMIM14, DNAJB11, MANF, SDF2L1, WIP11, ASNS, HYOU1, WARS, HYOU1, ASS1, WARS, ASS1, TRIB3, INHBE, HERPUD1, HERPUD1, BEX2, CRELD2, DDIT3
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| si[-] | + | - | - | - |
| si[IGLCR] | - | + | - | + |
| si[CHOP] | - | - | + | + |

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si[-]  +  -  -  -  -  
si[IgLCCR]  -  +  -  +  
si[NOXA]  -  -  +  +  

IgL
IgH
IRE-1α
GRP78
PERK
p-PERK T980
MCL-1
NOXA
β-Actin
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Relative IgL expression

5b
$r^2 = 0.56, P < 0.01$

Relative $IgL$ Expression

% MFI (si[GLCcr] / si[-])

5d
One siRNA pool targeting the $\lambda$ constant region stops $\lambda$ light chain production and causes terminal endoplasmic reticulum stress

Ping Zhou, Xun Ma, Lakshmanan Iyer, Chakra Chaulagain and Raymond L. Comenzo