Low neoantigen expression and poor T-cell priming underlie early immune escape in colorectal cancer

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Immune evasion is a hallmark of cancer and therapies that restore immune surveillance have proven highly effective in cancers with high tumor mutation burden (TMB) (for example, those with microsatellite instability). Whether low TMB cancers, which are largely refractory to immunotherapy, harbor potentially immunogenic neoantigens remains unclear. Here, we show that tumors from all patients with microsatellite stable colorectal cancer express clonal predicted neoantigens despite low TMB. Unexpectedly, these neoantigens are broadly expressed at lower levels compared to those in colorectal cancer with microsatellite instability. Using a versatile platform for modulating neoantigen expression in colorectal cancer organoids and transplantation into the distal colon of mice, we show that low expression precludes productive cross-priming and drives immediate T-cell dysfunction. Notably, experimental or therapeutic rescue of priming rendered T cells capable of controlling tumors with low neoantigen expression. These findings underscore a critical role of neoantigen expression level in immune evasion and therapy response.

Approximately 12% of colorectal cancer (CRC) has defects in DNA mismatch repair (MMR) resulting in microsatellite instability (MSI), with a high burden of mutation-derived tumor-specific antigens (neoantigens) that underlies favorable response to immune checkpoint blockade (ICB). The remaining majority of CRC is microsatellite stable (MSS) with lower TMB. However, MSSCRC has an average more mutations than some cancers that respond favorably to ICB. Presentation of neoantigen-derived epitopes (neoepitopes) on human leukocyte antigen class I (HLA-I) has also been observed in a small study of MSS CRC. This suggests that other factors, both tumor intrinsic and microenvironmental, likely contribute to the poor immunogenicity of these and other immune ‘cold’ cancers. Indeed, the intestinal microenvironment is tolerant to commensal bacteria and food-derived antigens and these immune ‘cold’ cancers. In addition, the vast majority of CRC is associated with aberrant WNT/β-catenin signaling, which can promote exclusion of dendritic cells and failure to prime productive T-cell responses in melanoma and liver cancer. To rigorously study processes underlying T-cell dysfunction and immunotherapy resistance, it is critical that models faithfully recapitulate the tissue microenvironment and genetics of the human disease. Models should also enable isolation of defined antigen-specific T cells. To our knowledge, no single model of CRC meets all these criteria. Therefore, we adapted a technique employing endoscope-guided submucosal injection to induce genetically defined tumors in the mouse colon harboring model CD8+ T-cell antigens.

An additional feature we sought to model is the role of neoantigen expression level in modulating the antitumor immune response. While mutations in cancer are generally enriched in poorly expressed genes due to reduced transcription-coupled repair, this pattern is absent in MMR-deficient tumors. This raises the possibility that poor immunogenicity of MSS CRC and other MMR-proficient cancers is not only due to lower burden but also lower expression of neoantigens. It is appreciated that antigen expression is a central determinant of the magnitude of T-cell response in viral infection and low neoantigen expression or affinity for MHC-I results in tumor immune evasion in flank transplant models. Clinical studies have also shown that tumors frequently evade immune recognition via loss of heterozygosity of HLA alleles and dysregulation of antigen processing and presentation. Despite these results, there remains some controversy surrounding the role of initial T-cell receptor (TCR) signaling strength in shaping T-cell fate and function. In cancer specifically, the impact of low neoantigen expression on T-cell dysfunction is poorly characterized.

Results
MSS CRC has lower burden and expression of neoantigens. To guide development of a mouse model of CRC enabling tracking of tumor-specific T-cell responses, we first developed a neoantigen prediction pipeline integrating HLA haplotype calling and affinity prediction algorithms and applied it to whole-exome and RNA-sequencing data from The Cancer Genome Atlas (TCGA) colorectal adenocarcinoma (COADREAD) cohort. Tumors from all
patients with MSS CRC (excluding rare cases with hypermutation) expressed at least 14 (median 121) single-nucleotide variant (SNV)- or insertion/deletion (indel)-derived neoantigens with predicted affinity to their respective HLA-I of half-maximum inhibitory concentration (IC_{50}) ≥ 500 nM, despite a lower TMB compared to patients with MSI high (MSI-H) CRC (Fig. 1a,b and Extended Data Fig. 1a). Notably, average expression of genes encoding predicted neoantigens was also significantly lower in MSS versus MSI-H tumors (Fig. 1b and Extended Data Fig. 1b), consistent with more mutations in highly expressed genes in MMR-deficient tumors.

We also observed significantly lower average predicted neoantigen clonality (ABSOLUTE purity) for estimation of clonality (adjVAF); n = 62 patients with MSI-H, 68 with MSI-L and 266 with nonhypermutant MSS. NS, not significant. b-e, Analysis of patients with available ABSOLUTE purity for estimation of clonality (adjVAF); n = 50 patients with MSI-H, 58 with MSI-L and 236 with nonhypermutant MSS. Spearman rank correlation matrix of MSS status (MSS versus MSI-H) and clonal neoantigen expression, predicted affinity, burden and clonality by patient (b). Strength of correlation is represented by color scale (red, positive; blue, negative) and significance is indicated by asterisk with P values displayed. Proportion of patients expressing at least one clonal (adjVAF ≥ 0.5) neoantigen with very strong predicted binding affinity (IC_{50} ≤ 10 nm) (c). Empirical cumulative distribution function of mean neoantigen expression by patient, showing enrichment of lower expression patients with MSS (d). Significance assessed by two-sided Kolmogorov–Smirnov test. Mean expression of clonal neoantigens by patient (FPKM, upper quartile-normalized) (e). FPKM, fragments per kilo base per million mapped reads. Significance in a and e was assessed by two-tailed Wilcoxon rank-sum test with Holm’s correction for multiple comparisons.

