Tumors escape CD4+ T cell-mediated immunosurveillance by impairing the ability of infiltrating macrophages to indirectly present tumor antigens

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Abstract

Tumors cells can escape cytotoxic CD8+ T cells by preventing MHC I display of tumor antigens. It is unknown how tumors evade CD4+ T cell responses, but since many tumor cells lack MHC II expression, novel mechanisms would be required. We have investigated this issue in a model where MHC II- myeloma cells secrete a monoclonal Ig containing a V region L chain epitope (V\textsubscript{L}) recognized by CD4+ T cells. Infiltrating macrophages process and present the secreted tumor antigen to Th1 cells, resulting in reciprocal induction of macrophage cytotoxicity and apparent rejection of the tumor. Despite long-term tumor protection in V\textsubscript{L}-specific T-cell-receptor-transgenic mice, we here describe that some myeloma cells persisted in a dormant state, and eventually formed expanding tumors. Escape tumor cells maintained their secretion of complete (H+L) monoclonal Ig with unchanged sequence, while secretion of surplus free L chain was severely diminished. While free L chains were efficiently processed and presented by tumor infiltrating macrophages to CD4+ T cells, complete (H+L) monoclonal Ig was not. Forced overexpression of free L chain secretion reinstated tumor rejection. These results show that tumors can escape CD4+ T cell-mediated rejection by impairing indirect presentation of tumor antigen by infiltrating macrophages. This occurs through a novel mechanism of immunoediting, in which modulation of the quaternary structure of the secreted tumor specific antigen reduces its immunogenicity.
**Introduction**

Previous strategies to induce effective anti-tumor immune responses have focused largely on cytotoxic CD8^+^ T cells that recognize tumor-specific peptides on MHC class I on the tumor cell surface. The efficacy of CD8^+^-mediated anti-tumor immune responses is limited by the commonly observed phenomenon of tumor escape, in which subsets of tumor cells become resistant to killing by effector CD8^+^ cells. Several mechanisms of tumor evasion have been described, including loss of tumor antigen synthesis or impaired loading on MHC class I molecules (1-3). This type of adaptation of residual tumor cells to an ongoing immune attack is commonly referred to as immunoediting (4).

Recent work has shown that tumor-reactive CD4^+^ T cells may significantly improve the efficacy of CD8^+^ T cells in adoptive T cell therapy (5, 6). Moreover, CD4^+^ T cell transfer may confer tumor eradication in the absence of CD8^+^ T cells (7-11). These results have lead to a growing interest in the use of CD4^+^ T cells in immunotherapy in humans (11, 12).

Antigen recognition by CD4^+^ T cell responses is dependent on antigen display on MHC class II, which is normally limited to professional antigen-presenting cells (APCs). Although some tumor cells express MHC class II (8, 9), and have been shown to be killed by direct interaction with cytotoxic CD4^+^ T cells by granzyme B/perforin-dependent (9) or Fas ligand-mediated (13) mechanisms, a large number of tumor cells lack detectable surface-bound MHC class II in vivo, and engagement of CD4^+^ T cells in such cases is therefore dependent on antigen uptake and display on APCs. Several groups have demonstrated that anti-tumor CD4^+^ T cell responses can protect against MHC II negative (MHC II^NEG^) tumor cell types (14, 15), supporting the notion that
indirect display of tumor-specific antigens on APCs can mediate tumor rejection. Several effector mechanisms have been implicated in such processes, including activation of macrophages (15, 16) or NK cells (14), or the induction of anti-angiogenic cytokines such as interferon gamma (IFNγ) (10).

We have previously demonstrated that CD4+-mediated rejection of MHC II\(^{NEG}\) myeloma cells is dependent on antigen secretion by the tumor cells, and is mediated by uptake and presentation of secreted tumor antigens on APCs within the tumor stroma, as well as draining lymph nodes (16-18). The killing of tumor cells occurs indirectly through Th1-mediated activation of tumor-infiltrating macrophages into M1-like cells with cytotoxic properties (16, 19). This indirect Th1/M1 mechanism of tumor cell killing is critically dependent on secretion of the tumor antigen (18). Since such indirect recognition by CD4\(^{+}\) T cells uncouples antigen presentation from the tumor cell itself, evasion of the immune response would be expected to involve either impaired secretion, mutations in the secreted tumor antigen, or impaired antigen uptake/presentation by host APCs. We here report evidence for the occurrence of the latter.

