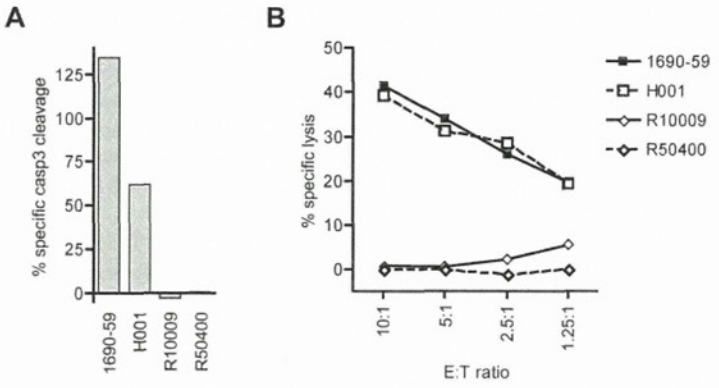

Figure 6. CD8 T-cell clone 2196.D11$_b$ exhibits specific cytotoxic activity for cyclin-A1–expressing primary AML samples. (A) Four-hour caspase-3 assay. Specific caspase-3 cleavage is shown after subtraction of spontaneous caspase-3 activation, and determined relative to staurosporine-induced cleavage as 100%. (B) Four-hour ^{51}Cr release assay at a range of E:T ratio with the same effector and target cells. H001 and 1690-59 are HLA A*0201 positive, and R10009 and R50400 are A*0201-negative.

drawbacks of a pure in silico approach. First, not all probe sets identified by the mathematical selection steps displayed the desired expression pattern. Second, after revising the target sequences of the probe sets, we found a high percentage of sequences to not represent a coding transcript. Finally, we found large differences between in silico data predictions and quantitative RT-PCR results. This might be because, as opposed to quantitative RT-PCR, microarrays do not provide linear data. Furthermore, the absence of a standardized protocol for sample processing and hybridization impacts the comparability of the different datasets.

Cyclin-A1 was highly expressed in AML cells, including the stem cell compartment of more than 50% of patients, and cyclin-A1 expression was highly restricted to malignant cells, a fact that was predescribed only based on semiquantitative data.[31] We previously noted that both WT1 and cyclin-A1 are expressed at significantly higher levels in LSCs than in HSCs,[14] but WT1 can also be detected in several nonhematopoietic organs, such as kidney, spleen, and ovary at levels potentially higher than in leukemic blasts (supplemental Figure 1C). In the present study, which is the first providing linear expression data of cyclin-A1 in AML, healthy hematopoiesis, and tissues in direct comparison, we now found that, except for AML, cyclin-A1 is expressed only in germ cells from testis, which is considered to be an immune-privileged site.[32] Minimal cyclin-A1 staining in healthy BM as described in immunohistochemistry in an earlier study[31] was not observed in our IF staining and might be the result of unspecific binding of the polyclonal antibodies used. Consequently, toxicities from recognition of normal cells while targeting cyclin-A1 appear unlikely.

Based on the expression pattern, cyclin-A1 falls into a group of antigens called cancer-testis-antigens (CTAs), which are characterized by expression in normal tissues restricted to immature germ cells in testis and by aberrant expression in cancers. There have been at least 2 systematic genome-wide studies to detect new cancer-testis genes (CTGs), but neither identified *CCNA1* as such a gene.[33,34] This is probably because hematologic malignancies, compared with solid tumors, have been found to infrequently express any of the known CTGs[35] and were therefore either not included or underrepresented in the cancer panels used in the 2 studies. Recently, a classification of CTGs based on a systematic analysis of in silico expression data differentiating CTGs as testis-restricted and testis-selective CTGs was proposed, with the latter displaying minimal expression in non-immune privileged tissues. Interestingly, testis-selective CTGs had a high probability of being autosomal, not belonging to a multigene family, and being associated with a known function in gametogenesis and/or meiosis.[33] Most of these characteristics of a testis-selective CTA apply to *CCNA1*, as it is located on chromosome 13q, is a single-copy gene, and has a known function in meiosis. The only predescribed leukemia-associated CTA PRAME was neither expressed in any LSC sample of our microarray datasets nor found overexpressed by quantitative RT-PCR in any of the AML samples (not shown). Thus, we think that cyclin-A1 can now be classified as the first described CTA associated with AML LSCs.

Cyclin-A1 is an alternative CDK2-associated type-A cyclin with an essential role in male gametogenesis, positively regulating the G_2/M phase transition in meiosis I.[17,36-38] The expression of meiotic genes in nonmeiotic non–germ cell malignancies has been observed with other CTGs, such as *SYCP-1* and *SPO11*, and expression of meiosis-associated genes has been hypothesized to contribute to chromosomal aberrations and therefore support the initiation and further cytogenetic clonal evolution of a malignancy.[39,40] In the case of cyclin-A1 in AML, this mechanism may not apply because, even though expression of cyclin-A1 has been shown to sustain the malignant phenotype,[21-23] our recently published data indicated higher cyclin-A1 expression in AML specimens with normal rather than aberrant karyotypes.[20] Furthermore, in case of APL, cyclin-A1 appears to be a target of the RAR-α fusion proteins rather than causal for the translocation.[41] Several lines of evidence suggest that cyclin-A1 can play a role in the cell cycle regulation of mitotic and especially malignant cells, with cyclin-A1 positively regulating the G_1/S transition independent of the particular cell type.[37,42] Therefore, the major leukemogenic mechanism resulting from overexpression of cyclin-A1 in myeloid cells might be accelerated S-phase entry rather than induction of chromosomal instability.[37]

Besides the expression pattern and physiologic function of a gene, another important characteristic of a broadly useful T-cell target is having a large number of available epitopes, and overexpressed LAAs would be predicted to have a much larger number of targetable AA sequences than the very limited distinct sequences present in unique leukemia antigens generated by translocations or mutations. Using 2 normal donors, we identified 8 immunogenic peptides restricted to at least 3 different HLA class I molecules. Such broad immunogenicity is probably facilitated by limited tolerance induction to CTAs.

The characterization of epitope 341-351 demonstrates one limitation of silico epitope prediction approaches for identifying immunogenic peptides. Whereas the observed binding to A*0201 of the 10-mer(341-350) from the immunogenic 15-mer could be predicted retrospectively by 3 of 3 tested prediction algorithms (SYFPEITHI,[43] BIMAS,[44] IEDB analysis resource[45]), no binding was predicted for the 11-mer; therefore, this epitope would have never been identified without the use of a peptide library. A possible

explanation for the fact that all clones generated to the larger 15-mer peptide were reactive exclusively against the 11-mer might be that T cells reactive with the 10-mer had been eliminated by negative selection because of cross-reactivity to a similar epitope on a high-abundance self-protein. Indeed, for the shorter epitope 341-350, which did bind to A*0201 more stably, similar though not identical epitopes were identified in cyclin-A2 and cyclin-B1, which are broadly expressed self-antigens.

The efficient cytotoxic activity of cyclin-A1-specific T-cell clones demonstrated in this study may reflect several factors: Cyclin-A1 expression appears significantly higher than other AML associated antigens such as WT1, PRAME, HMMR, and PRTN3 (Li et al[46]; S.O., unpublished data, May 2011). Moreover, the dramatic periodicity of cyclin-A1 expression is accompanied by high degradation rates regulated by the ubiquitin-proteasome-mediated pathway.[47] This suggests that epitopes are likely being made available for presentation. Finally, although the testis appears to provide peripheral immune privilege to germline cells,[32] little is known about negative selection during development of T cells reactive with gametogenesis-associated antigens, especially CTAs. However, reduced deletion and/or central tolerance for testicular antigens would explain both the high incidence of autoimmune mediated infertility observed in patients after traumatic disruption of the blood-testis barrier[32] and the high cytotoxic activity of the T-cell clones generated in this study. Further studies analyzing both the functionality and the TCR affinity of spontaneous T-cell responses in patients with cyclin-A1 expressing diseases are initiated to further investigate this assumption.

Recently, a National Cancer Institute pilot project to prioritize cancer antigens made an effort to not only develop a priority-ranked list of the known cancer antigens but to also provide a list of weighted "ideal" antigen criteria/characteristics for evaluating other antigens.[13] According to these criteria, cyclin-A1 would appear to be a highly suitable antigen for targeting AML based on: a very selective oncofetal expression pattern with uniformly high expression in leukemia in a large fraction of patients, being leukemogenic when overexpressed in mice, expression in the LSC compartment, having many epitopes available, and having an intracellular location. Thus, applying the weighted criteria of the National Cancer Institute pilot project to cyclin-A1 resulted in a ranking for cyclin-A1 between 3 to 5 among the 75 antigens that were ranked.[13] Therefore, cyclin-A1 appears to now be the first described non–X-linked leukemia-testis-antigen with optimal features for targeting of AML LSCs by T cell–based therapy approaches, such as vaccination and/or adoptive T-cell transfer.

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Authorship

Contribution: S.O. designed and performed the experiments and prepared the final manuscript; R.M. and I.L.W. provided and analyzed microarray data; T.S. performed FACS assays; D.S. provided and analyzed microarray data and provided patient samples; U.K., M.B., E.H.W., and B.W. provided patient samples; K.R.L. and Y.E.C. performed IF and FACS assays; M.H. performed FACS sorting; Y.A. performed molecular cloning and retroviral transfection; P.D.G. designed the concept, reviewed the data, and prepared the final manuscript; and all authors reviewed and edited the manuscript.

Conflict-of-interest disclosure: A patent application for the use of cyclin-A1 as a T-cell target antigen has been submitted by S.O., P.D.G., R.M., and I.L.W. The remaining authors declare no competing financial interests.

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Autophagy Creates a CTL Epitope That Mimics Tumor-Associated Antigens

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Abstract

The detailed mechanisms responsible for processing tumor-associated antigens and presenting them to CTLs remain to be fully elucidated. In this study, we demonstrate a unique CTL epitope generated from the ubiquitous protein puromycin-sensitive aminopeptidase, which is presented via HLA-A24 on leukemic and pancreatic cancer cells but not on normal fibroblasts or EBV-transformed B lymphoblastoid cells. The generation of this epitope requires proteasomal digestion and transportation from the endoplasmic reticulum to the Golgi apparatus and is sensitive to chloroquine-induced inhibition of acidification inside the endosome/lysosome. Epitope liberation depends on constitutively active autophagy, as confirmed with immunocytochemistry for the autophagosome marker LC3 as well as RNA interference targeting two different autophagy-related genes. Therefore, ubiquitously expressed proteins may be sources of specific tumor-associated antigens when processed through a unique mechanism involving autophagy.

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Introduction

Exploring the mechanisms underlying cancer-specific CTL recognition is important in the establishment of safe and effective immunotherapy. The discrimination of normal and malignant cells by CTLs depends on the repertoire of antigenic peptides displayed via the MHC class I molecules of these cells. As both normal and malignant cells possess antigen-processing machinery, the repertoire displayed depends on the expression level of the target proteins. These target proteins are degraded in the cytoplasm by the proteasome, with the resulting short peptides being translocated into the endoplasmic reticulum, where they bind to MHC class I molecules [1]. Therefore, tumor antigens are bascially determined by their expression pattern, not by the machinery responsible for the processing. If malignant cells possess unique antigen-processing machinery, they may create cancer-specific antigenic peptides, even from ubiquitously expressed proteins.

Autophagy is equally as important for peptide degradation as is proteasomal lysis of cytoplasmic proteins and organelles [2]. In macroautophagy (referred to as autophagy hereafter), autophagosomes are formed and then fused with lysosomes to produce autolysosomes, where proteins are degraded by lysosomal proteases. Upregulation of autophagy is an adaptation to stresses such as starvation, oxidant injury and genomic damage. Although autophagy normally functions under physiological conditions, degradation by the autophagosome is also important under aberrant conditions, such as cancer [3]. Thus, autophagy plays a pivotal role not only in suppressing tumorigenesis but also in promoting tumor progression. Autophagy is also known to affect both innate and adaptive immunity [4,5]. Particularly in the latter, autophagy participates in MHC class II antigen presentation [6,7], although reports regarding MHC class I presentation via autophagy are sparse.