Neoantigen expression level is a critical determinant of immunity. We first developed an autochthonous model in Apc^{−/−} mice initiated by lentivirus expressing Cre-recombinase and the chicken ovalbumin antigen linked to luciferase (LucOS) (Extended Data Fig. 2a), as we have previously conducted in models of lung cancer and soft-tissue sarcoma. Injection with LucOS dramatically reduced tumor incidence in a T-cell-mediated manner and tumors that did arise invariably lost antigen expression (Extended Data Fig. 2b–e). To assess effects of antigen expression in established tumors, T cells were continuously depleted for 5 weeks, at which point tumors retained antigen expression. However, 7 weeks after withdrawal of depleting antibodies, tumors had grown and lost antigen expression (Extended Data Fig. 2f,g). Given this potent immune editing and variability of antigen expression (Extended Data Fig. 2h), we developed a colon organoid model that maintains distinct levels of antigen expression throughout tumorigenesis. Organoid engineering also enables the use of a Kras mutant allele to model metastatic colon adenocarcinoma, which is confounded in the autochthonous model by concomitant Kras-driven fibrosarcoma formation (unpublished observations).

To enforce stable and continuous expression of antigen, we generated CRC organoids with SIINFEKL (H-2K^{b} IC_{50} ≤ 10 nm) (Fig. 1c). Additionally, average expression of genes encoding clonal predicted neoantigens was still significantly lower in tumors from patients with MSS versus MSI-H (Fig. 1d,e). Allele-specific expression of all SNV-derived clonal neoantigens, while limited by sparse coverage, recapitulated these results (Extended Data Fig. 1e).

Finally, a published immunopeptidomics study of human MSS CRC identified three clonal HLA-I neoepitopes in two of five patient-derived organoids analyzed by mass spectrometry (MS). While this study is small and the number of neoepitopes validated is substantially lower than predicted, the results suggest a lower limit of ~40% of MSS CRC presenting bona fide neoepitopes. Our re-analysis of this dataset located the neoepitopes in the 6th, 31st and 45th percentiles of abundance of all detected HLA-I epitopes (Extended Data Fig. 1f,g). While peptide-specific properties can influence the efficiency of separation and ionization in MS, this qualitative analysis suggests lower surface abundance than most self-epitopes. Given that the detection limit of MS in these types of experiments is poorly defined and the recovery rate between replicates is limited, it is highly likely that a number of bona fide neoepitopes were not recovered, particularly those of lowest abundance. Indeed, some neoepitopes that were undetected by MS have been shown to be presented by tumor cells via immunogenicity assays. Altogether, our integrated analysis of TCGA and the literature argue that many patients with MSS CRC may harbor therapeutically actionable neoantigens. However, it remains poorly understood how low expression of neoantigens shapes resulting T-cell responses in MSS CRC.

**Neoantigen burden**

| Clonality | MSS | MSI-L | MSI-H | | P = 3 × 10^{-10} |
|-----------|-----|-------|-------| |  |
| 0.25      | 0   | 1     | 4     | |  |
| 0.50      | 0   | 1     | 4     | |  |
| 0.75      | 0   | 1     | 4     | |  |
| 1.00      | 0   | 1     | 4     | |  |

**Mean expression (log2 FPKM)**

| MSS | MSI-L | MSI-H |
|-----|-------|-------|
| 12  | 20    | 22    |
| 14  | 16    | 18    |
| 16  | 18    | 20    |

**Affinity**

| Clonality | MSS | MSI-L | MSI-H |
|-----------|-----|-------|-------|
| 0.25      | 0   | 1     | 4     |
| 0.50      | 0   | 1     | 4     |
| 0.75      | 0   | 1     | 4     |
| 1.00      | 0   | 1     | 4     |

**Expression**

| Clonality | MSS | MSI-L | MSI-H |
|-----------|-----|-------|-------|
| 0.25      | 0   | 1     | 4     |
| 0.50      | 0   | 1     | 4     |
| 0.75      | 0   | 1     | 4     |
| 1.00      | 0   | 1     | 4     |

**Clonality**

| Expression | MSS | MSI-L | MSI-H |
|------------|-----|-------|-------|
| 0.25       | 0   | 1     | 4     |
| 0.50       | 0   | 1     | 4     |
| 0.75       | 0   | 1     | 4     |
| 1.00       | 0   | 1     | 4     |

**Neoantigen expression level is a critical determinant of immunity.** We first developed an autochthonous model in Apc^{−/−} mice initiated by lentivirus expressing Cre-recombinase and the chicken ovalbumin antigen linked to luciferase (LucOS) (Extended Data Fig. 2a), as we have previously conducted in models of lung cancer and soft-tissue sarcoma. Injection with LucOS dramatically reduced tumor incidence in a T-cell-mediated manner and tumors that did arise invariably lost antigen expression (Extended Data Fig. 2b–e). To assess effects of antigen expression in established tumors, T cells were continuously depleted for 5 weeks, at which point tumors retained antigen expression. However, 7 weeks after withdrawal of depleting antibodies, tumors had grown and lost antigen expression (Extended Data Fig. 2f,g). Given this potent immune editing and variability of antigen expression (Extended Data Fig. 2h), we developed a colon organoid model that maintains distinct levels of antigen expression throughout tumorigenesis. Organoid engineering also enables the use of a Kras mutant allele to model metastatic colon adenocarcinoma, which is confounded in the autochthonous model by concomitant Kras-driven fibrosarcoma formation (unpublished observations).
in regression\(^1\), dependence on shApC provides powerful selection against antigen loss (Extended Data Fig. 2i). Finally, deletion of Smad4, commonly mutated in CRC\(^2\), was achieved by CRISPR/Cas9 editing. Selection of organoids harboring complete mutation of all genes was performed following published protocols\(^{36,37}\). This resulted in isogenic quadruple-mutant (shAKPS) organoids modeling some of the most common genetic mutations in MSS CRC\(^2\) (Extended Data Fig. 2j), which are co-mutated with high frequency in metastatic disease and associated with poor prognosis\(^4\).

To investigate the importance of neoantigen expression level, we generated organoids with 400-fold range of mScarlet\(^\text{SIIN}\) expression via modifications to the shAPc-expressing lentivirus, including placement in reverse orientation to the promoter (EF\(\alpha\) initiates bidirectional transcription), removal of the Kozak consensus sequence and replacing 25% of codons with rare variants (Fig. 2a,b and Extended Data Fig. 2k). This flexible system is broadly applicable to other cancers via linkage to relevant essential events (for example, knockdown of Trp53) and is easily adapted to the study of other immune epitopes.