Multiple myeloma cells secrete monoclonal Ig (myeloma protein) that often contains unique amino acid sequences due to VDJ recombination and somatic hypermutation. Such non-germline sequences constitute attractive targets for immunotherapeutic interventions in B-cell-derived malignancies. We have previously described the generation of a T-cell receptor-transgenic (TCR-Tg) mouse that harbors CD4\(^{+}\) T cells specific for a mutated tumor-specific epitope (idiotope, Id) in the V\(_{L}\)-region of the M315 myeloma protein produced by the MOPC315 myeloma cell line (20, 21). The Id-specific CD4\(^{+}\) T cells recognize a CDR3 epitope on the \(\lambda2^{315}\) light chain of M315, when presented on I-E\(^{d}\) MHC class
Il molecules (7, 21). Id-specific TCR-Tg mice (7), even on a SCID background (15),
are resistant to s.c. challenge with MOPC315. Upon tumor challenge, such mice
fail to develop palpable tumors, and myeloma protein is only transiently
detectable, leading to the assumption that Id-specific CD4+ immune responses
cause a complete and lasting rejection of tumor cells (15).

Here, longitudinal monitoring of myeloma cells in Id-specific TCR-Tg SCID
mice revealed that a minute population of residual tumor cells remained in a
dormant state for a prolonged period in the absence of detectable myeloma
protein. Tumor dormancy was eventually followed by escape of tumor cells with
reduced secretion of free L chain, while the production of complete myeloma
protein was unchanged. Reduction of free light chain was causally related to
tumor escape.
Materials and methods

Cells and reagents

MOPC315 (IgA, λ\textsubscript{2}^{315}) is a BALB/c plasmacytoma obtained from the American Type Culture Collection (ATCC, Manassas, VA). mCherry-labeled MOPC315 cells were generated as previously described (22). The Ig non-producing variant MOPC315.36, and MOPC315.37, which retains free L-chains intracellularly due to a point mutation (Ala\textsubscript{15}→Arg\textsubscript{15}) in the L chain have been previously described.(18) The MOPC315.37α\textsubscript{315} variant was generated by stable transfection of MOPC315.37 cells with a pLNOH2 expression vector (23) containing a sequence encoding the H chain (V\textsubscript{H} + C\textsubscript{H}2) of M3 15. The identity of cell lines utilized in the study was confirmed by western blot using mAbs specific for the mutated L chain (24), and detection of CD138 and surface/intracellular M3 15 by flow cytometry. Cultured cells were propagated in RPMI1640-GlutaMAX medium (Sigma-Aldrich, St Louis, MO) supplemented with 10%FCS and penicillin/gentamycin (Invitrogen, Carlsbad, CA). The following factors were used for in vitro propagation of Id-specific T cells: 20U/mL rmIL-2 (PeproTech, Rocky Hill, NJ), 4ug/mL Id peptide (aa 89-107 of λ\textsubscript{2}^{315} sequence; NeoMPS, Strasbourg, France).

Plasmid constructs and lentiviral transduction

The pLJM1-EGFP vector was provided by dr. David Sabatini through the Addgene repository (#19319). pLJM1-L2_315 was constructed by replacement of EGFP with the coding sequence of λ\textsubscript{2}^{315}. Lentiviral transduction was performed by co-transfection of HEK293T cells with the pLJM1 vector and the packaging vectors pCMV-VSV-G (#8454) and pCMV-dR8.2 dvpr (#8455), provided by dr. Bob
Weinberg through Addgene.

**Mice, tumor challenge and tissue preparation**

Id-specific T cell receptor transgenic (TCR-Tg) mice were generated from (CBA/Br x C57Bl6/6LiA)F2 embryos, backcrossed for 10 generations to BALB/c (21), and crossed onto a BALB/c SCID background (15). For the current study, TCR-Tg SCID mice or SCID littermates on BALB/c background were bred in a (nontransgenic SCID x heterozygous TCR-Tg SCID) fashion. Mice used in experiments were 4-6 week old females weighing 16-25g. Mice were injected subcutaneously with $1.6 \times 10^5$ tumor cells in 100 μL phosphate-buffered saline (PBS) or in 100 μL Matrigel (BD Biosciences, San Diego, CA). Tumor growth was followed using the IVIS200 Imaging System (Caliper LifeSciences, Hopkington, MA). Mice were euthanized when tumor diameter reached >15mm. Bromodeoxyuridine (BrdU) incorporation assays were performed as previously described (16). Preparation of cells for flow cytometry was performed as previously described (19). The sources of antibodies used is given in Table S1.