Herein, we provide evidence of a cancer-specific CTL epitope created through both autophagy and proteasomal action, derived from the ubiquitously expressed protein puromycin-sensitive aminopeptidase (PSA). The data suggest that unique processing accounts for differential epitope liberation between normal and cancer cells.

Results

Artificial Antigen Presenting Cells (aAPCs) Effectively Induce Tumor-specific CTLs

Generating tumor-specific CTLs generally requires autologous tumor cell lines. To bypass the substantial difficulties in establishing such lines, we have sought to use aAPCs to express endogenous tumor-associated peptides on given HLA molecules. The K562 cell line is an ideal platform for this use due to the absence of HLA expression on their cell surface; thus, K562 cells

could serve as APCs when an HLA needs to be exogenously expressed [8]. To establish the K562-based aAPCs, K562 cells were stably transduced with HLA-A24, CD86 and 4-1BBL with lentiviral vectors, and a positive population was isolated (Figure 1A) to generate tumor-specific T cells from HLA-A24-positive donors. After 2 rounds of stimulation, polyclonal T cells secreted IFN-γ in an antigen-specific manner. Using a limiting dilution culture of the bulk CTL line, we established a T-cell clone, designated as 16F3, that produced IFN-γ against HLA-A24-expressing K562 cells (referred to as A24-K562) but not against dermal fibroblast cells, normal bronchial epithelial cells or EBV-transformed B lymphocytes (B-LCLs) (Figure 1B, 1C). Moreover, 16F3 recognized three out of five pancreatic cancer cell lines (Figure 1D) in an effect that was blocked by anti-HLA class I Abs (data not shown). These cell lines were positive for HLA-A24 expression (data not shown). However, 16F3 did not produce IFN-γ in response to T2-A24 cells pulsed with the 22 HLA-A24-restricted peptides derived from previously reported tumor antignes (data not shown).

PSA-derived Epitope Induces a Specific CTL

As 16F3 recognized antigens specifically expressed on cancer cells (Figure 1B–D), we explored its nature with a cDNA library derived from K562 cellular mRNA. The library was cloned into an expression vector and divided into 960 pools, each containing 100 cDNA clones. Plasmid DNA was extracted from each pool and then transfected into HLA-A24-expressing HEK-293T (referred to as A24-293T) cells. In the first screening, one of the pools induced 16F3 to produce IFN-γ (data not shown). The positive pool was subcloned into individual cDNA clones and screened (Figure S1A). The single clone, 8G, that induced 16F3 to produce IFN-γ proved to be a variant cDNA of PSA (NM_006310). This variant PSA cDNA is generated via intronic polyadenylation [9] and consists of exons 1–12 with part of an intron located directly downstream of exon 12 (Figure S1B). To identify the epitope recognized by 16F3, A24-293T cells were transfected with plasmids encoding truncated forms of PSA. Upon analysis, the antigenicity disappeared with the following mutations: C-terminal truncation between amino acid residues 200 and 300 and N-terminal truncation between 241 and 251 (Figure S1C). To define the N- and C-terminal ends more precisely, further truncation was performed within this region. As inclusion of isoleucine at position 261 was essential for the stimulation of the clone (Figure S1D), this amino acid was indicated to be located in the C-terminus. Exclusion of aspartic acid at position 250 abolished antigenicity; thus, we considered an unusually long 12–mer peptide, DYFNVPYPLPKI (residues 250–261), having tyrosine at the second position as a primary anchor for HLA-A24 binding [10], to be the minimal epitope. A synthesized 12-mer peptide (residue 250–261) was demonstrated to bind HLA-A24 molecules more efficiently than a CMV pp65 peptide (Figure S1E) and to be recognized by 16F3 (Figure S1F). Failure of recognition of KP-2 and SUIT-2 cells by 16F3 was attributable to the absence of epitope presentation because both are recognized by 16F3 when pulsed with the peptide (Figure S2). A 9–mer peptide, DYFNVPYPL (residue 250–258), which was highly scored for HLA-A24 binding by the BIMAS software (http://www-bimas.cit.nih.gov/molbio/hla_bind/), was not recognized by 16F3 (Figure S1D).

PSA has Two Variant Forms to be Presented

The PSA protein is expressed ubiquitously [11]. Thus, we investigated the expression levels of both full-length and variant PSA mRNA with RT-PCR. The primers for detecting the full-length forms corresponded to exon 11 and exon 13, and those for the variant forms corresponded to exon 11 and part of the intron located directly downstream of exon 12 (Figure 2A, top). Both forms of PSA mRNA were detected in all of the samples (Figure 2A, bottom). To assess protein expression, Western blot analysis was performed using monoclonal antibodies against full-length PSA. The PSA protein was also expressed in all samples (Figure 2B). As the expression pattern of PSA was not concordant with that of sensitivity to 16F3, we speculated that 16F3 might recognize a distinct peptide derived from a separate protein that is antigenically close to the PSA-derived peptide. To examine this hypothesis, we investigated the CTL response against cells treated with PSA-specific siRNAs. The target sequences of three siRNAs were chosen outside of the PSA variant coding regions using BLOCK-iT™ RNAi Designer (Invitrogen). Because two pancreatic cancer cell lines, KP-3 and MIA PaCa-2, were able to accept siRNA efficiently, these cells were used in these experiments. The expression of the PSA protein was reduced in the cells treated with siRNAs specific for PSA (Figure 2C), which led to much lower capacities to stimulate 16F3 than with a negative control siRNA (Figure 2D), indicating that 16F3-mediated IFN-γ production occurs through recognition of PSA.

The Processing of the PSA Epitope with the MHC Class I Involves the Vacuolar Pathway

Next, we addressed the mechanisms underlying the epitope generation using specific inhibitors, as no difference in the mRNA expression of TAP1, TAP2, LMP2 or LMP7 was evident between 16F3-sensitive and 16F3-insensitive cells (data not shown). For this purpose, we prepared a CMV pp65-expressing aAPC [12]. Fixed APCs are typically used to examine the effects of drugs on antigen-processing mechanisms [13]. However, 16F3 did not recognize paraformaldehyde- or glutaraldehyde-fixed A24-K562 cells (Figure S3A), and further treatment of quenching with glycine did not recover the epitope recognition (Figure S3B). Therefore, IFN-γ production was measured in the presence of reversible inhibitors, such as BFA, CQ and BafA, to avoid the restoration of the epitope following inhibitor removal during co-culture with T-cells (Figure 3A–C). IFN-γ production was detected via intracellular staining with BFA, as this drug causes the accumulation of IFN-γ within the cytosol (Figure 3A), and via secretion assays with other inhibitors (Figure 3B, 3C). IFN-γ production by 16F3 was decreased after treatment with both a proteasome inhibitor, lactacystin and with BFA, inhibiting the endoplasmic reticulum to Golgi transport (Figure 3A, 3B). This effect was also observed for another HLA-A24-restricted CTL clone specific to CMV pp65 [14], indicating the involvement of the classical MHC class I presentation pathway. Unexpectedly, CQ, an inhibitor of acidification inside the endosome/lysosome, decreased epitope processing from PSA, but not from the CMV protein (Figure 3B). In addition, KP-3 cells treated with either CQ or BafA, another acidification inhibitor, decreased PSA epitope presentation (Figure 3C). CQ and BafA did not affect T-cell function during co-culture, as demonstrated by intact IFN-γ secretion by cognate peptide-pulsed target cells in the presence of the inhibitors (Figure 3B, 3C). These data indicate that a vacuolar pathway mediates the processing of the PSA epitope, contrasting with the classical MHC class I pathway for the CMV epitope, although both utilize the same HLA-A24 molecules for presentation.

Two examples have been reported with regard to MHC class I molecules loading epitopes via CQ-sensitive vacuolar pathways. One example indicates that recycled MHC class I molecules, as with to MHC class II, can assemble with peptides in the acidic environment of the late endosome [15]. The other example involves the intracellular trafficking of peptides to the endosome mediated by specific signals within the cytoplasmic domain of class

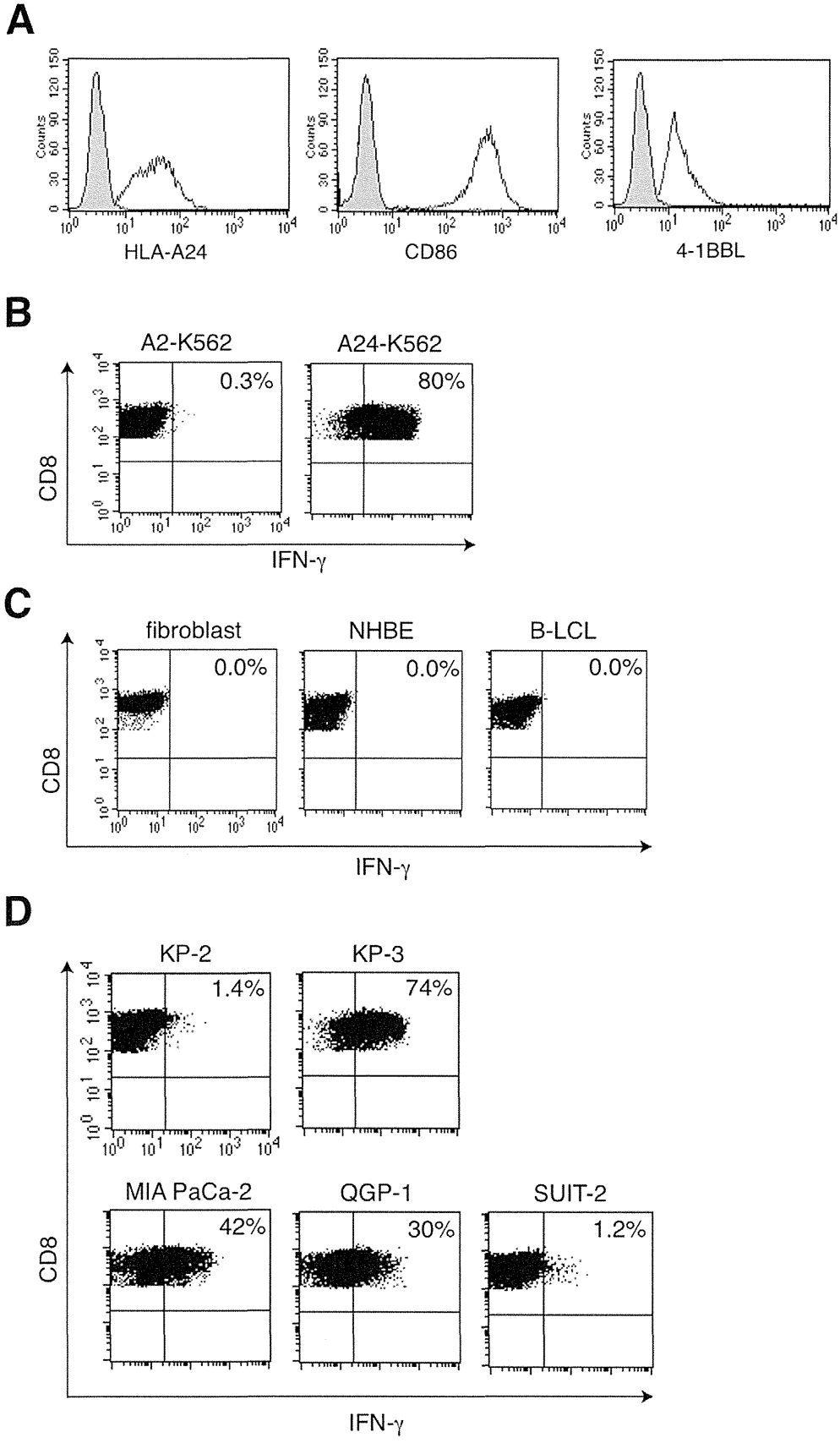
A
Counts
HLA-A24
CD86
4-1BBL
B
A2-K562
A24-K562
0.3%
80%
CD8
IFN-γ
C
fibroblast
NHBE
B-LCL
0.0%
0.0%
0.0%
CD8
IFN-γ
D
KP-2
KP-3
1.4%
74%
MIA PaCa-2
QGP-1
SUIT-2
42%
30%
1.2%
CD8
IFN-γ