To compare surface MHC-I presentation of SIINFEKL across the expression series of organoids quantitatively, we performed H-2K\(\alpha\) immunoprecipitation and multiplexed tandem mass tag mass spectrometry (TMT–MS) on eluted peptides. While lo\(^\text{SIIN}\) through med\(^\text{SIIN}\) showed near perfect correlation between SIINFEKL abundance and mScarlet\(^\text{SIIN}\) protein expression, hi\(^\text{SIIN}\) unexpectedly showed intermediate abundance (Fig. 2c and Extended Data Fig. 2l). Given that antigen expression in mid\(^\text{SIIN}\) and med\(^\text{SIIN}\) was adjusted by altering translation efficiency, it is possible that proteosomal degradation of mScarlet\(^\text{SIIN}\) is enhanced in these lines, resulting in greater surface presentation. Alternatively, high expression of mScarlet\(^\text{SIIN}\) in hi\(^\text{SIIN}\) may lead to insoluble protein aggregates that are sequenced from antigen-processing machinery. Regardless, these results validate the flexibility of our system to modulate surface presentation levels of neoantigen. While the multiplexed format of TMT–MS precludes meaningful intrasample comparisons of the abundance of SIINFEKL versus other epitopes, SIINFEKL abundance in lo\(^\text{SIIN}\) was only 1.4-fold above background in hi\(^\text{SIIN}\) (which lacks SIINFEKL), consistent with a low level of surface presentation.

Endoscope-guided transplant of shAKPS organoids without antigen (no\(^\text{SIIN}\)) efficiently induced tumors (Fig. 2d,e) and spontaneous metastases to liver and lung, with histology remarkably like human CRC (Extended Data Fig. 2m). In contrast, transplant of the highest expression variant (hi\(^\text{SIIN}\)) resulted in CD8\(^+\) T-cell-mediated rejection in all animals (Fig. 1h,i). While transplant of med\(^\text{SIIN}\) also resulted in complete rejection, mid\(^\text{SIIN}\) and dim\(^\text{SIIN}\) formed tumors with intermediate efficiency (Fig. 2e and Extended Data Level 2n,o), suggesting a nondiscrete effect of neoantigen expression level in tumorigenesis. We also generated shAKPS organoids harboring different epitopes with high affinity for MHC-I, including SIYRYGL (hi\(^\text{SIIN}\)), ITTYTWTRL (hi\(^\text{VGF}\)) and VGFNFRTL (hi\(^\text{VGF}\)) (Extended Data Fig. 2p). The latter two are mutant epitopes of Alg8 (A506T) and Lamek (G1254V) that arose in a methylcholanthrene-induced mouse sarcoma, which were reported to be insufficient for tumor rejection but critical for ICB response in a syngeneic flank transplant model\(^4\). Here, high expression of all three epitopes resulted in tumor rejection (Fig. 2e), demonstrating that immunogenicity is not idiosyncratic to SIINFEKL, but a general feature associated with high expression of high-affinity epitopes. This also argues that the major genetic features of MSS CRC do not confer cell-autonomous resistance to T-cell killing.

Notably, transplant of the lowest expression variant (lo\(^\text{SIIN}\)) induced tumors with similar efficiency, histology and infiltration as no\(^\text{SIIN}\) organoids (Fig. 2d–i). This was also true of organoids with low expression of ITTYTWTRL (lo\(^\text{VGF}\)) and VGFNFRTL (lo\(^\text{VGF}\)), which formed tumors with high efficiency (Fig. 2e and Extended Data Fig. 2p). CD8\(^+\) T cells were sparse and only modestly increased in lo\(^\text{SIIN}\) tumors, whereas helper and regulatory T-cell infiltration was not significantly different (Fig. 2h–l and Extended Data Fig. 2m). This is characteristic of the immune ‘cold’ landscape of MSS CRC in humans\(^4\). Notably, the immune escape in lo\(^\text{SIIN}\), lo\(^\text{VGF}\) and hi\(^\text{VGF}\) tumors did not result from neoantigen ignorance, as advanced tumors were infiltrated by antigen-experienced (CD44\(^+\)) and specific (H-2K\(\alpha\) tetramer\(^+\)) CD8\(^+\) T cells (Fig. 2m,n and Extended Data Fig. 2q). Altogether, these results demonstrate that MSS tumors can harbor high-affinity neoantigens despite poor T-cell infiltration and low neoantigen expression is an important mechanism of tumor immune evasion.

**Low neoantigen expression drives early T-cell dysfunction.** To investigate why lo\(^\text{SIIN}\) tumors escaped immune rejection, we first compared the kinetics of the antigen-specific T-cell response in lo\(^\text{SIIN}\) versus hi\(^\text{SIIN}\) lesions. Low neoantigen expression resulted in both delayed and lower magnitude response (Fig. 3a). Notably, this difference was far less pronounced in the caudal and iliac draining lymph nodes (DLNs) (Extended Data Fig. 3a), suggesting that early T cells in lo\(^\text{SIIN}\) animals are either impaired in their ability to traffic to or proliferate within the tumor. The latter is unlikely, however, as lo\(^\text{SIIN}\) and hi\(^\text{SIIN}\) T cells within tumors and DLNs showed no difference in proliferation (Extended Data Fig. 3b). Alternatively, T cells arriving at the tumor may have undergone deletional tolerance\(^4\). A critical step in the early maturation of functional T-cell responses is effector differentiation, characterized by production of cytokines and cytolytic granzymes, particularly granzyme B (GZMB) and loss of progenitor potential. TCF1 is a marker of progenitor potential and is expressed in naive, memory precursor and memory T cells\(^43,44\). Consistent with impaired effector
differentiation, at 8d significantly more antigen-specific T cells from loSIIN tumors and DLNs were TCF1+/GZMB− and significantly fewer were TCF1−/GZMB+(Fig. 3b,d,e and Extended Data Fig. 3c). This is unlikely a result of delayed kinetics, as the percentage of TCF1−/GZMB+ T cells in loSIIN tumors at peak response (14d) remained significantly lower (Fig. 3c,f and Extended Data Fig. 3d).