**Real-time PCR**

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany), and cDNA generated using the 1st Strand cDNA Synthesis Kit (Life Technologies, Grand Island, NY) with oligo-(dT) primers. RT-PCR was performed using TaqMan Universal PCR Master Mix on an ABI-Prism 7000 Sequence Detection system (Applied Biosystems, Foster City, CA) using pre-designed FAM/TAMRA primer/probe-sets (IDT Technologies, Coralville, IO). Results were quantified using ddCt method with normalization against TATA-box binding protein (Tbp).
In vitro T cell proliferation and tumor cell growth inhibition assays

Matrigel-infiltrating CD11b+ cells were isolated using CD11b MicroBeads (Miltenyi Biotech, Gladbach, Germany). Sorted CD11b+ cells (≥95% pure) were irradiated (2,000 rad) before utilization in T cell proliferation assays. Splenocytes isolated from SCID mice or tumor-derived CD11b+ cells were irradiated (2000 rad) and utilized as stimulators (2x10^5 cells per well). Responders (2×10^4 cells per well) were short-term cultured Th1-polarized lymphocytes from TCR-Tg SCID mice, prepared as previously described (25). Supernatant collected from 24h culture of 1x10^6 irradiated tumor cells were used as an antigen source. An optimal stimulatory concentration of synthetic 91-101 Id (λ2^{315}) peptide (4 µg/ml) was added to the positive controls. M315, λ2^{315} and λ2^{T952} for use in T cell proliferation assays were purified as previously described (26). For tumor growth inhibition assays, 1,5x10^4 tumor cells were co-incubated with 1,5x10^5 irradiated CD11b+ cells or splenocytes and 5x10^4 Id-specific T cells prepared as described above. Cultures were pulsed with [3H]-thymidine (Montebello Diagnostics, Oslo, Norway) after 24 h, harvested 48 h later and counted using a TopCount NXT microplate counter (PerkinElmer, Waltham, MA).

Imunoaffinity chromatography and metabolic labeling

For the separation of complete M315 and free λ2^{315} light chains from MOPC315, samples were passed through a column containing sepharose-conjugated anti-Id Ab2-1.4 mAb, which requires association of the λ2^{315} with H^{315} in a complete (H+L) M315 molecule for binding (27). Thus, free λ2^{315} L chain passes through the column. To obtain free L chain/M315 (H+L) ratios, pre-/post-adsorption
samples were analyzed in parallel by ELISA, as previously described (18). For high-throughput screening, cell culture supernatants were centrifuged in 96-well filter plates containing a 100kDa cut-off membrane (AcroPrep Advance; Pall Corporation, Port Washington, NY) and the flow-through collected for ELISA analysis as described above. Metabolic labeling of tumor cells was performed as previously described (28). Briefly, cells were washed in labeling medium (methionine-free RPMI1640 + 10% FCS and 15mg/L methionine) and pulsed for 1h in labeling medium containing 350μCi/mL L-(³⁵S)-methionine (Hartmann Analytic GmbH, Braunschweig, Germany). Non-reduced lysates and supernatants were immunoprecipitated for 12h at 4°C using sepharose-conjugated mAb specific for the C-domain of λ2 (2B6). Bound protein was eluted using 0,1M Tris-glycine buffer and separated on 10% SDS-PAGE gels. Gels were soaked in 0,5M sodium salicylate (Sigma-Aldrich) and developed using Amersham Hyperfilm MP (GE Healthcare, Piscataway, NJ).

**Mass spectrometry (MS) analysis and Protein Identification**

Affinity-purified M315 in solution was digested with trypsin prior to MS analysis, and data was acquired as previously described (29). Acquired MS data was submitted to de novo sequencing using PEAKS, using trypsin with no proline restriction as enzyme. Only peptide sequences with more than 85% average coverage certainty were submitted to BLAST analysis using the antibody sequence previously obtained by nucleotide sequencing of MOPC315 using the PEAKS DB option.

**Statistical analysis**
The Mann–Whitney U test was used for statistical analysis unless stated otherwise. For tumor challenge experiments, differences in survival were analyzed using the log-rank test. Statistical analysis was performed using Prism 5.0 software (GraphPad Software, La Jolla, CA). p<0.05 was considered statistically significant.
Results

Myeloma cells subject to CD4+ T cell immune responses undergo immunoediting with eventual tumor escape

Utilizing fluorescently labeled MOPC315 cells, we monitored the fate of injected tumor cells by *in vivo* imaging. In accordance with previous reports (15), tumor-challenged SCID mice developed rapidly expanding tumors within 2-3 weeks after challenge (*Fig. 1A*). By contrast, the TCR-Tg SCID mice (hereafter referred to as TCR-Tg) showed a rapid decrease in fluorescent signal intensity. Nonetheless, by long time monitoring, we observed that the TCR-Tg mice retained a weak but detectable fluorescent signal at the injection site (*Fig. 1B-C*), even though serum myeloma protein concentration was below the detection limit (*Fig. 1D*). Eventually, palpable tumors developed after a mean observation time of **12±4** weeks (*Fig. 1A*). Concomitant with tumor development, M315 myeloma protein became detectable at levels comparable to that of similarly sized tumors in SCID mice (*Fig. 1D*).