Figure 1. Characterization of a CTL clone, designated as 16F3, from in vitro culture with aAPCs. A, Surface expression of HLA-A24, CD86 and 4-1BBL molecules on the K562 cells used for stimulation. The shaded and solid area show non-transduced and lentiviral-transduced cells, respectively. B–D, IFN-γ secretion of 16F3 upon incubation with various cells. The 16F3 cells were incubated with K562 cells expressing either HLA-A2 or HLA-A24 (B) for 4 h, and the IFN-γ secreting cells were detected and analyzed. The frequency is shown as the percentage of the total living $CD8^+$ T cells. HLA-A24-positive fibroblast cells, normal human bronchial epithelial cells and B-LCLs were used as representative non-cancerous cells (C). Five HLA-A24-positive pancreatic carcinoma cell lines were also used as stimulator cells (D). The data are representative of three independent experiments.
doi:10.1371/journal.pone.0047126.g001

I molecules [16]. To examine the above possibilities, we generated mutant HLA-A24 molecules that were reported to lack any capacity for internalization and endosomal/lysosomal trafficking [16–18]. HLA-A24-YA contains a single point mutation replacing a tyrosine of exon 6 with alanine, and HLA-A24-YAΔ7 has a deletion of exon 7 and the same tyrosine substitution (Figure S4A). CMV pp65-expressing K562 was stably transduced with lentiviral vectors expressing those mutated HLA-A24 forms. All of the constructs similarly expressed HLA on the cell surfaces (Figure S4B). Both HLA mutants were recognized via either 16F3 or CMV clones as efficiently as wild type HLA-A24 (Figure S4C). In addition, primaquine, an inhibitor of the recycling of HLA class I from the endosome to the cell surface [19], did not inhibit PSA epitope presentation (data not shown). These data indicate that both MHC class I molecule recycling and an endosomal trafficking pathway of MHC class I molecules are irrelevant to the generation of the epitope.

Constitutively Active Autophagosomes are Indispensable for the PSA Epitope Generation

As CQ and BafA are known to inhibit lysosomal function, we evaluated the level of autophagy using an immunofluorescence assay to detect the autophagosomal marker LC3. KP-3 and MIA PaCa-2 cells, which process and present the PSA epitope, had strong punctate LC3 staining, whereas SUIT-2, KP-2 and normal fibroblast cells that were not sensitive to CTL recognition showed weak staining (Figure 4A). Moreover, colocalization of autophagosomes and PSA was observed with BafA treatment for 4 h (Figure 4B), indicating that the protein might become a substrate of autophagic digestion in KP-3 and MIA PaCa-2 cells. To examine whether autophagic puncta could progress to autolysosomes in these cells, the autophagic flux was measured using a difference in the sensitivity of GFP and mRFP to the lysosomal environment [20]. When mRFP-GFP-LC3 tandem-tagged fluorescent protein entered the autolysosome from the autophagosome, the GFP fluorescence signals would be diminished, but the mRFP would remain fluorescent. KP-3 and MIA PaCa-2 cells transfected with the plasmid had many mRFP puncta in autolysosomes, with few colocalized signals of GFP/mRFP in autophagosomes (Figure 4C), implying that these cells have a high autophagic flux state and that the LC3 puncta were not mere aggregates. Furthermore, we measured the flux status by evaluating an autophagosomal marker, LC3-II, which digests itself in the autolysosome. The status of the autophagosome is accurately represented by differences in the amount of LC3-II between samples in the presence and absence of lysosomal inhibitors, such as CQ, that block the degradation of the autolysosome [21]. KP-2 cells and B-LCLs expressed LC3-II at low levels, and changes in the LC3-II band were limited on the addition of CQ (Figure 4D). However, LC3-II levels were markedly increased in K562, KP-3 and MIA PaCa-2 cells that were recognized efficiently by 16F3 (Figure 4D). Fibroblasts and SUIT-2 cells had low or moderate autophagic flux. These data suggest that the epitope presentation requires constitutively active autophagosomes and autophagic flux.

PSA Epitope Processing and Presentation in Pancreatic Cancer Cells Engages the Autophagic Pathway

Recently, autophagy has been shown to deliver cytosolic proteins continuously to endosomes/lysosomes for antigen loading onto MHC class II molecules [22]. To address the involvement of an active autophagic pathway in antigen presentation onto MHC class I molecules, we first treated CMV pp65-expressing A24-K562 cells with 3-MA, a commonly used inhibitor of autophagy. We observed a decrease in IFN-γ production by 16F3 (Figure 5A) but not by CMV-specific CTLs. To confirm that autophagic processing is critical for epitope generation, siRNAs specific for two atgs, atg5 and atg7, were independently transfected into KP-3 and MIA PaCa-2 cells to suppress the protein expression and resultant autophagosome formation [21,23]. After the siRNA treatment, atg5 and atg7 mRNA expression levels were reduced in KP-3 and MIA PaCa-2 (Figure 5B). Knockdown of each gene in both cells resulted in a decreased IFN-γ production by 16F3 (Figure 5C). HLA-A24 expression was not significantly changed on the surfaces of either 3-MA- or siRNA-treated cells compared with the control (Figure S5, S6). These results suggest that constitutively active autophagosomes play a significant role in epitope generation with MHC class I in cancer cells that have an elevated autophagic activity.

Degradation of Full-length PSA Protein is Inhibited by CQ but Not by Lactacystin

To provide an insight into the hierarchy of autophagy and proteasomal digestion to process PSA, K562 cells were treated with CQ, and the expression levels of the full-length PSA protein were examined by Western blot analysis. As shown in Figure 6A and C, the level of the PSA protein increased in K562 following CQ treatment. The increase was not observed in KP-2 cells with less autophagy. The increase in p62 protein, an efficient substrate of autophagy [24], was comparable in K562. Next, these cells were treated with lactacystin, and the levels of PSA along with HIF-2α protein, a substrate of proteasomal digestion [25], were examined. The level of PSA in K562 cells was not changed, whereas that of HIF-2α was increased (Figure 6B, 6C).

Autophagy-induced Fibroblast Cells can Not Generate the PSA Epitope

Finally, we tested whether rapamycin, an m-TOR inhibitor being well documented as an inducer of autophagy, or low nutrient culture conditions could induce normal fibroblast cells to create the epitope. As shown in Figure 7A, autophagosomes increased by the drug treatment and by low nutrient culture conditions. However, these cells ere not recognized by 16F3 (Figure 7B). The same treatments did not induce B-LCLs to present the epitope to 16F3 (data not shown). These data demonstrate that, in normal cells, induced autophagy can not create the epitope, suggesting that constitutively active autophagy in cancerous cells is required for the epitope creation.

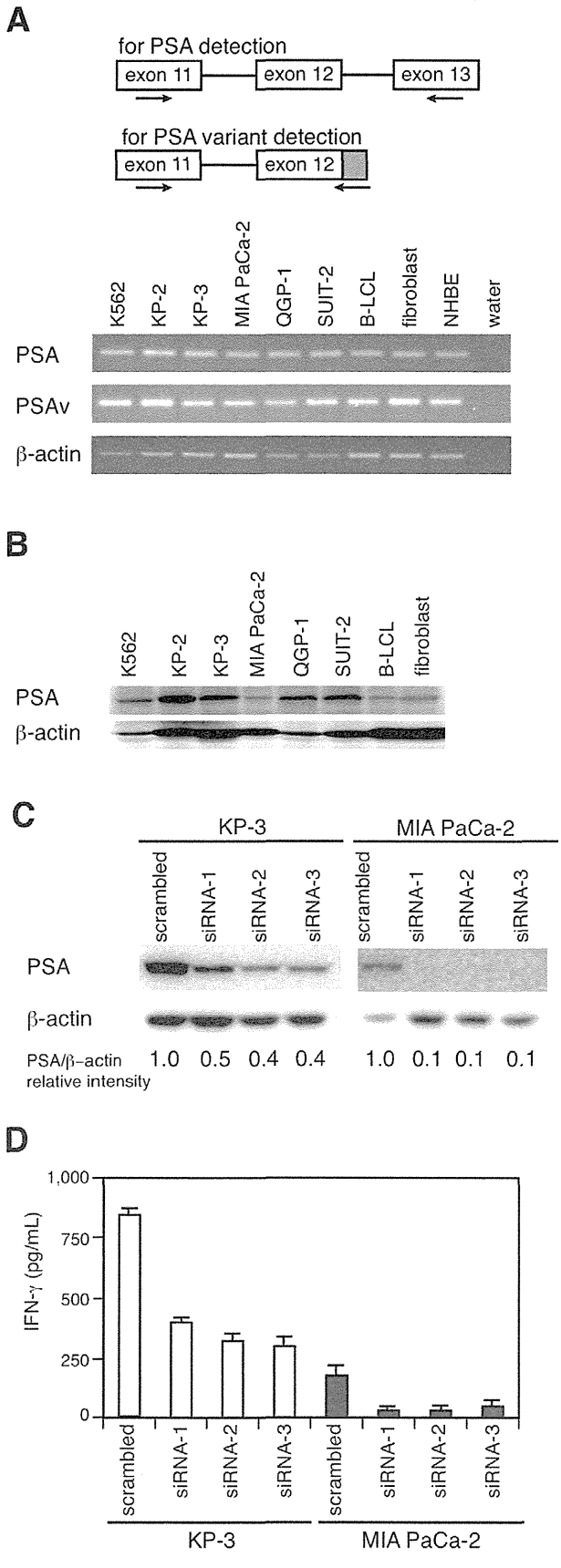

Figure 2. Ubiquitous expression of the PSA mRNA and protein within various cells irrespective of clone recognition. A, Two RT-PCR primer sets are designed to discriminate between wild type and variant PSA (PSAv), indicated by horizontal arrows (top). RT-PCR analysis was performed using the PSA primer sets and those for β-actin (bottom). B, The expression of the PSA and β-actin proteins was analyzed using Western blot analysis. C, KP-3 and MIA PaCa-2 cells were transfected for 70 h with scrambled or PSA-specific siRNA. Three different siRNA targets were chosen for the PSA gene, and Western blot analysis of the PSA protein in siRNA-treated cells was performed. The intensities of the bands were calculated using the ImageJ software. D, CTL responses against siRNA-treated cells were examined using an IFN-γ ELISA. The results are expressed as the means ± SD of triplicate values. Similar results were obtained in three separate experiments.
doi:10.1371/journal.pone.0047126.g002

Discussion

The present study demonstrated, using cDNA expression cloning, that a variant PSA was an antigen recognized by 16F3, although full-length PSA also stimulated 16F3. Remarkably, despite the ubiquitous expression of both the variant and full-length forms, the 16F3 CTL was found preferentially to recognize cancer cells but not normal cells. As other examples of this phenomenon, transcripts of p15 and cDNA31.2 are ubiquitously expressed, yet relevant CTLs only recognize tumor cells and not normal cells, including B-LCLs [26,27]. Herein, we demonstrate a new mechanism of antigen processing and presentation that emphasizes the differences between cancer and normal cells with respect to ubiquitously expressed proteins. Based on the present results with specific inhibitory chemicals and siRNA targeting autophagy, and monitoring the results of autophagic activities, epitope liberation is caused by differences in the autophagic status, and therefore, a ubiquitously expressed protein, PSA, mimics tumor-associated antigens through a unique mechanism involving autophagy.

Our data indicate that both autophagosomes and proteasomes are necessary for the processing of the epitope peptide from PSA. Taking into consideration that lactacystin considerably decreases PSA epitope presentation to CTLs (Figure 3B) without changing the level of full-length PSA protein (Figure 6C), it is likely that proteasomes cleave smaller fragments of PSA to create the epitope. We speculate that these data suggest that PSA is degraded through the autophagosome-lysosome pathway prior to digestion in the proteasome.