Unexpectedly, the percentage of antigen-specific T cells capable of secreting both tumor necrosis factor (TNF)-α and interferon (IFN)-γ effector cytokines was higher in loSIIN versus hiSIIN DLNs and not different in tumors at 8d (Extended Data Fig. 3e). However, this cytokine-proficient population also showed higher TCF1 and lower GZMB levels (Extended Data Fig. 3f), suggesting a similar lack of effector differentiation. Of note, the percentage of double-negative (TCF1−/GZMB−) T cells was greater in loSIIN versus hiSIIN animals at 8d (Fig. 3b,g) and became even more pronounced by 14d (Fig. 3c,h). Absence of TCF1 and GZMB implies lack of progenitor and effector functionality and indicates dysfunction. Indeed, by 14d TCF1−/GZMB− antigen-specific T cells in loSIIN versus hiSIIN tumors showed higher expression of co-inhibitory receptors PD-1, TIM3, LAG3 and 2B4 (Extended Data Fig. 3g) and an increased fraction coexpressing...
Fig. 3 | Low neoantigen expression drives impaired T-cell effector commitment and dysfunction. a, Total CD44+/CD8+ antigen-specific T cells isolated from lesions at indicated days post-transplant of hiSIIN (red) and loSIIN (blue) organoids by flow cytometry; n = 5–9 independent animals per line and time point. b, c, Antigen-specific T-cell expression of TCF1 versus GZMB in tumors at 8 d (b) and 14 d (c). Representative of animals in a, d, e, f. Percent of antigen-specific T cells from DLNs and tumors at 8 d positive for TCF1 and negative for GZMB (d), negative for TCF1 and positive for GZMB (e) and double-negative for TCF1 and GZMB (f); n = 10 hiSIIN and 9 loSIIN-transplanted animals. g, h, Percent of antigen-specific T cells from DLNs and tumors at 14 d negative for TCF1 and positive for GZMB (g) and double-negative for TCF1 and GZMB (h); n = 6 hiSIIN and 7 loSIIN-transplanted animals. i, Median percentage of TCF1+/GZMB−-antigen-specific T cells from tumors at 14 d coexpressing 0 through 4 inhibitory receptors (PD-1, TIM3, LAG3 and 2B4); n = 6 hiSIIN and 7 loSIIN-transplanted mice. Bars, s.d., j–m, In vivo killing assay of transferred control (weak CTV stain) and SIINFEKL-loaded ‘target’ (strong CTV stain) splenocytes and flow plots of antigen-specific T cells recovered from DLNs at 8 d (j) and 14 d (l, m) post-transplant of hiSIIN (red) and loSIIN (blue) organoids. Representative of n = 6–7 independent animals per line and time point. CTV, CellTrace Violet. n, Target killing normalized to total antigen-specific T cells recovered in 14-d killing assay; n = 7 independent animals per line. o, p, Total antigen-specific T cells isolated from lesions at 8 d across all expression variant lines (o) and versus mScarletSIIN mean fluorescence intensity (MFI) (p). q, r, Percent of TCF1+/GZMB− antigen-specific T cells from 14-d lesions across expression variants (q) and versus mScarletSIIN MFI (r). Dashed lines connect medians. Significance assessed by Spearman’s rank correlation; n = 5–9 independent animals per line in e–r. Significance in a, d, h, n, and q assessed by two-tailed Wilcoxon rank-sum. Holm’s correction applied in o and q.

three or all four (Fig. 3i, Extended Data Fig. 3h). To further interrogate functionality of the loSIIN T-cell response, we performed an in vivo killing assay45 by transferring SIINFEKL-loaded ‘target’ splenocytes into tumor-bearing mice. Targets were eliminated in the DLNs and spleen 6 h post-transfer in hiSIIN, but not loSIIN, animals at 8 d (Fig. 3j,k and Extended Data Fig. 3i). Even at the peak of
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To determine whether these effects are mediated by discrete or continuous levels of neoantigen expression, we characterized the T-cell responses to dimSIIN and hiSIIN lesions, we performed TCR-β sequencing. Consistent with an attenuated T-cell response to dimSIIN, mid SIIN and med SIIN organoids at 8 d continuous levels of neoantigen expression, we characterized the dysfunctional T-cell response with reduced magnitude, diversity and per-cell functionality.

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Neoantigen expression level is limiting for cross-priming. Impaired effector differentiation and early dysfunction are indicative of poor priming, such as that which occurs in the absence of CD4+ T-cell help. While loSIIN and hiSIIN organoids lack a defined MHC-II-restricted model neoantigen, depletion of CD4+ T cells completely rescued formation of hiSIIN tumors (Fig. 5a). Therefore, absence of help is unlikely to be the mechanism of dysfunction in the loSIIN model. It is possible that CD4+ T cells are primed against uncharacterized neoantigens in mScarlet, tumor-associated self-antigens or microbial antigens in the colon microenvironment. Consistent with the importance of neoantigen expression level in priming, hiSIIN tumor formation was partially rescued in Batf3−/− mice, which lack conventional cross-presenting dendritic cells (DC1s) (Fig. 5a). To directly test the role of neoantigen expression level in cross presentation, we co-cultured bone-marrow-derived dendritic cells (BM-DCs), consisting of ~13% CD103+ DC1s, with naïve TCR-transgenic T cells specific to SIINFEKL (OT-1) (Extended Data Fig. 5a,b). Compared to BM-DCs loaded with hiSIIN organoids, those loaded with loSIIN were markedly less capable of promoting OT-1 proliferation and effector differentiation (Fig. 5b–e and Extended Data Fig. 5c). On the other hand, in vitro-primed OT-1 cells were equally capable of killing loSIIN as hiSIIN organoids when co-cultured (Fig. 5f,g). These results argue that neoantigen expression is limiting for T-cell cross-priming, but not tumor cell recognition by effector T cells.