Depletion of CD4+ cells in TCR-Tg mice harboring dormant tumor cells resulted in rapid tumor escape, confirming the importance of continued presence of Id-specific CD4+ T cells for immnosurveillance (*Fig. S1A*). Consistent with the recurrence of serum myeloma protein upon tumor escape, *in vitro* secretion of M315 by escape tumor cells (MOPC315.E) was unchanged compared to the parental MOPC315 cell line (*Data not shown*), and peptide sequencing of the M315 L chain gene of MOPC315.E cells revealed no mutational changes within the antigen coding sequence (*Data not shown*). Moreover, M315 isolated from cultures of parental or escape tumor cells showed comparable ability to stimulate proliferation of Id-specific T cells (*Fig. S1B*).
To further identify the mechanisms of immunoevasion, tumor cells were isolated from escape tumors, cloned and re-injected into Id-specific TCR-Tg mice after 1-2 weeks of culture. A complete loss of immunoprotection against such cells was consistently observed (Fig. 1E).

**Tumor escape is caused by impaired intratumoral presentation of tumor specific antigen**

Based on preserved secretion of the tumor-specific myeloma protein by the escape tumor cells, we hypothesized that immunoevasion was caused by functional disturbances in the tumor-specific T cell response. Arguing against this possibility, draining lymph nodes (DLNs) from TCR-Tg mice harboring escape tumors still contained Id-specific T cells that proliferated upon in vitro re-stimulation at increased levels compared to naïve TCR-Tg T cells (Fig. 2A). When syngeneic TCR-Tg mice were injected with escape tumor cells (MOPC315.E), a decreased Id-specific T cell proliferation (Fig. 2B) and CD69 expression (Fig. 2C) was observed in DLNs, even though Th1 polarization (Tbet+IFNγ+) was maintained (Fig. 2D). There was a corresponding decrease in the number of tumor-infiltrating Id-specific T cells (Fig. 2E). Based on the reduced numbers of responding T cells in DNL and tumors of MOPC315.E-challenged mice, it seemed possible that tumor escape could be caused by impaired local antigen presentation on tumor-infiltrating APCs. It was previously demonstrated that the bulk of tumor-infiltrating APCs in our model is formed by CD11b+ cells with macrophage characteristics (16). To assay the antigen-presenting ability of these cells, CD11b+ cells were isolated from tumors of SCID mice challenged with MOPC315 or MOPC315.E cells, and used in *in vitro* T cell
proliferation assays. CD11b+ cells derived from escape tumors had an impaired ability to induce T cell proliferation, but stimulatory capacity was restored upon addition of Id peptide (Fig. 3A), suggestive of reduced intratumoral antigen availability. Consistent with this, supernatants (SN) from escape tumor cells had a dramatically impaired ability to induce proliferation of Id-specific T cells in the presence of APC (Fig. 3B). This loss of T cell proliferation could be reversed by reconstitution with 50% MOPC315 conditioned medium or by addition of Id peptide (Fig. 3B), suggesting that impaired T cell activation was caused by inefficient antigen presentation in escape tumors.

Inflammation (19) associated with cytotoxic M1-like macrophage activation (16) has been implicated as a key mediator of successful tumor immunosurveillance. The number of Matrigel-infiltrating CD11b+ cells was similar in mice challenged with MOPC315 and MOPC315.E cells (Fig. 3C), but CD11b+ cells from escape tumors were poorly activated, as evidenced by decreased expression of activation markers MHC class II and CD86 (Fig. 3D and data not shown).

**Id-specific CD4+ T cell proliferation is dependent on secretion of free immunoglobulin light chains**

Based on the above results, we reasoned that loss of T cell activation might be dependent on the tumor antigen being present in another form than complete M315 Ig.

MOPC315 cells, similar to MM cells in general, are known to produce excess amounts of Ig L chains detectable as free L chains (FLC). We therefore assayed the secretion of FLC by MOPC315 and MOPC315.E cells into cell culture
supernatants. A significant reduction in free $\lambda 2$ L chain secretion was found for all escape tumors (Fig. 4A, $p < 0.001$), while complete (H+L) Ig was present at levels comparable to that of parental cells (Fig. 4A). Assays of clones derived by limiting dilution revealed homogenous FLC secretion by the parental MOPC315 cells, and a relatively uniform decrease in FLC secretion by escape tumor clones obtained by limiting dilution from escape tumors (Fig. 4B). Western blots of tumor cell lysates revealed a selective decrease in the amount of free L chains in escape tumors, whereas the level of the complete M315 Ig was present at comparable levels in parental and escape tumor cells (Fig. 4C). Correspondingly, mRNA expression of the $\lambda 2$ light chain was significantly decreased in escape tumor clones (Fig. 4D), with a significant correlation between $\lambda 2$ mRNA expression level and the amount of free L chains secreted (Fig. S2A). In contrast, IgA heavy chain mRNA levels remained unchanged (Fig. 4D). Metabolic labeling experiments revealed similar kinetics of Ig secretion by parental and escape tumor cells (Fig. 4E and S2B), with no evidence of L chain retention in escape tumor cells (Fig. 4E). L chains appeared in the culture supernatant primarily in monomeric form, in accordance with previous reports (30), but was present at significantly lower levels in cultures of escape tumor cells than in parental cells (Fig. 4A). In summary, these results demonstrate a selective decrease in light chain transcription, reflected in impaired secretion of FLC, whereas synthesis remains sufficient to allow secretion of complete L+H Ig.