Autophagy plays an important role in tumor initiation and progression in contrasting manners. Cancer cells use autophagy to survive and propagate in hypoxic and low-nutrient microenvironments, whereas autophagy is suppressive of tumor initiation because of the clearance of mutations. Increased autophagy has been shown in late-stage colon cancer [28], breast cancer [29], melanoma [30], hepatoma [31] and malignant glioma [32]. Additionally, for pancreatic cancer, autophagy correlates with a poor outcome [33] and is required for tumor growth [34]. Moreover, autophagy is associated with mutated K-ras-induced malignant transformation [35,36]. Taken together, most pancreatic cancer cells with a K-ras mutation at codon 12 should have constitutively active autophagy. We demonstrated that an autophagosomal marker LC3 was strongly expressed in KP-3 and MIA Paca-2 cells. These pancreatic ductal cancer cell lines have a K-ras mutation at codon 12, whereas autophagosome is basal level in KP-2 cells having no K-ras mutation [37]. Remarkably, rapamycin treatment and starvation did not induce normal fibroblast cells to create the epitope, although autophagosome was observed. Our data suggest that aberrant increases in autophagosomes in association with K-ras mutation create distinct peptide repertoires displayed on the MHC class I molecules of

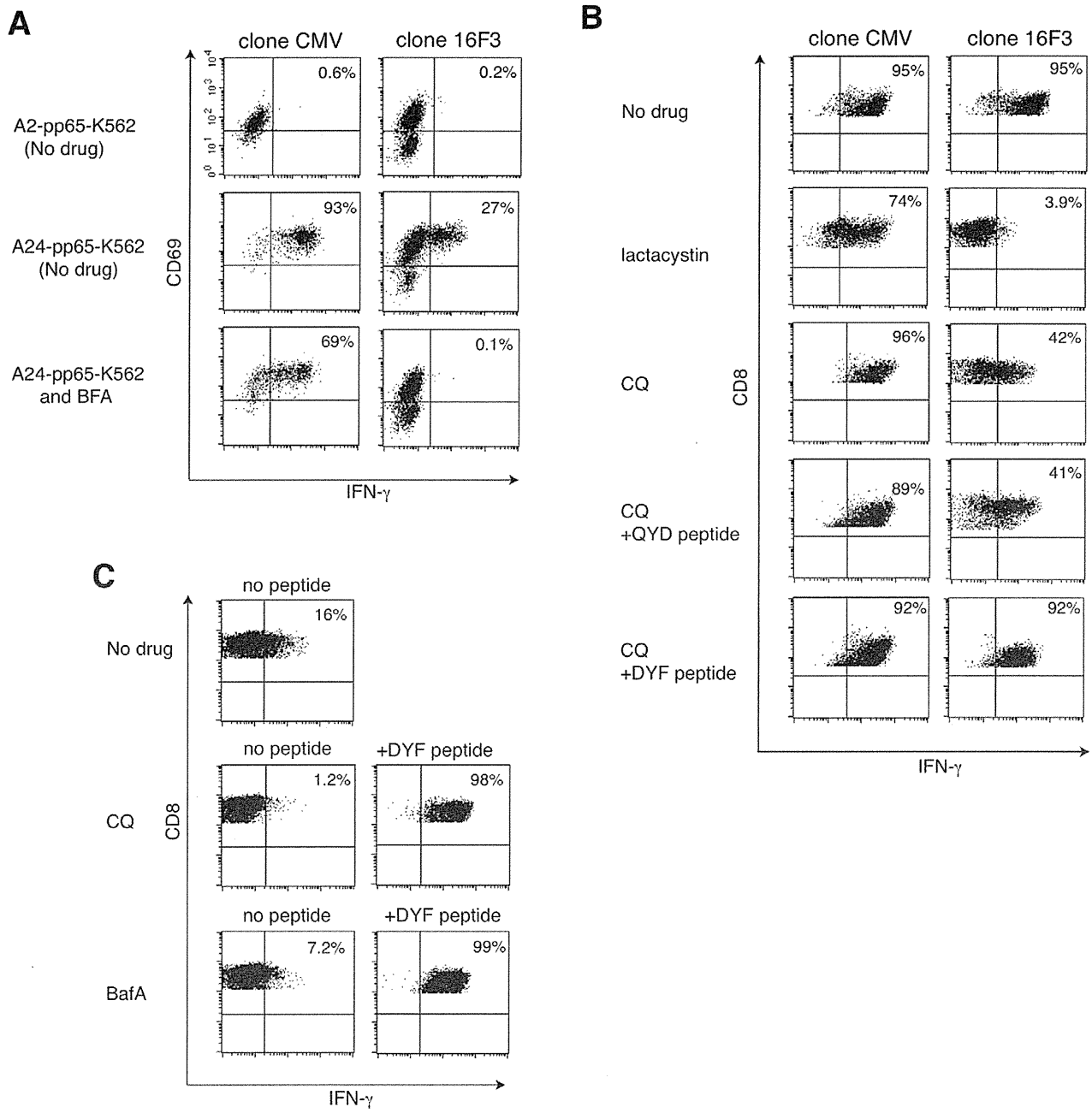

Figure 3. The epitope is presented and processed through a vacuolar pathway. A, K562 cells expressing both HLA-A2 or A24 and CMV pp65 (A2-pp65-K562 or A24-pp65-K562) were acid-stripped and incubated at 37°C for 9 h in the presence or absence of BFA. Then, the cells were co-cultured with either 16F3 or an HLA-A24-restricted CMV pp65-specific CTL clone for an additional 5 h. BFA was also added during the co-culture. After fixation and permeabilization, the cells were stained for CD3, CD8, CD69 and IFN-γ. $CD3^+$ and $CD8^+$ T cells were gated and analyzed using a flow cytometer. The frequency of IFN-γ producing cells is shown as the percentage of the total $CD3^+$ $CD8^+$ T cells. B-C, IFN-γ secretions of clones for 4 h after stimulation with A24-pp65-K562 cells (B) or KP-3 cells (C) treated with acid buffer for peptide stripping and/or inhibitors for 14 h was detected using an IFN-γ catch assay. 7-AAD^- alive $CD8^+$ T cells were gated and analyzed using a flow cytometer. The frequency of IFN-γ secreting cells is shown as the percentage of the total alive $CD8^+$ T cells. Whereas irreversibly acting lactacystin was removed during co-culture period (B), CQ and Baf A were retained in the media (B, C) because of their reversible nature. To exclude the possibility that CQ and Baf A could be inhibitory for 16F3 to produce IFN-γ, cognate or irrelevant peptides were added at concentrations of 1 μg/ml (B, C) and the T-cell response was examined.
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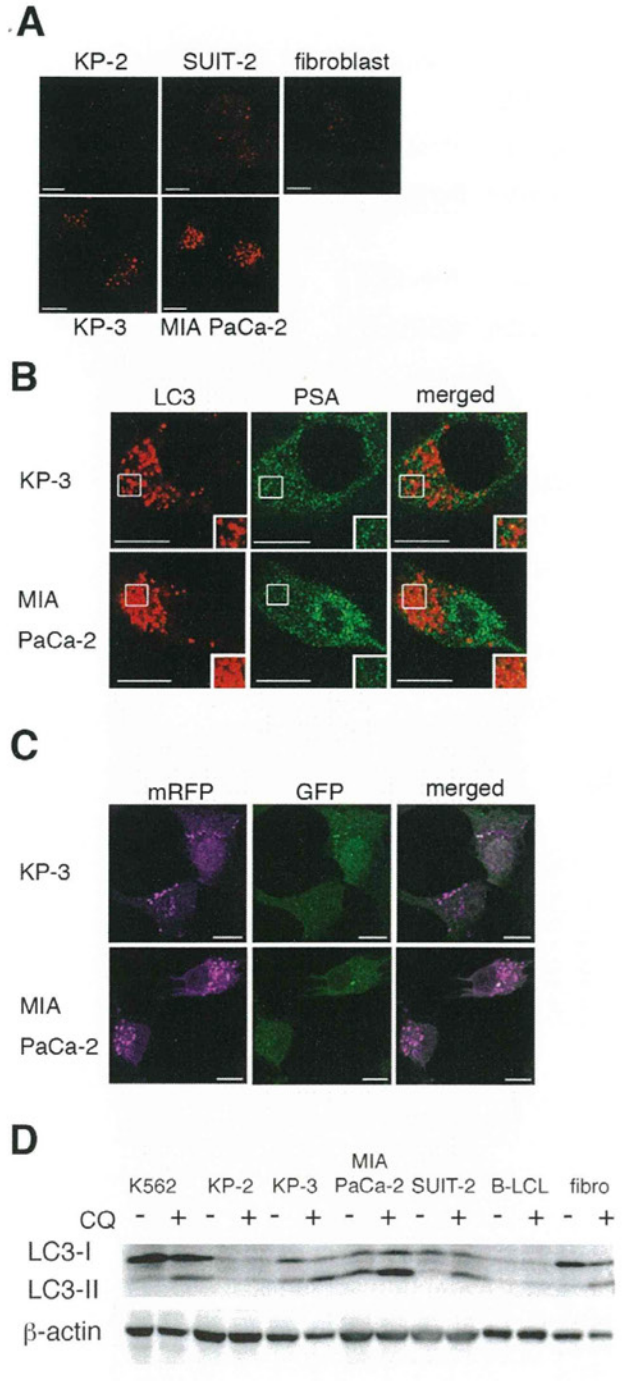
cancer cells, potentially evoking CTL responses while sparing normal cell damage.

In this study, a long 12-mer epitope antigen derived from PSA was identified, consistent with an earlier investigation that revealed an unusually long epitope presented on MHC class I [38]. Not only viral antigens but also tumor antigens are naturally processed into longer epitopes, and higher immunogenicities are indicated even in cancer patients [39]. The structures of the long peptides on



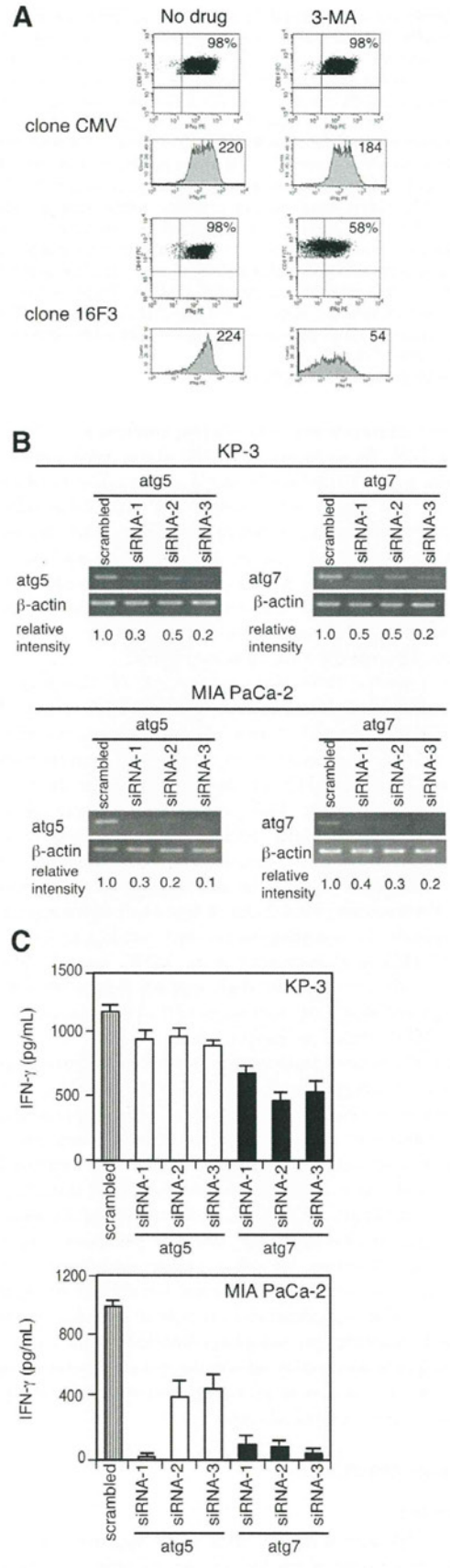


Figure 4. Constitutively active autophagy is involved in the 16F3 epitope processing of cancer cells. A, An immunofluorescence assay was performed to examine the expression of endogenous LC3. B, Double staining for endogenous LC3 and PSA was performed. Cells were cultured with BafA for 4 h, and then immunocytochemistry was performed. A yellow signal indicates colocalization. C, KP-3 and MIA PaCa-2 cells were transfected with plasmids expressing an mRFP-GFP-LC3 tandem-tagged fluorescent protein. Forty hours after transfection, the cells were fixed and analyzed via microscopy. A white signal indicates colocalization. The bars indicate 10 μm (A–C). D, The status of autophagic flux was measured via the LC3-II expression level. Cells were cultured with or without CQ for 2 h, and then the cell lysates were subjected to Western blot analysis for LC3.
doi:10.1371/journal.pone.0047126.g004