To interrogate priming in vivo, we performed: (1) re-challenge with loSIIN organoids 28 d after transplant of hiSIIN organoids; (2) co-injection of loSIIN and hiSIIN organoids in the same animals; and (3) transfer of in vitro-activated OT-1 cells concurrent with transplant of loSIIN organoids. All these approaches resulted in complete rejection of loSIIN organoids (Fig. 5h), demonstrating unequivocally that efficiently primed T cells are capable of killing tumors with low neoantigen expression in vivo. Priming in the context of high neoantigen expression also rescued the phenotype of T cells infiltrating loSIIN lesions. Notably, antigen-specific T cells infiltrating loSIIN and hiSIIN lesions from the same animals (Fig. 5i) showed similar abundance (Fig. 5j) and overlapping expression of TCF1 and GZMB that is indistinguishable from that of animals transplanted with only hiSIIN organoids (Fig. 5k and Extended Data Fig. 5d–f). To determine whether these effects were SIINFEKL-specific, we performed co-injection of loSIIN and hiGFP or hiFITY organoids in the same animals. While hiGFP and hiFITY did not form tumors in any animals, 100% of animals developed loSIIN tumors (Fig. 5l and Extended Data Fig. 5g), demonstrating that rescue of priming by hiSIIN is mediated through increased SIINFEKL expression.

The incomplete penetration of hiSIIN tumor formation in Batf3−/− animals implicates additional mechanisms of priming, such as direct priming by tumor cells or cross-priming by non-DC1s. Consistent with this, we detected activated antigen-specific T cells in the colons and DLNs of most of these animals 6 weeks post-transplantation, albeit at very low numbers (Extended Data Fig. 5j–l). To assess the role of direct priming, we used CRISPR/Cas9 to generate hiSIIN organoids lacking H-2Kb (Kb-KOSIIN) (Fig. 5m and Extended Data Fig. 5k). Consistent with failure to present SIINFEKL, these organoids formed tumors with 100% efficiency in immunocompetent hosts. However, co-transplantation of loSIIN with Kb-KOSIIN resulted in complete rejection of loSIIN in all but one animal (Fig. 5n,o), despite outgrowth of Kb-KOSIIN in all animals, demonstrating that SIINFEKL from Kb-KOSIIN is efficiently cross presented and that this is the dominant mechanism of priming in our model. To further interrogate any potential contribution of direct tumor cell priming, we repeated the co-transplantation experiments in Batf3−/− animals. In this DC1 deficient context, loSIIN formed tumors with ~40% efficiency when co-transplanted with Kb-KOSIIN (Fig. 5n,p), indistinguishable from the efficiency of tumor formation with hiSIIN transplanted alone. These results strongly suggest that direct priming by tumor cells is not operative and that non-DC1s play an important role in antitumor T-cell cross-priming in the colon.

Therapeutic priming rescues the poorly primed T-cell response. Given the central role of T-cell priming in our model, we tested the therapeutic potential of neoantigen vaccination in animals with established tumors. Mice with GFP-expressing tumors, as determined by colonoscopy, were randomly enrolled to receive SIINFEKL-containing OVA250–270 (CGLEQLESINFEKLEWTSS) or nonspecific mutant gp100(250–269) (CAVGAGEPFRNKQDVLGVPQL) peptide-based vaccines consisting of a peptide-amphiphile and adjuvant amphiphile-CpG53 administered at 14 d and 21 d post-transplant (Extended Data Fig. 6a). Vaccination with OVA250–270, but not nonspecific peptide, induced profound expansion of tumor-specific T cells in peripheral blood (Fig. 6a,b and Extended Data Fig. 6b,c). One week following the second dose, tumors in the OVA250–270 vaccine arm trended toward greater reduction in size (Fig. 6c) and at termination (6 weeks), tumor burden was significantly reduced, with four complete regressions (Fig. 6d). These proof-of-principle results suggest that it may be therapeutically tractable to vaccinate against poorly expressed, albeit clonal neoantigens and that strict neoantigen expression cutoffs in antitumor vaccine pipelines should be re-evaluated.

We next asked whether more readily deployable antibody-based immunotherapies are efficacious in our model. Agonistic antibodies against the CD40 receptor (anti-CD40) enhance priming by potentiating the co-stimulatory function of antigen-presenting cells. Anti-CD40 is efficacious in preclinical mouse models of pancreatic ductal adenocarcinoma (PDAC), particularly when combined with IC8 and immunogenic chemotherapies. This is notable...
in light of the low TMB and immunogenicity of PDAC, which, like MSS CRC, is refractory to ICB. Therapeutic combinations with anti-CD40 may be able to rescue or generate new T-cell responses against weak affinity or poorly expressed neoantigens, or against tumor-associated self-antigens that lack high-affinity T-cell clones due to central tolerance. However, clinical studies in CRC are lacking.

We performed preclinical trials in mice bearing loSIIN colon tumors starting 14 d post-transplant (Extended Data Fig. 6a) with single agents anti-CD40, anti-PD-1 and anti-CTLA-4 and combinations.
anti-CD40/anti-PD-1, anti-CD40/anti-CTLA-4 and anti-CD40/anti-PD-1/anti-CTLA-4. Response was evaluated by colonoscopy at 28 d post-transplant following Response Evaluation Criteria in Solid Tumors. All animals in the no-treatment arm presented with progressive disease, whereas 2 of 12 (17%), 3 of 12 (25%) and 5 of 16 (31%) showed complete responses in the anti-PD-1, anti-CTLA-4 and anti-CD40 arms, respectively. Response was notably better in all combination arms, with 9 of 12 (75%), 8 of 12 (67%) and 12 of 17 (71%) complete responses in the anti-CD40/anti-PD-1, anti-CD40/anti-CTLA-4 and anti-CD40/anti-PD-1/anti-CTLA-4 arms, respectively (Fig. 6e–m and Extended Data Fig. 6d). Comparing all combination arms against single agent anti-CD40 showed significantly more objective responses ($P = 0.02$) and complete responses ($P = 0.01$, Fisher’s exact test). Of note, adoptive cell transfer (ACT) of one million ex vivo-activated OT-1 T cells at 14 d post-transplant significantly delayed tumor growth but only resulted in one complete response (31%) showed complete responses in the anti-PD-1, anti-CTLA-4 and anti-CD40 arms, respectively. Response was notably better in all combination arms, with 9 of 12 (75%), 8 of 12 (67%) and 12 of 17 (71%) complete responses in the anti-CD40/anti-PD-1, anti-CD40/anti-CTLA-4 and anti-CD40/anti-PD-1/anti-CTLA-4 arms, respectively (Fig. 6e–m and Extended Data Fig. 6d). Comparing all combination arms against single agent anti-CD40 showed significantly more objective responses ($P = 0.02$) and complete responses ($P = 0.01$, Fisher’s exact test). Of note, adoptive cell transfer (ACT) of one million ex vivo-activated OT-1 T cells at 14 d post-transplant significantly delayed tumor growth but only resulted in one complete.
response (Fig. 6m and Extended Data Fig. 6e), suggesting that transferred T cells rapidly become dysfunctional. Despite initially delayed tumor growth in the single ICB arms, no significant difference in final tumor burden was observed at necropsy, suggesting only a transient effect in most tumors (Fig. 6m). In addition, incidence of metastasis was not significantly decreased in single ICB arms (Fig. 6n–r). These results are reminiscent of the poor response to ICB seen in MSS CRC and demonstrate that ICB is only modestly effective at rescuing a poorly primed T-cell response. In contrast, single agent anti-CD40 significantly decreased primary tumor size at end point, whereas combination with ICB significantly reduced tumor size further (Fig. 6m). All treatment arms with anti-CD40 resulted in significantly reduced rates of metastasis (Fig. 6n), although this could reflect the absence of primary tumors in many of these animals. However, the combined rate of metastasis in animals with progressive disease across all anti-CD40 arms was