Given the selective reduction in FLC in escape tumors, with secretion of complete (H+L) M315 myeloma protein remaining essentially unchanged, we reasoned that the two different quaternary forms of the tumor specific antigen could differ in their ability to stimulate tumor-specific CD4$^+$ T cells. In
accordance with a previous report (20), in vitro T cell proliferation assays
demonstrated that isolated λ2 FLC had a 100-fold greater ability of to induce T
cell proliferation compared to equimolar amounts of complete M315 (Fig. 4F).

Id-driven CD4+ T cell immunoprotection against MOPC315 myeloma cells is
dependent on secretion of free L chains

Overexpression of λ2315 L chain under the control of a viral promoter in escape
tumor cells increased light chain synthesis (Fig. 5A) and restored
immunoprotection (Fig. 5B). In contrast, overexpression of the mutant λ27952
light chain, which differs from λ2315 in the amino acid composition within the
idiotype (aa 94-99) in MOPC315.E cells, failed to reverse the escape phenotype
(Fig. 5B). These results conclusively demonstrate that tumor escape is caused by
impaired secretion of λ2315 L chain.

To further confirm the importance of FLC secretion for immunoprotection, we
utilized the previously described MOPC315.37 variant cell line. These cells
produce no heavy (H) chain, and contain a point mutation within the L chain
variable (V) region (Ala15→Arg15) that causes retention of FLC within the ER,
preventing its secretion (31, 32). We found that by transfecting MOPC315.37
cells with a plasmid expressing the IgA M315 H chain, secretion of a complete
H+L Ig was obtained even though FLC secretion was still absent (presumably,
upon association with an H chain, the ER retention signal on the mutated L chain
is masked). This ensures secretion of only the complete M315-like molecules by
the resultant MOPC315.37α315 cell line (Fig. 5C). Tumor challenge experiments
in TCR-Tg mice revealed that injection of MOPC315.37α315 cells led to rapid
tumor development in both SCID and TCR-Tg mice, similar to MOPC315.E cells, confirming that assembled Ig is a poorly stimulatory tumor antigen (Fig. 5D).

**Loss of λ2 free light chain secretion is a consequence of T-cell-mediated immunooediting**

In order to establish whether loss of FLC secretion occurs as the result of *in vivo* immunooediting rather than through selection of pre-existing clones, MOPC315 clones were derived by limiting dilution. Tumor challenge experiments were performed using clones with high levels of FLC secretion. Clonally derived tumor cells were subject to immunooediting, causing tumor escape within the same timeframe as for the heterogeneous parental MOPC315 population (Fig. 5E), and escape tumor cells displayed similarly suppressed L chain transcription and impaired FLC secretion (Fig. 5F).

A possible explanation for these results could be that variants expressing low levels of FLC might arise during in vitro expansion of cloned cells, required to obtain sufficient number of tumor cells for injection. To test this, cells from the injection inoculum were re-cloned and assayed for FLC secretion. Light chain secretion in the clonally derived cells was found to be homogenous, with no FLC loss variants identified amongst 900 screened cells (Fig. S2C), essentially excluding the possibility of FLC loss occurring during the in vitro expansion phase prior to tumor challenge.
Discussion

The present results demonstrate that an initially successful CD4+/-restricted T cell response against Id-secreting myeloma cells is eventually overcome by residual tumor cells that persist in a dormant state for several months. The escape tumor cells retain their ability to resist T-cell mediated killing upon re-injection into Id-specific TCR-transgenic mice, even after cloning and prolonged culture, suggesting that the underlying mechanisms represent stable changes intrinsic to the tumor cells.

Further characterization of escape tumor cells revealed that the antigen responsible for T cell activation is not, as previously assumed, the complete M315 Ig molecule, but rather a surplus of L chains that are secreted in a free form, not assembled with H- chains, by the myeloma cells. Tumor escape was associated with a dramatic reduction in secretion of free L chains (FLC), despite continued secretion of the complete myeloma protein. Importantly, L chain assembled in the H+L myeloma protein still expressed the tumor specific Id-epitope, since no mutations were found, and since myeloma protein from escape tumors were fully stimulatory to Id-specific CD4+ T cells. Experimental reintroduction of free L chain secretion in escape tumor cells resulted in prompt rejection by Id-specific TCR-Tg mice, providing a causal link between the presence of FLC and successful tumor rejection. Further evidence was obtained by H chain transfection of a MOPC315 tumor cell variant that normally retains a mutated L-chain. Again, secretion of FLC was needed for rejection to occur. Taken together, these findings demonstrate that the presence of the tumor antigen in the form of a protein complex (assembled M315 IgA) impairs the accessibility of the epitope for presentation on host APCs compared to free, non-
complexed L chains. Thus, a change in the abundance of one protein conformation allows tumor escape from CD4\(^+\) T cells even though the peptide sequence recognized by T cells is unchanged and remains available in a complexed form.