Figure 5. Autophagy is involved in the 16F3 epitope processing in cancer cells. A, K562 cells expressing both HLA-A2 or A24 and CMV pp65 were treated with an acid buffer for peptide stripping and incubated for 14 h in the presence or absence of 3-MA. Next, the cells were co-cultured with each clone for 4 h for IFN-γ secretion detected via the IFN-γ catch assay. The frequency of IFN-γ-secreting cells is shown as the percentage of the total living $CD8^+$ T cells. 3-MA was not added during the co-culture period. In the lower panels, histograms of the IFN-γ signal and their mean fluorescence intensity are shown. B, RT-PCR analysis of scrambled, atg5- or atg7-specific siRNA-treated cells performed 70 h after transfection. The intensities of the bands were calculated using the ImageJ software. C, The CTL response against KP-3 and MIA PaCa-2 cells transfected with scrambled, atg5- or atg7-specific siRNA for 70 h was examined using an IFN-γ ELISA. Three different siRNA targets were chosen for each autophagy-associated gene. The results are the means ± SD of triplicates. Similar results were obtained in three separate experiments.
doi:10.1371/journal.pone.0047126.g005

MHC have been characterized, and bulging conformations have been observed [40]. In particular, HLA-B alleles preferentially bind to peptides over 11 residues in length, rather than to other HLA alleles. In this study, we demonstrated that HLA-A24-binding 12-mer peptides were created by autophagy. The epitope DYFNVPYPLKI has anchor residues for HLA-A24, such as a tyrosine at the second position and an isoleucine at the carboxyl-terminus, assuming a bulged formation in the middle of the epitope peptides. The unique processing pathway through autophagy may generate the long epitope peptide.

Concerning adaptive immunity, the report of autophagy-enhancing endogenous presentation on MHC class I is clearly of interest [13]. HSV type 1 (HSV-1) was found to trigger a vacuolar response that increased the presentation of a peptide derived from HSV-1 glycoprotein B to CTL on MHC class I. In HSV-1-infected murine macrophages, LC3-positive four-layered membrane structures emerged from nuclear envelopes and were uniquely found in "classical" autophagosomes, consisting of double-layered structures formed in the cytosol of uninfected macrophages. Furthermore, virus-induced four-layer forms appear to function similarly to autophagosomes and participate in the presentation of HSV-1 glycoprotein B on MHC class I. The difference in our data is that our data support a constitutively active autophagic pathway that contributes to the presentation of a self antigen by MHC class I on cancer cells.

In conclusion, the present investigation provides information on the contribution of autophagy to MHC class I processing and presentation in cancer cells but not in normal cells. Importantly, PSA displays differential susceptibilities to antigen presentation between cancer and normal cells, although it is expressed ubiquitously in both cases. Epitope creation from PSA unambiguously requires autophagic pathways, but elucidation of the more detailed molecular mechanisms and essential proteases awaits further studies. Furthermore, to what extent autophagy may participate in the generation of MHC class I-restricted epitopes remains unknown. The comprehensive analysis of peptides bound to MHC class I molecules on autophagy-knocked down cancer cells using mass spectrometry may answer the question, promoting a better understanding of cancer immunity and adding information for effective cancer immunotherapy.

Materials and Methods

Ethics Statement

The study design and purpose, after prior approval by the Institutional Review Board of the Aichi Cancer Center were fully

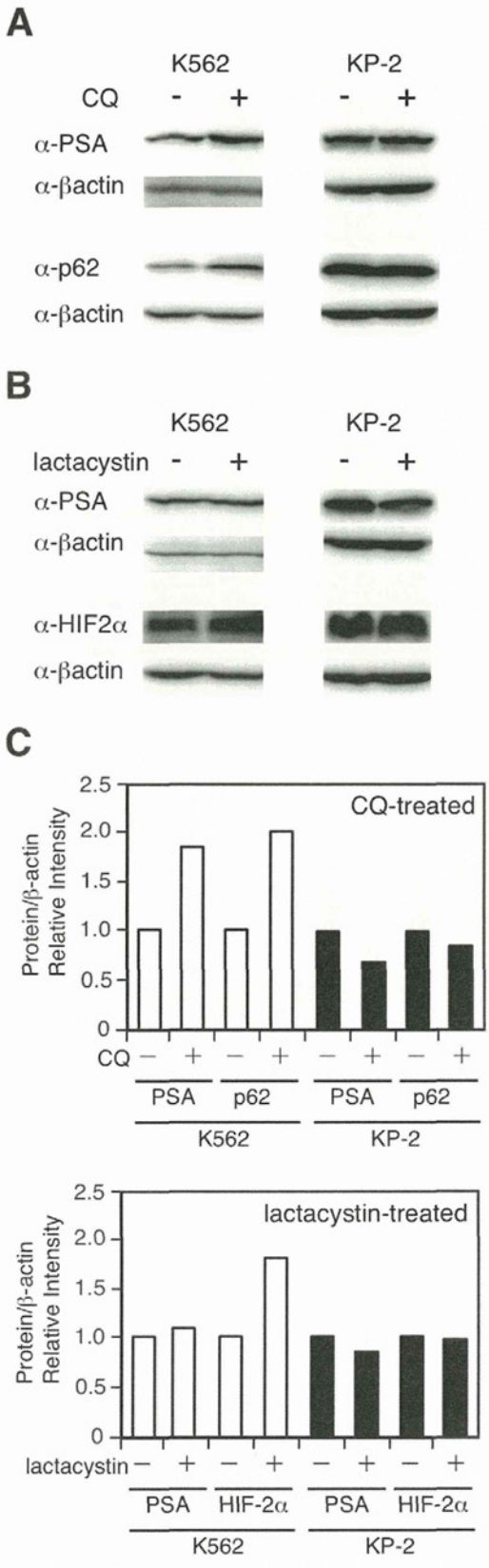


Figure 6. Degradation of full-length PSA protein is inhibited by CQ but not by lactacystin. A, Cells were cultured with or without CQ (50 μM) overnight, and then the cell lysates were subjected to Western

blot analysis for PSA and p62 as a positive control for autophagic digestion. B, Cells were cultured with or without lactacystin (5 μM) overnight, and the cell lysates were subjected to Western blot analysis for PSA and HIF-2α as a positive control for proteasomal digestion. C, The intensities of the bands were calculated using the ImageJ software.
doi:10.1371/journal.pone.0047126.g006

explained, and written consent was obtained from healthy blood donors.

Cell Lines

The human pancreatic cancer cell lines were purchased from the Japanese Collection of Research Bioresources. HLA-A24-positive normal human bronchial epithelial cells were cultured according to the supplier's recommendations (CC2540, Takara, Ohtsu, Japan). An HLA-A24-positive dermal fibroblast line and TAP-deficient T2-A24 cells were cultured as previously described [14]. The retroviral transduction of CMV 65 kDa phosphoprotein (pp65) was performed as previously described [12].

Construction of Artificial Antigen-presenting Cells (aAPCs)

Vectors carrying HLA-A*02:06, -A*24:02, mutated HLA-A*24:02, human CD86 and human 4-1BBL genes under the EF-1α promoter were constructed (CSII-EF-MCS) [41]. Lentiviral transduction was performed with similar retroviral methods [12].

CTL Induction

Naïve $CD8^+$ T cells were negatively isolated from PBMCs of A24-positive donors using the $CD8^+$ T cell isolation kit II and anti-CD45RO microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The isolated cells were more than 95% pure $CD45RO^-$ $CD8^+$ populations. These $CD8^+$ T cells (1×10^6 cells/well) were cocultured with irradiated (100 Gy) aAPCs (1×10^5 cells/well) in 2 mL X-VIVO20 (CAMBREX, East Rutherford, NJ) supplemented with 5% human AB serum (MP Biomedicals, Solon, OH) in wells of a 12-well plate in the presence of 10 ng/mL IL-12 (Peprotech, Rocky Hill, NJ)). On day 3, 10 ng/mL IL-7 (Peprotech) and IL-15 (Peprotech) were added. Every 3 days, half the medium was exchanged for fresh medium containing 10 ng/mL IL-15. On day 12, the T cells were restimulated with γ-irradiated aAPCs. One day thereafter, IL-2 (Primmune, Kobe, Japan) was added, to a final concentration of 20 U/mL. To establish T cell clones, a limiting dilution of the polyclonal CTL was performed as previously described [14].

Detection of IFN-γ Producing $CD8^+$ T Cells

An IFN-γ secretion assay to detect IFN-γ release with a PE-labeled antibody was performed using a kit (Miltenyi Biotec) and following the manufacturer's recommendations. In inhibition assays, after an acid-stripping treatment, as described previously [42], the stimulators were incubated with drugs at the following concentrations overnight: brefeldin A (BFA, Sigma-Aldrich, St. Louis, MO), 10 μg/mL; lactacystin (Calbiochem, Darmstadt, Germany), 20 μM; chloroquine (CQ, Sigma-Aldrich), 100 μM; bafilomycin A1 (BafA, Sigma-Aldrich), 250 nM; and 3-methyladenine (3-MA, Sigma-Aldrich), 10 mM. BFA, CQ and BafA were added during co-culture with T-cells, while lactacystin and 3-MA were not. After staining, flow cytometric analysis of the stained cells was performed using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ), and the data were analyzed with the help of CellQuest Pro software (BD Biosciences). Synthetic epitope peptides (Greiner, Frickenhausen, Germany) were added at concentration of 1 μg/mL to confirm intact IFN-γ production by T-cells in the presence of the drugs. In certain experiments, intracellular cytokine assessment using flow cytometry was performed instead of an IFN-γ secretion assay, as previously described [43].

Construction of a cDNA Library and Expression Screening by ELISA

The preparation of a cDNA library and expression screening were performed as described previously [44]. After overnight culture with 16F3, IFN-γ in the supernatant was measured by ELISA.

MHC Stabilization Assay

An MHC stabilization assay was performed to test the peptide for HLA-A24 binding efficiency using T2-A24 cells, as described earlier [14]. Briefly, T2-A24 cells were incubated with RPMI 1640 containing 0.1% FCS and each of the peptides at a concentration of 10 μg/mL at 26°C for 16 h, followed by incubation at 37°C for 3 h. After incubation, surface HLA-A24 molecules were stained with the anti-A24 mAb and anti-mouse FITC-conjugated Ab. HLA-A24 expression was measured using a FACSCalibur (BD Biosciences), and the mean fluorescence intensity was analyzed using the Cellquest Pro software (BD Biosciences).

IFN-γ ELISA for the Evaluation of Antigen Recognition by 16F3 with Fixed Stimulators

K562 cells expressing both HLA-A24 and CMV pp65 were fixed by various methods previously described [45,46] with slight modification as follows: fixation with 0.2% paraformaldehyde for 10 min, fixation with 0.008% glutaraldehyde for 3 min or fixation with 0.05% glutaraldehyde for 30 sec. After fixation, cells were washed with RPMI 1640 medium plus 10% FCS five times. For quenching, the cells were further washed with PBS containing 10 mM glycine two times. Following overnight co-culture of T-cell and the stimulators, the expression of IFN-γ in the cell culture supernatant was determined using an ELISA.