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**Fig. 7 | Immunotherapy refractory low neoantigen-expressing tumors remain vulnerable to antigen-specific T-cell killing.** a–d, Flow cytometric analysis of H-2Kb and PD-L1 MFI (a, b) and representative histograms of expression (c, d) following 24 h of IFN-γ stimulation (10 ng ml⁻¹) in ex vivo loSIIN tumor-derived organoids. Each organoid line was derived from a treatment refractory tumor taken from an independent animal in the indicated treatment arms in Fig. 6. Parental, un-transplanted loSIIN organoids. n = 3 no treatment, 3 anti-PD-1- and 2 anti-CD40/anti-PD-1-independent organoid lines. e, f, Flow cytometric analysis of mScarlet (e) and eGFP (f) expression in ex vivo tumor-derived organoids. n depicts same as above. g, Images of co-cultures with ex vivo tumor-derived organoids and activated OT-1s at an effector-to-target ratio of 5:1 at day 4. h, Schematic representation of the role of neoantigen expression level in immune evasion and response to therapeutic priming.
still significantly reduced (Extended Data Fig. 6f). Notably, while ACT had no effect on reducing primary tumor size at end point, it resulted in complete control of metastatic tumor burden (Fig. 6n).

To determine whether therapy resistance is mediated by down-regulation of antigen expression or MHC-I, we isolated ex vivo IgH tumor-derived organoids from two anti-CD40/anti-PD-1 escapers, three anti-CD40-1 escapers and three untreated mice. All lines showed comparable sensitivity to IFN-γ stimulation, expression of H-2Kd and mScarlet and were similarly sensitive to killing when co-cultured with activated OT-1s (Fig. 7a–g). These results suggest that low neoantigen expression obviates the need for dysregulation of antigen presentation and are consistent with the lower frequency of such events in MSS versus MSI cancers57. Critically, MSS tumors may remain sensitive to T-cell killing if priming against poorly expressed neoantigens can be rescued (Fig. 7h).

Discussion

The poor response of most CRC to immunotherapy represents a major unmet clinical need. Mouse models have provided invaluable insights into T-cell dysfunction in cancer, but none to our knowledge recapitulates essential features of human CRC while facilitating detailed study of antigen-specific T cells. Here, we developed colonoscopy-guided models enabling comparison of functional versus dysfunctional tumor-specific T-cell responses in a context highly faithful to the microenvironment, genetics, histopathology and metastatic progression of human disease.

We found that tumors from all analyzed patients with MSS CRC harbored clonal neoantigens with high predicted HLA-A affinity, but these were broadly expressed at lower levels compared to those from MSI CRC. This raises the possibility that poor immunogenicity in MSS CRC and other immune cold cancers is driven by both lower burden and lower expression of neoantigens. Indeed, our low neoantigen-expressing model, like MSS CRC, demonstrated poor T-cell infiltration and ICβ response. We showed that neoantigen expression is an analog input that tunes the quality of antitumor T-cell cross-priming. Low expression shifts priming toward a tolerogenic response characterized by reduced magnitude, diversity, effector commitment and per T-cell functionality. A general feature of early immune evasion in cancer may be that T-cell dysfunction begins as a tolerogenic program initiated during priming with insufficient antigen stimulation, in addition to a lack of local inflammatory and/or co-stimulatory cues. By extension, it is likely that immune responses against clonal neoantigens, at least those acquired early in tumorigenesis, are poorly primed and tolerogenic, axiomatic to their failure to restrain tumor outgrowth. We showed that rescuing priming is sufficient to prevent tumor initiation in our model, suggesting that early neoplasia lack immunosuppressive mechanisms to evade functional T cells. We also showed that poorly primed T cells undergo progressive exhaustion, in line with prevailing literature and suggesting that T-cell dysfunction is a heterogeneous state shaped by multiple processes operative early and late in tumorigenesis.

Therapeutically targeting priming via anti-CD40 was highly efficacious in our model, particularly in combination with ICβ. While ICβ alone had no effect on the rate of metastasis, anti-CD40 and ACT almost completely prevented metastases, even in mice with progressive primary disease. Therefore, targeting priming may be efficacious against early metastatic lesions that may not be detected at time of treatment. These results establish the preclinical utility of our model and highlight the therapeutic promise of combined anti-CD40 and ICβ for MSS CRC and other immune cold cancers. Given that no adequately powered clinical trials of anti-CD40 in CRC have been initiated to date, these results warrant clinical evaluation. Our demonstration that antitumor immunity against a poorly expressed neoantigen can be rescued by therapeutic vaccination is particularly relevant to analogous efforts ongoing in humans57,58.