These observations most likely relate to differences in antigen processing of complete Ig and FLC within the APC. Accordingly, we demonstrate that FLC is \(100\times\) more efficient than complete (H+L) Ig at stimulating Id-specific T cells. This finding is in agreement with previous experiments demonstrating that complete (H+L) M315 in either native or reduced/alkylated form, as well as Fab\(^{315}\), fed to spleen APCs were all poorly stimulatory to cloned Id-specific CD4\(^+\) T cells. By contrast, “preprocessed” fragments such as free \(\lambda2^{315}\) chains, \(V_L^{315}\) and \(Fv^{315}\) fragments were all highly stimulatory (20). It could thus be speculated that properties of the assembled Fab region of the Ig molecule might prevent complete antigen processing, possibly due to effects of the non-covalent interaction between Fd fragments (VH+CH1) and to the L chain. This would limit availability of short V region sequences for binding to MHC class II molecules in endosomes, resulting in diminished Id presentation. Previous experiments have indicated that processing of complete Ig in macrophages results in accumulation of Fab-like molecules, suggestive of a relative resistance of Ig to proteolytic enzymes (33).

The issue of differences in processing of various forms of Ig light chains, either in free form or associated with H-chains, in APCs is the subject of ongoing investigations.

The influence of conformation of processing of proteins is not unique to Ig but may be extended to such as insulin (34), hemoglobin (35) and lysozyme (36).
Modulation of the form of the antigen produced by the tumor cells may constitute an under-appreciated means of immunoediting. Hence, our findings of altered FLC availability might also be relevant for other tumor antigens that form complexes. This sort of analysis requires insight into the forms in which the antigen is present, its secretory status and, most likely, the mode of T-cell mediated killing. Further evaluation of this issue is complicated by the limited number of presently available models of CD4+ T cell responses against bona fide tumor antigens, as recently reviewed (37). Nonetheless, our results underscore the importance of considering tertiary and quaternary protein structure when looking at T cell responses against such antigens.

MHC class II expression is normally limited to professional APCs, although IFNγ has been shown to induce surface expression of MHC class II in some types of malignant cells. Similar to most myeloma cells, MOPC315 cells are MHC class II negative, even in the presence of IFNγ (16, 18). Hence, recognition of Id by CD4+ T cells is critically dependent on display on host APCs (18). Other groups have similarly shown a dependence of anti-tumor CD4+ T cell responses on host MHC II expression (14), even when tumor cells themselves are MHC IIPOS (8, 9). Thus, display of tumor antigen-derived peptides on host APC may be universally required for tumor protective CD4+ T cell responses. It thus seems possible that malignant cells may circumvent CD4+ T cell responses by changing the expression of tumor-specific antigens so that display on host APCs is impaired. The present data exemplifies this type of evasion strategy, and further shows that such effects can be obtained by restricting antigen availability to a poorly
immunogenic multi-protein complex, most likely influencing antigen uptake and processing within the APC.

Concerning the mechanism underlying the escape phenotype, the loss of FLC secretion was associated with impaired mRNA expression levels, and a decreased abundance of FLC in cell lysates. The levels of complete M315 secretion by escape tumor cells were not significantly decreased compared to parental cells, although some heterogeneity in the levels of secreted M315 was observed among the escape tumor clones. The basis for the resulting balanced synthesis of H and L chains in escape tumor cells is not known. Previous reports have demonstrated that unbound H chains have cytotoxic effects (38, 39), which provides a selective pressure against excess synthesis of H chains. At the same time, the high protein turnover in myeloma cells imposes a considerable endoplasmic reticulum stress (reviewed in (40)), which increases susceptibility to cell death by added stressors such as reactive oxygen species. Thus, the balance of L and H chain synthesis might represent a tradeoff between a need to avoid heavy chain-mediated cytotoxicity (41) and beneficial effects of reduced L chain availability in the face of the Id-specific T cell response. Whereas outgrowth of tumor cells secreting only L-chains is observed in a subset of myeloma patients during disease progression (so-called "free light chain escape") (42, 43), the occurrence of loss of FLC secretion has not been reported. It should be underscored that this phenomenon of "free light-chain escape" represents an entity with no obvious relation to the present findings. Although CD4+ T cell reactivity against myeloma cells in patients receiving conventional treatment cannot be excluded, selective outgrowth of tumor cells with impaired FLC secretion seems unlikely to occur in the absence of immunotherapeutic
interventions targeting Id. Nonetheless, it seems plausible that the mechanisms driving the loss of FLC secretion could be related to tumor cell adaptation to other kinds of cellular stress, e.g. development of resistance to cytotoxic drugs. This issue is being addressed through ongoing experiments.