Plasmid Construction of HLA-A24 Mutants

A plasmid expressing HLA-A24, pcDNA3.1(+)/HLA-A24, was constructed as previously described [12]. To construct an HLA-A24-YA mutant [16] containing a single point mutation substituting an alanine residue for the tyrosine residue of exon 6, overlapping PCR [47] was performed, using pcDNA3.1(+)/HLA-A24 containing a T7 promoter primer binding site and BGH reverse priming site as a template. The following primers were used: sense primer for the T7 promoter primer-binding site, 5′-TAATACGACTCACTATAGGG-3′ (T7pp primer); internal antisense primer, 5′-CAGCCTGA-GAGGCGCTCCCTCCTTTTCTATCTGAG-3′; an internal sense primer, 5′-AAGGAGGGAGCGCCTCTCAGGCTG-CAAGCAGTGA-3′; an antisense primer for the BGH reverse priming site (BGHrp primer), 5′- TAGAAGGCACAGTC-GAGG-3′. The next PCR was performed with the first PCR products as templates, using the T7pp primer and the BGHrp primer, and produced the fusion product. To construct the HLA-A24-YA-Δ7 mutant [16] containing a complete deletion of exon 7 and a single point mutation substituting an alanine residue for the tyrosine residue of exon 6, PCR was performed with pcDNA3.1(+)/HLA-A24 as a template using the T7pp primer and an antisense primer, 5′-AAGCGGCCGCTCACACTG-CAGCCTGAGAGGCGCTCCCTCC-3′. The resultant PCR fragments were cloned into lentiviral expression vectors (CSII-

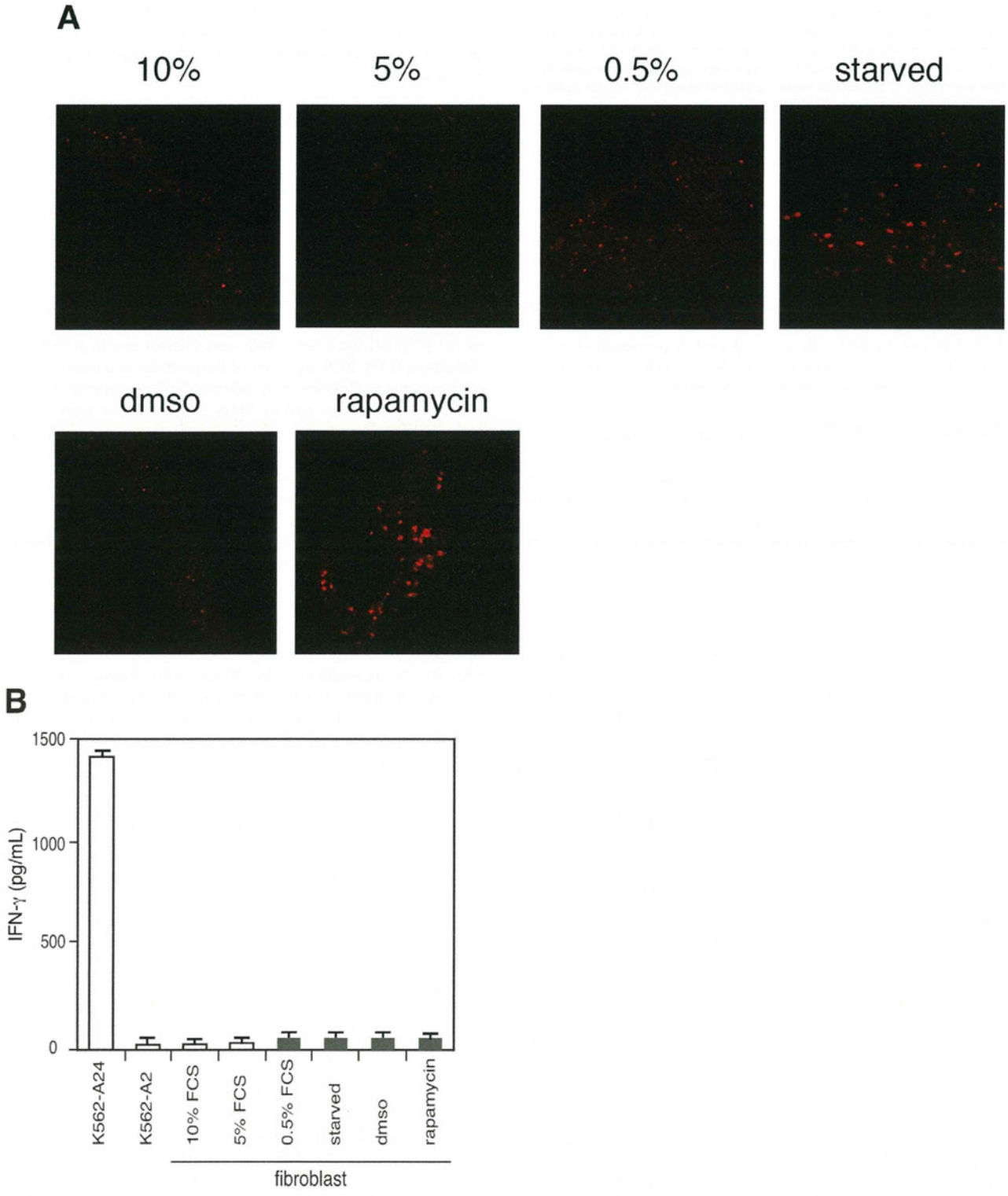


Figure 7. Induced autophagy does not result in PSA epitope presentation in fibrobast cells. A. Immunofluorescence assays for endogenous LC3 in fibroblast cells after low nutrient culture or rapamycin treatment. In low nutrient conditions, fibroblast cells were cultured in medium supplemented with 10%, 5%, 0,5% FCS or in Hank's Balanced Salt Solution (starved). B, CTL response to autophagy-induced fibroblast cells treated with low nutrient culture conditions or rapamycin. Target cells were treated with low nutrient culture conditions or rapamycin for 4h, washed twice and cultured with CTL overnight. Next day, supernatants were harvested and IFN-γ measured by ELISA. K562-A24 cells and K562-A2 cells were used as positive and negative control, respectively. The results show means ± SD of triplicates.
doi:10.1371/journal.pone.0047126.g007

EF-MCS). The cloned genes were sequenced to verify their identity.

Immunofluorescence Microscopy

Cells on glass coverslips were fixed for 10 min with PBS containing 3% paraformaldehyde. After washing, the cells were permeabilized with 50 μg/mL digitonin (Sigma-Aldrich) in PBS for 5 min at room temperature. The cells were then washed and blocked with 3% BSA in PBS for 10 min. After probing with a rabbit Ab specific to human PSA (GeneTex, Irvine, CA) and a mouse mAb specific to human LC3 (4E12, MBL, Nagoya, Japan) for 1 h, secondary Abs conjugated to Alexa 488 and 594 (Invitrogen, Carlsbad, CA) were applied for 30 min. All of the immunochemical assays were performed at room temperature. The slides were mounted in ProLong Gold antifade reagent (Invitrogen). Confocal fluorescence images were obtained using a confocal laser-scanning microscope (LSM510 META; Carl Zeiss, Göttingen, Germany) with a Plan-Apochromat 63x/1.40- or 100x/1.40- numerical -aperture oil immersion lens and LSM Image Browser software (Carl Zeiss).

Western Blot Analysis

Western blot analysis was performed as described previously [48]. A mouse mAb specific to human PSA (#2649C4a; Cosmo Bio, Tokyo, Japan), p62 (5F2; MBL) HIF2α (#NB100-132; NOVUS, Littleton, CO) or β-actin (Sigma-Aldrich), or a rabbit Ab specific to human LC3 (MBL) was used. All of the images were processed by Lumi Vision Pro 400EX (Aisin/Taitec, Inc., Kariya, Japan). The signal intensity was quantified with the ImageJ software.

Transfection with Small Interfering RNAs (siRNAs)

SiRNAs specific for PSA, autophagy-related gene (atg)5 and atg7 were created using Stealth™ RNAi preparations and obtained from Invitrogen. The target sequences are as follows: PSA-siRNA-1, GGAGGAUUCUUAAUAUCCAGACUAA; PSA-siRNA-2, CAGCUCUGCCAUGCUGGAAAGUUUA; PSA-siNRA-3, CCAAACCUGGAGAAGGUCAUCUCGA; atg5-siRNA-1, UCGAGAUGUGUGGUUUGGACGAAUU; atg5-siRNA-2, AAGCAACUCUGGAUGGGAUUGCAAA; atg5-siRNA-3, CCUCAAAGAAGUUUGUCCUUCUGCU; atg7-siRNA-1, CAGAAGGAGUCACAGCUCUUCCUUA; atg7-siRNA-2, GAAAGCCAUGAUGUCGUCUUCCUAU; and atg7-siRNA-3, GGCCGUGGAAUUGAUGGUAUCUGUU. A scrambled siRNA (Invitrogen) was used for negative control. The KP-3 and MIA PaCa-2 cells (2×10^5 cells/well) were transfected with each siRNA (100 nM) using Lipofectamine 2000 (Invitrogen) in 24-well plates. After 70 h, the cells were harvested and used in an ELISA, or the total RNA was extracted using the RNAeasy mini kit (Qiagen, Tokyo, Japan) with DNase I treatment, and reverse transcription was performed in 20-ul reactions containing oligo $(dT)_{20}$ primers and 0.5 μg aliquots. The alignments of specific primer sets were as follows: PSA and PSA variant common forward, 5′-TCCATCTGAGGTTGATGAGAT-3′; PSA reverse, 5′-TCCACATAAATGAGGGGAAATC-3′; PSA variant reverse, 5′-GTCCCAGCCTGGATAGAGTG-3′; atg5 forward, 5′-TGGGATTGCAAAATGACAGA-3′; atg5 reverse, 5′-CACTGCAGAGGTGTTTCCAA-3′; atg7 forward, 5′-GAAACCAAAGCAGCAAGGAG-3′; and atg7 reverse, 5′-CATTCATCCGATCGTCACTG-3′. The PCR products were separated in 2.0% agarose and visualized with ethidium bromide staining.