In a recent consortium study integrating neoantigen prediction pipelines from 28 research teams, it was concluded that expression, among other variables, is an important predictor of neoepitope immunogenicity and a minimum threshold of >33 transcripts per million was imposed59. However, neoepitope immunogenicity in this and other studies was validated by experimentally measuring reactivity of existing T cells in patient blood or tumor57-59. Given that tumor-specific T cells in advanced tumors are dysfunctional, it is likely that functional readouts of these assays are limited by low sensitivity. Additionally, it is possible that therapeutic priming against neoantigens overlooked by these assays could unleash productive T-cell responses from naive T cells or reservoirs of clonally expanded precursors in lymphoid tissues. Indeed, a recent phase 1b trial (NEO-PV-01) found that while personalized vaccines elicited de novo T-cell responses in all patients, the vast majority showed no detectable responses in peripheral blood before vaccination58. Our findings argue that RNA sequencing in these trials should be performed at sufficient depth to distinguish lack of expression from dropout due to poor coverage and that any detectable allele-specific expression of clonal neoantigens is sufficient to nominate them for vaccination. It has been shown in vitro that effector T cells can lyse targets presenting only three cognate MHC-I–epitope complexes60. Therefore, tumor cells likely must undergo near absolute loss of neoepitope presentation to render responding T cells truly ignorant.

Altogether, the results of our study describe a model in which tumors harboring poorly expressed neoantigens elicit tolerogenic responses and evade deletion, while remaining vulnerable to those same T-cell responses following therapeutic priming (Fig. 7h). It will be important to determine whether therapies that potentiate priming mediate their effects predominantly through naive T cells or reservoirs of antigen-experienced T cells in the tumor bed or lymphoid tissues. The flexible organoid-based system developed here should facilitate a broad range of future studies in faithful models of cancer. Our use of single clonal neoantigens is a simplification of most human tumors, which are mutational heterogeneously. It will therefore be important to interrogate the impact of intratumoral heterogeneity of neoantigens in future studies. Notably, organoids can be rapidly engineered to express multiple antigens with a range of affinities and mixed to varying degrees of clonality. Finally, our model should provide a powerful preclinical platform for future studies of emerging immunotherapies.

Methods

TCGA neoantigen prediction analysis. A total of 350 patients with colon adenocarcinoma and 76 with rectum adenocarcinoma were analyzed from TCGA COAD and READ studies. These represented all samples with tumor (−01A) and matched-normal whole-exome sequencing (WES), RNA-seq and mutation annotation format (MAF) files available. Sequencing data were obtained as binary alignment map (BAM) files aligned to GRCh38. HLA-A, HLA-B and HLA-C alleles were called using OptiType, v.1.3.1 (ref. 16). Tumor/normal WES BAMs were used to create inputs to OptiType. Reads were filtered to those mapping to the HLA region (chr6:28510120–33480577 in GRCh38) with Samtools v.1.10 (ref. 17), converted to FASTQ and filtered with Rayser v3.5.8 (ref. 18), as recommended in the OptiType documentation. OptiType was run with default parameters.

A custom Python v.2.7.13 script was employed to evaluate concordance between normal and tumor HLA allele calls. Overall, 1,917 out of 2,100 alleles (91.3%) in the COAD cohort and 428 out of 456 (93.9%) alleles in the READ cohort were consistent between tumor and normal WES-based calls; the tumor allele was accepted as the final call to resolve discrepancies between calls from tumor and normal sample sequencing data. Patient MAFs were converted to variant call format (VCF) and filtered to SNVs only. All non-PASS variants were removed except for some in KRAS and TP53 that had been marked as either panel_of_normals, clustered_events or homologous_mapping_event in the TCGA MAF files.

Indels were called using Strelka2 v.2.9.2 (ref. 19) and Scalpel v.0.5.4 (ref. 20). Scalpel was run with default parameters, with a bed file derived from the CGHub bitbucket account (https://cghub.ucsc.edu:8080/washington_east_alameda_1.1/refsseq_pplus_3_boosters.targetIntervals.bed), with coordinates converted to GRCh38. A panel of 426 patients was used for standardization of call set and data analysis. Sequencing reads of each sample were aligned to the GRCh38 genome using BWA-MEM (ref. 5).
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Neoantigen clonality was estimated by dividing WES level V AF by was calculated as the product of RNA V AF and corresponding gene expression parameters:–symbol,–terms=SO,–cache,–offline,–transcript_version,–pick. The–pick parameter was reordered from default to report transcript with most extreme consequence for each variant: rank, canonical, appris, tsl, biotype, ccds, and the median value across all affinity predictions was taken.

Only neoantigens with evidence of expression (RNA-seq FPKM upper quartile-normalized (FPKM-UQ)) were included in analyses. Tumor purity estimates (ABSOLUTE algorithm) for TCGA COADREAD were acquired from a previous publication. Neoantigen clonality was estimated by dividing WES level VAF by ABSOLUTE purity (vADF), with vADF ≥ 0.5 considered as clonal. This is an estimate of clonality only, as other factors not considered here can also influence VAF.

Tumor RNA-seq BAMs were analyzed to detect and quantify SNV expression at the transcriptional level. For all SNV's corresponding to predicted for all peptide–MHC allele pairs with NetMHC-4.0, NetMHCpan-4.0, predicted for all peptide–MHC allele pairs with NetMHC-4.0, NetMHCpan-4.0, normalized (FPKM-UQ) affinity predictions was taken.

Tandem mass tag mass spectrometry. Dried down MHC-1 eluted peptides were resuspended in 100 μl triethylammonium bicarbonate buffer and labeled with TMT16plex (Pierce). Samples were then mixed and cleaned with C18 ZipTip (Millipore Sigma). One-fifth of the sample was used for one LC–MS/MS analysis. Samples were again re-dried and reconstituted in 2% formic acid for MS analysis. Peptides were loaded with directly into the on-column trap and directly on the column followed by 15% B (100 μl of 10% acetic acid and purified with 10 kDa MWCO spin filters (PALL Life Science).

Organoid isolation and transformation. Normal colon crypts were isolated from WT (female), Apc−/− (female), and Apc−/− (male) animals, as previously described75 and functional titers (Cre activity, mScarlet/eGFP expression) confirmed by PCR using published primers68,69. Next, organoids were infected with a D64V mutant psPax2 packaging vector and absence of integration was confirmed by was observed for Cas9 protein by western blot and sensitivity to puromycin killing.