It should be underscored that the escape phenotype appears not to be due to pre-existing variants within the tumor cell inoculum. Clonally derived MOPC315 cells with stable expression of both H+L and FLC secretion consistently underwent down-regulation of FLC secretion in the presence of an ongoing Id-specific CD4+ T cell response. These results argue for an acquisition of the escape phenotype in vivo as a consequence of Th1/M1-mediated immune pressure on tumor cells.

The present study identifies a novel mechanism of tumor immunoevasion from an anti-tumor CD4+ T cell response. Immune escape develops through a modulation of secretion of a natural tumor-specific antigen, limiting availability of the antigen in the conformation that is required for efficient display on tumor-infiltrating APCs. Consequently, T cell activation and induction of cytotoxic macrophages is dampened. This underscores the importance of efficient local antigen display within the tumor microenvironment for potent CD4+ T-cell mediated immunotherapy of MHCII<sup>NEG</sup> tumor cells, and extends the framework for investigations into mechanisms of tumor immunoediting.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
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**Figure legends**

**Figure 1 - Tumor escape from CD4+ T cell immunoprotection**

**(A)** Survival of Id-specific TCR-Tg SCID (n=45; hereafter referred to as TCR-Tg) and SCID mice (n=8) challenged with 1.6x10^5 MOPC315 cells. **(B-C)** Bioluminescence imaging of tumor development. Quantitative data are in **(B)** and representative examples in **(C)**. Mice were challenged s.c. with 1.6x10^5 MOPC315-mCherry cells. White arrows **(C)** in top left panel indicate the injection site. Lanes 1 and 4 show SCID mice; lanes 2, 3 and 5 show TCR-Tg mice. Color scale was set at a radiance ([photons/second/cm^2/steradian]/[μW/cm^2]) of 6.7x10^8-1.6x10^9. **(D)** Serum M315 levels in TCR-Tg mice. Timepoint “Escape” represents the time of tumor escape, ranging from 10-20 weeks after challenge. Dotted line (n.d.) indicates detection threshold. Graph shows pooled data from 2 independent experiments (n=10 mice). **(E)** Survival of TCR-Tg and SCID mice injected with MOPC315 or MOPC315. E cells (1.6x10^5). Results are pooled from 3 independent experiments with MOPC315.E clones derived from three different primary tumors established in TCR-Tg mice, n=6-8 per treatment group. Bars indicate mean ± SD.

**Figure 2 - Impaired T cell responses in escape tumors**

**(A)** *In vitro* proliferation of 5x10^4 lymphocytes from DLN of unchallenged (naïve) or tumor-challenged TCR-Tg mice at the time of tumor escape (MOPC315.E) in the presence of syngeneic APCs and synthetic Id peptide (n=8-12 per treatment group) **(B)** BrdU incorporation at d+11 after challenge in TCR-Tg mice. Dot plots show representative results of intracellular BrdU staining in Id-specific Vβ8+ T cells from DLN. **(C)** CD69+ Id-specific CD4+ T cells in DLN of TCR-Tg mice at d+11 following challenge. Results are expressed in percentage
of the total T cell population (n=12 per treatment group). CD4+ cells were gated using TCR-clonotypic mAb. (D) Representative staining for Tbet and IFNγ in tumor-infiltrating Id-specific CD4+ T cells at d+11 following s.c. challenge with Matrigel-embedded MOPC315 or MOPC315.E cells. Dotted lines shows isotype control. Results are representative of two independent assays using cells derived from different tumors, n=6 per treatment group. (E) Quantitation of tumor-infiltrating Id-specific T cells on day +11 following challenge. Bars indicate mean ± SD.

**Figure 3 - Impaired T cell priming by escape tumor cells**

(A) *In vitro* proliferation of short-term cultured Id-specific T cells in the presence of CD11b+ cells isolated from Matrigels at day +11 following tumor challenge of SCID mice with MOPC315, MOPC315.E or the Id non-producing MOPC315.36. The rightmost bar shows proliferation with addition of synthetic Id-peptide. (B) *In vitro* proliferation of Id-specific T cells cultured with SCID splenocytes in the presence of conditioned medium (CM) from 3-day cultures of MOPC315 or MOPC315.E cells, 1:1 mixture of CM, or MOPC315.E-CM spiked with synthetic Id peptide. (C) Mean number of CD11b+ macrophages per Matrigel plug on day +11 following challenge determined by flow cytometry. Data in (A-C) represent the pooled results of two independent assays using cells from different primary tumors, n=6 per treatment group. (D) MFI-values for MHC II expression on tumor-infiltrating CD11b+ cells from TCR-Tg and SCID mice at day +8 following challenge with Matrigel-embedded MOPC315 or MOPC315.E cells. Results represent pooled data from two independent experiments, n=12 per treatment group. Bars indicate mean ± SD.
Figure 4 – Selective loss of myeloma protein light chain secretion in tumor escape