Supporting Information

Figure S1 Identification of PSA variant cDNA encoding an epitope recognized by 16F3. A, HLA-A24-expressing HEK293T (A24-293T) cells were transfected with each plasmid from a cDNA library constructed from mRNA extracted K562 cells. IFN-γ was measured by ELISA. Arrow indicates a well of 8G containing an antigenic plasmid. B, The cDNA clone was a variant PSA (NM_006310). The schematic drawing of full length (top) and variant (bottom) PSA is shown. The variant is an intronic polyadenylated mRNA ending with the exon 12 and a following intron. C-D, Identification of an epitope peptide recognized by 16F3. A24-293T cells were transfected with each plasmid encoding truncated fragments. The constructs shown as open boxes were recognized by 16F3, while that shown as filled boxes were not. Numbers indicate amino acid positions (C). Minigenes were cloned into pcDNA3.1(+) plasmid and transfected into A24-293T cells. The amino acid sequences were shown in one-letter code, and defined epitope is underlined (D). E, MHC stabilization assay was executed without peptide or with either synthetic 12mer peptide, DYFNVPYPLPKI or control CMV peptide, QYDP-VAALF. F, T2-A24 cells were pulsed with serial concentrations of either synthetic 12mer peptide, DYFNVPYPLPKI (diamond) or control CMV peptide, QYDPVAALF (circle). Release of IFN-γ was measured by ELISA. The results are the mean of triplicate values.
(TIF)

Figure S2 Peptide-pulsed KP-2 and SUIT-2 cells were recognized by 16F3. KP-3 (white), KP-2 (black) and SUIT-2 (vertical stripe) cells were pulsed with either synthetic 12mer peptide, DYFNVPYPLPKI (DYF) or control CMV peptide, QYDPVAALF (QYD) ate a concentration of 1 μM. Release of IFN-γ was measured by ELISA.
(TIF)

Figure S3 Fixation of target cells abolish recognition by 16F3, which is not recovered by glycine quenching. A–B, K562 cells transfected with CMV pp65 and HLA-A24 were used as stimulators for either 16F3 or an HLA-A24-restricted CMV pp65-specific CTL clone. A, Stimulators were fixed in three ways; 0.2% paraformaldehyde (PFA) for 10 min, 0.008% glutaraldehyde (GA) for 3 min, or 0.05% glutaraldehyde (GA) for 30 sec. After fixation, cells were washed 5 times with RPMI medium containing 10% FCS. B, Fixed and washed stimulators were further washed two times with PBS containing 10 mM glycine for quenching. The stimulators were cultured with each clone and IFN-γ in the supernatants was measured by ELISA. The data express mead of duplicates.
(TIF)

Figure S4 The epitope is not presented by recycling HLA-A24 molecules. A, The amino acid sequences of the cytoplasmic portion of the wild-type HLA-A24 (WT-A24) and two endosome recycling-compromised mutants, designated as A24-YA and A24-YAΔ7, are shown as a single letter code. S, signal sequence; TM, transmembrane domain; CY, cytoplasmic domain. B, Surface expression of HLA-A24 or its mutant molecules on lentiviral-transfected K562 cells was measured using a flow cytometer. C, K562 cells transfected with CMV pp65 accompanied by HLA-A02, HLA-A24 or its mutant were incubated with either 16F3 or an HLA-A24-restricted CMV pp65-specific CTL clone, and an IFN-γ ELISA was performed. The results are expressed as the means of triplicate experiments.
(TIF)

Figure S5 Surface HLA expression of HLA-A24-expressing K562 cells treated with acid buffer and 3-methyladenine (3-MA). The surface expression of the HLA class I molecule of HLA-A24-expressing K562 cells was examined with a flow cytometer after the cells were treated with acid buffer for peptide stripping and incubated with or without 3-MA for 14 h. The white and shaded areas show samples incubated with FITC-labeled anti-HLA class I and isotype control mAb, respectively. Stained cells were analyzed using a flow cytometer.
(TIF)

Figure S6 The surface HLA expression of siRNA-transfected pancreatic carcinoma cells. KP-3 (A) and MIA PaCa-2 (B) cells were transfected with scrambled, atg5, or atg7-specific siRNA for 70 h. The white and shaded areas show samples incubated with FITC-labeled anti-HLA class I and isotype control mAb, respectively. The stained cells were analyzed using a flow cytometer.
(TIF)

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Author Contributions

Conceived and designed the experiments: ADO KK. Performed the experiments: ADO. Analyzed the data: ADO KK. Contributed reagents/materials/analysis tools: HT YA HM TY. Wrote the paper: ADO KK.

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Long-term feto-maternal microchimerism revisited

Microchimerism and tolerance in hematopoietic stem cell transplantation

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Key words: fetal microchimerism, maternal microchimerism, hematopoietic stem cell transplantation, graft-versus-host disease, acquired tolerance, non-inherited maternal antigens, inherited paternal antigens, T regulatory cells, trans-vivo delayed-type hypersensitivity assay

Abbreviations: DTH, delayed-type hypersensitivity; GVHD, graft-versus-host disease; HSC, hematopoietic stem cell; IPA, inherited paternal antigen; mHAg, minor histocompatibility antigen; NIMA, noninherited maternal antigen; PBMC, peripheral blood mononuclear cell; TGF, transforming growth factor; T_E cells, T effector cells; T_R cells, T regulatory cells

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Bidirectional fetal-maternal cell traffic during pregnancy gives rise to stable persistence of minute amounts of allogeneic cells both in the mother and in her offspring, a phenomenon called long-term fetal or maternal microchimerism. Over the past decade, increasing attention has been devoted to elucidating the biological relevance of such reciprocal microchimerism, unveiling its conflicting roles in either immune sensitization or tolerance induction against fetal or maternal alloantigens. Recent studies in mice and humans have highlighted the significance of fetal-maternal microchimerism in the induction and maintenance of $CD4^+CD25^+$ and $CD8^+$ T regulatory cells that counterbalance the immune responses to fetal or maternal antigens mediated by T effector cells. Consistent with these observations, T-cell-replete hematopoietic stem cell transplantation between mutually microchimeric mothers and their HLA-haploidentical offspring has been shown to be feasible, although the degree of microchimerism-associated tolerance appears to substantially differ among the cases. Since in vitro or trans-vivo assays to detect antigen-specific tolerance in the context of the T regulator versus T effector balance are now available, future clinical studies incorporating these tests into the criteria for donor selection are warranted to more precisely define the relevance of fetal-maternal microchimerism in allotolerance and immune homeostasis after hematopoietic stem cell transplantation.

Introduction

Long-lasting bidirectional tolerance between the donor and recipient without excessive pharmacologic immunosuppression has been one of the ultimate but unfeasible goals in allogeneic hematopoietic stem cell (HSC) transplantation. Especially in the setting of HSC transplants from genetically HLA-disparate donors, substantial proportions of patients receiving intensive immunosuppressive agents still suffer from graft rejection or severe graft-versus-host disease (GVHD), either of which significantly compromises post-transplant survival outcomes. Given the ever-increasing number of HSC transplants with the use of donors other than HLA-identical siblings, it is crucially important to further elucidate the immunologic mechanisms by which HLA-incompatible HSC allografts are successfully accepted by some recipients but not by others.

Fetal-maternal microchimerism is a form of naturally acquired microchimerism that commonly occurs among eutherian mammals through two-way nucleated cell exchanges between the mother and fetus during the course of normal pregnancy.[1-3] With the help of flow cytometric cell sorting and highly sensitive polymerase chain reactions, minute amounts of hematopoietic cells and/or DNA of fetal origin can be detected in the blood and tissues of the respective mothers as long as decades postpartum,[4,5] while low levels of maternal cells can be identified not only in the various tissues of the fetus but in the peripheral circulation of the

immunocompetent adult offspring.[6,7] Thus far the biological relevance of such long-term persistent microchimerism is not fully characterized, although earlier observations suggested its possible roles in immune sensitization against fetal or maternal alloantigens.[1-3] Long-term fetal microchimerism in parous women has been reported to be associated with susceptibility to a variety of autoimmune disorders and protection from certain cancers.[1,2,8,9] Similarly, persistence of maternal microchimerism in immunocompetent offspring was suggested to be involved in the pathogenic mechanisms underlying immune-mediated pediatric diseases such as neonatal lupus syndrome and biliary atresia.[10,11]

In addition to functioning as sources for alloantigens to prime immune effectors, fetal-maternal microchimerism is now emerging as a crucial player involved in the induction and maintenance of pregnancy-associated immune tolerance toward fetal inherited paternal antigens (IPAs) in mothers as well as toward noninherited maternal antigens (NIMAs) in their progeny.[12] In the past several years, the feasibility of HSC transplants from HLA-haploidentical microchimeric donors has been explored to improve donor availability and transplant outcomes based on the hypothesis that maternal donors with fetal microchimerism are rendered immunologically hyporesponsive to their offspring and vice versa, offspring donors harboring maternal microchimerism are rendered tolerant to both their mothers and HLA-haploidentical siblings expressing NIMAs as mismatched histocompatibility antigens. In this brief article, we will discuss the promises and challenges of such HSC transplants from donors with fetal or maternal microchimerism in the light of future directions to take more advantage of microchimerism-associated immune equilibrium by employing assays to evaluate and means to enhance the robustness of IPA- or NIMA-specific tolerance probably determined by balance between T regulator and T effector cells responsive to fetal or maternal alloantigens.

Clinical and Experimental Observations Linking Long-Term Fetal-Maternal Microchimerism to Hematopoietic Cell Allograft Tolerance

A possible linkage between fetal microchimerism and HSC allograft tolerance was initially suggested by Tokita et al. who reported a case of a woman with refractory thymic carcinoma successfully treated with an infusion of allogeneic peripheral blood stem cells from her HLA-haploidentical daughter.[13] This woman was revealed to harbor microchimerism presumed to have originated from the daughter prior to and 11 months after the stem cell infusion. It was speculated that the infused donor cells could survive in face of the maternal immune system probably because the recipient mother had already become rendered hyporesponsive to IPAs of her daughter. Furthermore, the mother had no clinical manifestations of GVHD throughout the treatment period, implyng the presence of reciprocal tolerance to NIMAs in the daughter. Soon thereafter, Ochiai et al. reported a case of a young male patient with refractory leukemia who underwent successful non-T-cell-depleted peripheral blood stem cell transplantation from his microchimeric mother, setting the first milestone for the clinical development of HSC donor selection based on fetal-maternal microchimerism.[14] Notably, it was recently reported that HLA-haploidentical lymphocyte infusions from mothers harboring long-term fetal microchimerism were also effective in eradicating Epstein-Barr virus-positive T-cell lymphoproliferative diseases developed in their offspring without causing obvious GVHD.[15]

A close association of maternal microchimerism with acquisition and maintenance of specific tolerance to NIMAs has been more clearly demonstrated through a series of studies in mice and humans.[16-18] With the use of the murine F_1 x P backcross breeding model (B6 x BDF1) to generate H-$2^{b/b}$ offspring mice exposed to H-2^{d} as NIMA in utero and/or via breastfeeding, Zhang and Miller for the first time demonstrated that NIMA-exposed mice showed prolonged acceptance of maternal skin allografts while the period of graft survival was positively correlated with the amount of naturally transferred maternal T cells in their lymph nodes.[16] Andrassy and Kusaka et al. employed the same murine model and showed that NIMA-exposed offspring achieving tolerance to fully allogeneic H-$2^{d/d}$ heart allografts had relatively higher levels of maternal microchimerism in the lymphoid organs.[17] Importantly, human fetal lymphid organs also contain microchimeric maternal cells,[7,18] and subsequent experiments in mice and humans revealed that the tolerogenic NIMA effects were dependent on $CD4^+CD25^+$ T cells capable of suppressing alloreactive T-cell responses to maternal antigens.[18,19] These observations strongly suggested the crucial role of microchimeric maternal cells to generate NIMA-specific $CD4^+CD25^{high}Foxp3^+$ T regulatory cells in the offspring. It is also an intriguing and important question whether a similar mechanism can operate in parous women harboring fetal microchimerism because $CD4^+CD25^+$ T regulatory cells were also demonstrated to be involved in the induction and maintenance of maternal tolerance to the fetus in a murine model.[20]

Clinical Results of HLA-haploidentical Hematopoietic Stem Cell Transplantation from Donors Harboring Fetal or Maternal Microchimerism

In support of the above-mentioned observations, it has been reported that HSC transplants from mothers or NIMA-mismatched siblings confer superior outcomes as compared to those from fathers or siblings without NIMA mismatch as HSC donors,[21-23] probably suggesting better posttransplant immune reconstitution in the presence of IPA or NIMA mismatch. To further extend the availability of HLA-incompatible donors with the help of remote influence of fetal-maternal tolerance, we have explored the feasibility of T-cell-replete HSC transplantation from HLA-haploidentical family donors harboring persistent fetal or maternal microchimerism in the peripheral circulation.[24] These transplants involved three different types of donor-recipient combination: (i) transplantation from mothers

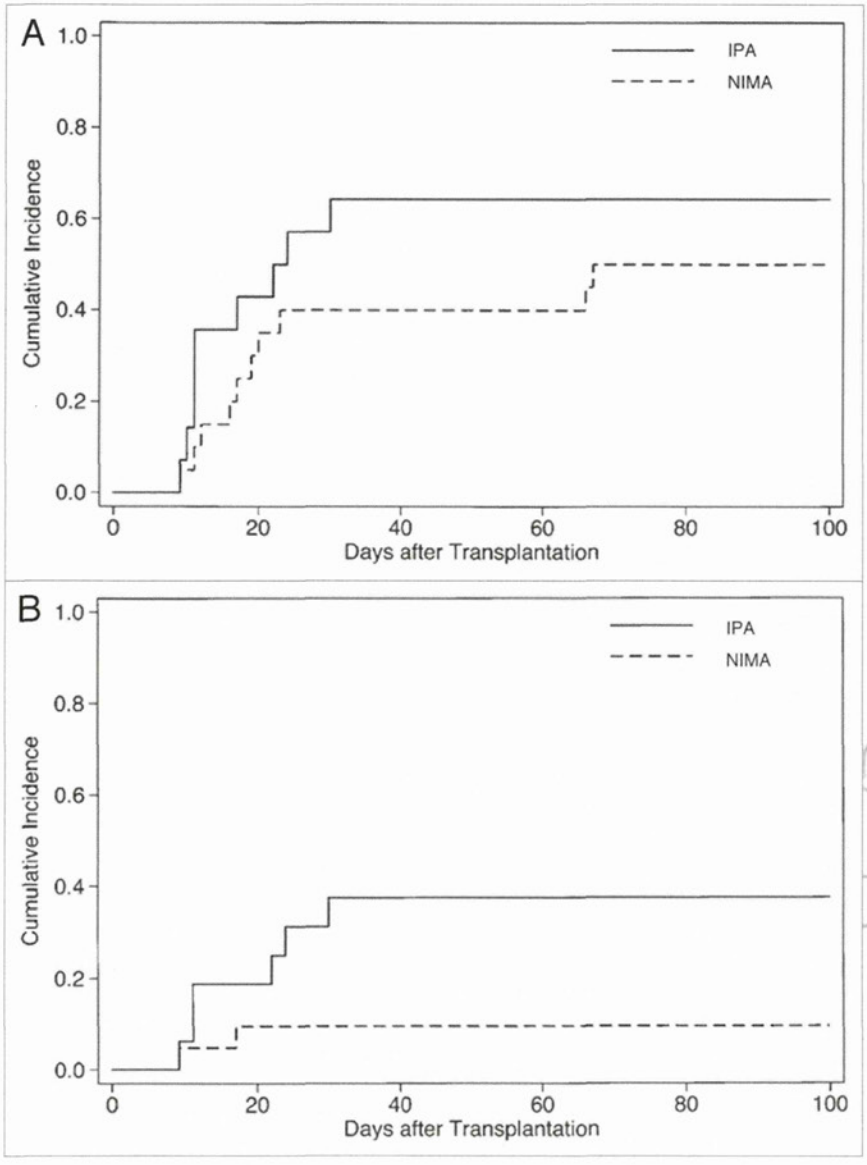

Figure 1. Cumulative incidence of acute GVHD in patients who underwent hematopoietic cell transplantation from an HLA-haploidentical related donor harboring fetal or maternal microchimerism. Cumulative incidence of moderate-to-severe (grade 2–4) (A) and severe (grade 3 or 4) (B) acute GVHD according to the type of HLA mismatch among 34 evaluable patients who received T-cell-replete HSC transplantation from an HLA-haploidentical related donor harboring fetal or maternal microchimerism. Solid line, transplants from mothers to their offspring (IPA mismatch in the GVH vector); dashed line, transplants from offspring to their mothers or to NIMA-mismatched siblings (NIMA mismatch in the GVH vector). This research was originally published in Blood.[24] Ichinohe T, et al. Feasibility of HLA-haploidentical hematopoietic stem cell transplantation between noninherited maternal antigen (NIMA)-mismatched family members linked with long-term feto-maternal microchimerism. Blood 2004; 104:3821–8. © The American Society of Hematology.

carrying microchimerism of fetal origin to their respective offspring, a setting that microchimerism-associated allotolerance in the donor might favorably modulate GVH responses against IPAs; (ii) transplantation from offspring carrying maternal microchimerism to their mothers or (iii) to their NIMA-mismatched HLA-haploidentical siblings, a setting that microchimerism-associated allotolerance in the donor might ameliorate GVH responses against NIMAs.[3]

Based on this scheme, we analyzed clinical outcomes of 35 patients with high-risk hematologic malignancy who received T-cell-replete HSC allografts from donors with fetal or maternal microchimerism.[24] With the use of conventional tacrolimus-based GVHD prophylaxis, all patients achieved durable engraftment and 19 (56%) of 34 evaluable patients developed grade 2–4 acute GVHD (**Fig. 1A**). In this study, the presence of NIMA mismatch in the GVH direction was associated with lower risk of developing severe acute GVHD as compared with IPA mismatch (**Fig. 1B**), although this observation should be reevaluated in a larger prospective cohort. Recently, we have updated information regarding survival of these patients and confirmed that such transplants conferred acceptable probabilities of long-term disease-free survival (**Fig. 2**). A series of case reports have also shown the feasibility of HLA-mismatched HSC donor selection based on fetal-maternal microchimerism,[12] but some cases are still associated with graft rejection and severe acute GVHD.[25] Furthermore, longer follow-up of these patients lead to a paradoxical observation that substantial proportions of survivors could discontinue administration of immunosuppressive agents despite the frequent occurrence of chronic GVHD requiring systemic treatment,[26] raising a new question as to the role of fetal-maternal microchimerism as an indicator of clinically relevant tolerance toward IPAs or NIMAs.

Equilibrium between T Regulatory Cells and T Effector Cells as a Mechanism to Determine the Net Immune Responses against Fetal or Maternal Alloantigens

Apparently conflicting results with respect to HSC transplants from microchimeric donors can be clearly reconciled by recent studies demonstrating that the tolerance associated with long-term fetal-maternal microchimerism would be determined by the balance between T regulatory cells (T_R) and T effector (T_E) cells specific for IPAs or NIMAs.[27-29] Cai et al. explored the role of minor histocompatibility antigen (mHAg) HA-1 in the allograft tolerance of three patients who received kidney transplants from an HLA-matched but HA-1-mismatched family member.[27] By

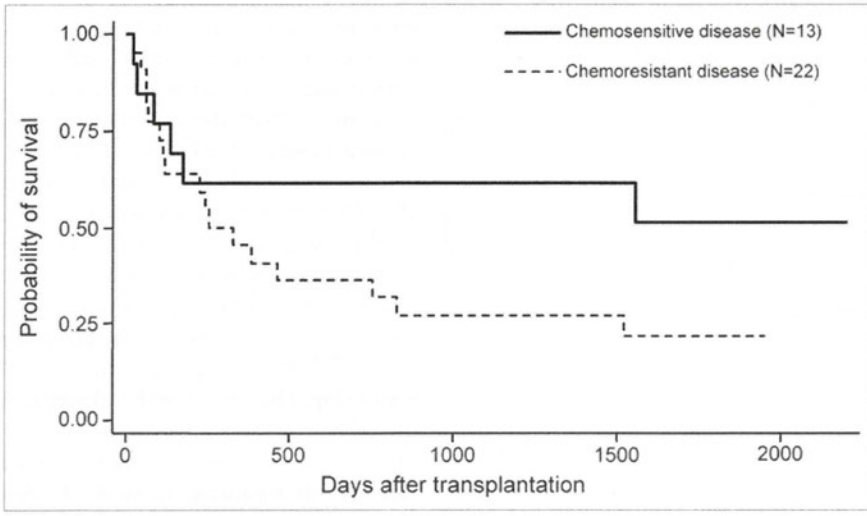

Figure 2. Long-term survival of patients who underwent hematopoietic cell transplantation from an HLA-haploidentical related donor harboring fetal or maternal microchimerism. Kaplan-Meier estimates of overall survival with a median follow-up of 4.6 years in 35 patients who received T-cell-replete HSC transplantation from an HLA-haploidentical related donor harboring fetal or maternal microchimerism for advanced hematologic malignancies. Solid line, transplants in remission; dashed line, transplants in chemotherapy-refractory disease.

analyzing peripheral blood mononuclear cells (PBMCs) obtained from these recipients with the use of HA-1-specific tetramer staining, they were able to detect two distinct tetramer-positive $CD8^+$ T-cell populations: the tetramer brightly positive population with characteristics of T_E cells that produces IFNγ and the tetramer dimly positive population with characteristics of T_R cells that produces IL-10 and transforming growth factor (TGF)β. Trans-vivo injection of PBMCs of these three patients into the footpads of severe combined immunodeficiency mice lead to IL-10 and/or TGFβ-regulated delayed-type hypersensitivity (DTH) responses to HA-1 peptide. The suppression of these T_E cell functions by HA-1 tetramer dimly positive $CD8^+$ T_R cells was dependent on IL-10, TGFβ and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). Intriguingly, these recipients were proven to harbor microchimerism of HA-1-positive dendritic cells. This report was the first demonstration of coexistence of microchimeric cells carrying mHAg, T_E cells responding to mHAg and T_R cells that are capable of suppressing mHAg-specific T_E cell functions, thus suggesting an essential role of microchimeric cells as a reservoir of alloantigens that are required for maintenance of both mHAg-specific T_E and T_R cells.

Recently, van Halteren et al. provided more robust evidence that parous women and their healthy offspring can persistently harbor mHAg-specific $CD8^+$ T_R cells bearing low-avidity T-cell receptors as well as $CD8^+$ cytotoxic T cells reactive to the same mHAg. With the use of PBMCs of healthy mother-offspring pairs mismatched for HY and/or HA-1 antigens, the presence of mHAg-specific $CD8^+$ T_R cells was analyzed in the trans-vivo DTH assays and a substantial proportion of mothers and offspring were found to harbor mHAg tetramer-dimly positive T_R cells functioning in a CTLA-4-dependent manner. Importantly, they also showed that the functional balance between T_R cells versus T_E cells significantly differ among individuals, providing a clue to explain the differential immunogenicity against IPAs or NIMAs observed in the setting of clinical HSC transplantation.

In a subsequent study using a murine model, Dutta et al. showed that the levels and tissue distribution of maternal microchimerism are strongly correlated with maintenance of NIMA-specific T_R cells in mice developmentally and neonatally exposed to NIMA.[29] Levels of maternal microchimerism were positively correlated with NIMA-specific suppression of both DTH and in vivo mixed lymphoproliferative reactions. Remarkably, when mice were exposed to NIMA only in utero and lacked oral exposure to NIMA via breastfeeding, maternal microchimerism was lost in association with emergence of sensitized DTH responses against NIMA. Collectively, these findings support the scenario that modes of immune responses toward IPAs in parous females as well as those toward NIMAs in offspring are determined by the positive or negative net balance between IPA- or NIMA-specific T_R cells and T_E cells, which might be correlated with levels of persistent fetal or maternal microchimerism and specific donor-recipient combinations of histocompatibility antigens.[30]

Future Directions and Conclusions

Accumulating lines of evidence from mice and humans have indicated the close association between long-term fetal-maternal microchimerism and the development of IPA- or NIMA-specific $CD4^+CD25^+$ and $CD8^+$ T_R cells. Since the immune equilibrium in favor of T_R cells versus T_E cells appears to be necessary for the maintenance of IPA- or NIMA-specific tolerance, future studies to improve the outcome of HSC transplants should aim at identifying means to enhance such tolerance in HSC donors harboring long-term fetal or maternal microchimerism. Because breastfeeding appears to be an indispensable mechanism to establish robust NIMA-specific tolerance, it is intriguing to investigate whether oral administration of NIMA peptide can promote the generation of NIMA-specific T_R cells in adult offspring previously exposed to NIMAs. In a similar fashion, whether oral or non-oral exposure to IPAs may affect IPA-specific immune responses in mothers harboring fetal microchimerism remains an open question. Since laboratory tests to evaluate T_R versus T_E balance such as allopeptide-specific tetramer staining or trans-vivo DTH assays have now become available, further clinical studies incorporating these tests into the criteria for microchimeric HSC donor selection are warranted to clarify the relevance of fetal-maternal microchimerism in establishing allospecific tolerance after hematopoietic stem cell transplantation.