(A) Levels of complete (H+L) M315 and free L chains (FLC) in cell culture supernatants of in vitro cultured MOPC315 and MOPC315.E (n=6). An L chain-only producing variant (MOPC315.26), a non-producing variant (MOPC315.36), and a variant that retains FLC intracellularly (MOPC315.37) were included as controls. (B) Secretion of complete M315 and FLC by short-term cultured clones of MOPC315 and MOPC315.E tumor cells (derived from three different escape tumors) obtained by limiting dilution. (C) Western blot detection of FLC (anti-\(\lambda\)2 mAb) and complete M315 (anti-\(\alpha\) mAb) in cell lysates of MOPC315, MOPC315.E, L chain-only producing MOPC315.26 and non-producing MOPC315.36 cells under non-reducing conditions. (D) Gene expression levels of L chain and IgA H chain in clones of either MOPC315 (n=16) or MOPC315.E cells (n=16). Fold change values were calculated relative to mean expression in MOPC315 tumor cells. (E) Pulse-chase experiments of cell lysates of MOPC315 and MOPC315.E tumor cells pulsed for 1h with \(^{35}\)S-labeled methionine and chased for the indicated amount of time. Non-reduced samples were immunoprecipitated using the 2B6 mAb specific for the L chain C-region. 2B6 mAb recognizes both assembled (H+L) and free L chain of M315. (F) Proliferation of Id-specific T cells in the presence of peritoneal macrophages and increasing concentration of either affinity-purified (H+L) M315 IgA myeloma protein, free L chain purified from M315 (\(\lambda_2^{315}\); Phe\(^{94}\)Arg\(^{95}\)Asn\(^{96}\)) or free L chains purified from T952 myeloma protein (\(\lambda_2^{T952}\); Tyr\(^{94}\)Ser\(^{95}\)Thr\(^{96}\); lacking the stimulatory CDR3 Id sequence) (26). Data is presented as mean \(\pm\) SD.

Figure 5 – Free L chain secretion is sufficient for Id-driven T cell immunoprotection against myeloma cells

(A) Non-reducing western blot showing free \(\lambda_2\) levels (\(\lambda_2^{315}\); anti-\(\lambda_2\) mAb 2B6) in cell
lysates of Id non-producing cells (MOPC315.36), L-chain only producing cells (MOPC315.26), clones of the parental MOPC315 cell line, escape tumor cells (MOPC315.E), mock-transduced escape tumor cells (MOPC315.E Ctr) and escape tumor cells overexpressing \( \lambda^2 \) under the control of a viral promoter (MOPC315.E-\( \lambda^2 \) c1 and c2, derived from two separate escape tumors). (B) Survival of TCR-Tg and SCID mice challenged with 1.6x10^5 MOPC315.E cells transduced with vector expressing \( \lambda^2 \) L chain (MOPC315.E-\( \lambda^2 \)) or the unrelated \( \lambda^2 \) L chain (MOPC315.E-\( \lambda^2 \)). (C) Concentration of M315 IgA and free \( \lambda^2 \) L chains in culture medium from the following MOPC315 variants; wild-type MOPC315, MOPC315.36, MOPC315.37 producing mutated \( \lambda^2 \) variant that is retained intracellularly and no heavy chain or M315-only-secreting MOPC315.37\( \alpha^3 \) (MOPC315.37 transfected with rearranged VDJH\( \alpha \) chain gene of MOPC315) (D) Survival of TCR-Tg and SCID mice (n=8) challenged with MOPC315.37\( \alpha \) cells which secrete complete M315 but no FLC, and control groups of TCR-Tg mice challenged with MOPC315 or MOPC315.37 cells. (E) Survival of TCR-Tg and SCID mice challenged with clonally derived MOPC315 cells. Results were pooled from independent experiments with three different MOPC315 clones (n=5-6 per clone). (F) \( \lambda^2 \) mRNA levels in escape tumor cells isolated from clones obtained in experiments shown in Fig 4C. Expression level was calculated relative to mean of parental tumor cells. Data is presented as mean ± SD.
Figure 3
Figure 4

A. 

B. 

C. 

D. 

E. 

F.
Tumors escape CD4+ T cell-mediated immunosurveillance by impairing the ability of infiltrating macrophages to indirectly present tumor antigens

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