MHC-I immunoprecipitation and peptide isolation. MHC-I (H-2Kβ) peptide isolation was performed on 210 × 20 μl plugs for triplicate for each organoid line using a modified immunoprecipitation and protein filtration protocol, as described previously76. Organoids were grown to confluence over 3 d before stimulation with 70 μl human IFN-γ (R&D Tech) for 18h before collection. Organoids were washed with PBS and mechanically liberated by vigorous pipetting in PBS. Cells were washed twice in 50 ml PBS and pellets were snap frozen in liquid nitrogen. Pellets were lysed in 2 ml of lysis buffer containing 50 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 60 mM octylglucosanide (Sigma), 20 mM iodacetamide, 10 U DNase and 1x Halt protease inhibitors (Pierce). Lysates were cleared by centrifugation and loaded onto a 30% EASY-Spray C18 column (ES803a, Thermo Scientific). Peptides were eluted from the column using a Dionex Ultimate 3000 Nano LC system with a 5-min gradient from 1% buffer B to 5% buffer B (100 % acetonitrile, 0.1 % formic acid), followed by an 84.8-min gradient to 25% and a 15.2-min gradient to 35% B, followed by a 12-min gradient to 60% B, followed by a 4-min gradient to 80% B and held constant for 4 min. Finally, the gradient was changed from 80% buffer B to 99% buffer A (0.1% formic acid in water) over 0.1 min and held constant at 99% buffer A for a further 19.9 min. The application of a 2.2 κV distal voltage electro sprayed the eluting peptides directly into the Thermo Exporis480 mass spectrometer equipped with a FAIMS and an EASY-Spray source (Thermo Scientific). Mass spectrometer-scanning functions and HPLC gradients were controlled by the Xcalibur data system (Thermo Scientific). MS1 scan parameters were 60,000 resolution, scan range m/z 350-1900, AGC at 300%, IT at 50 ms. MS2 scan parameters were either at 45,000 or 60,000 resolution, isolation width at 0.7, HCD collision energy at 30%, AGC target at 300% and IT set to 300 ms. Cycle time for MS1 was 1 s for each MS1 scan. The scan cycle MS1/MS2 was repeated for FAIMS voltages at –40V, 60V and +80V.

Tandem mass spectra were searched with Sequest (Thermo Fisher Scientific; version IsoeNode in Proteome Discoverer 2.5.4.000). Sequest was set up to search a mouse uniprot database (database v.July 3, 2020; 55650 entries containing common contaminants and the three proteins mScarlet-SIINFEKL, mScarlet-VGFNFRTL and eGFP) assuming no digestion enzyme (unspecific). Sequest was searched with a fragment ion mass tolerance of 0.02 Da and a parent ion tolerance of 10.0 ppm. TMIPpro was added as a fixed modification on the K and N terminus of peptides. Oxidation of methionine was specified in Sequest as a variable modification. Resulting peptides were filtered to exclude peptides with an isolation interference of ≥ 40% and median mass error of all peptide-spectrum matches (PSMs). SIINFEKL intensity across samples was normalized by the overall abundance of all peptides detected in each sample.

Organoid CRISPR/Cas9 RNP electroporation. Confluent hiAfc organooids were dissociated to single cells as described above and resuspended in 100 μl OPTI-MEM. Ribonucleoprotein (RNP) complexes were formed by mixing 1.64 μl (0.1 nmol) Alt-R Cas9 (IDT) with 3 μl (0.3 nmol) synthetic sgRNA (Synthego) and incubating for 10–20 min at room temperature. Cells were then added to the RNP mix, 100 μl transferred to a 2-mm gap cuvette (Bulldog Bio) and electroporated using a NEPA21 electroporator (Bulldog Bio) with the following pulse parameters: 200 μs, 400 V, + polarity; and transfer pulse parameters: 20 V, 50 ms, 50 ms interval, 5 pulses, 40% decay rate, + polarity. Electroporated organoids were resuspended gently in pre-warmed minimal medium and incubated at 37 °C for 15 min before plating in Matrigel. The sgRNAs used for electroporation were H-2Kβ, 5′-CAAGAGCAGAGAGUCAGGAG-3′; previously published B2m sequence, 5′-UUCAAUUUGAGGGGUTUUUG-3′.

Colonoscopy-guided injections. Orthotopic injection of lentivirus and organoids was performed similarly to previously described methods77. Intact organoids were always collected 2 d post-passagin by washing in PBS and dissociating in Dispase. Matrigel was broken up by vigorous pipetting and pipetting four times using a 1 ml pipette and incubating at 37 °C for 15 min. Organoids were washed thoroughly in PBS and resuspended in OPTI-MEM with 10% Matrigel at 50 organoids per ml. Intact organoids and lentivirus (20,000 or 100,000 TU ml−1) were injected via Hamilton syringe (Hamilton, 7656-01) and custom injection needle (Hamilton, 33-gauge, small Hub RN NDL, 16 inches, point 4, 45-degree bevel, like 7803-05).
fed through the working channel of the colonoscope and inserted into the colonic mucosa at ~30°. Approximately 50 μl was delivered per injection, resulting in large ‘blebs’ within the mucosa.

**Tissue preparation and flow cytometry.** Colon DLNs (caudal and iliac) were collected and mechanically dissociated in RPMI-1640 (Corning) with 5% heat-inactivated fetal bovine serum (HI-FBS) (collection medium). Tumors were identified using a Dual Fluorescent Protein Flashlight, Model DFP-1 (Nightsea), dissected and placed in a digestion buffer containing 500 U ml⁻¹ Collagenase Type 1 (Worthington) and 20 μg ml⁻¹ DNase (Sigma-Aldrich) in collection medium, minced using surgical scissors and digested at 37°C for 40 min with gentle agitation. Tumors were then further dissociated with a gentleMACS Octo Dissociator (Miltenyi Biotec) on the tumor_imp1.1 setting and filtered through a 100-μm filter. DLNs and tumor preparations were divided for immediate staining or peptide stimulation. Intratumoral staining before killing animals (to differentiate tissue-infiltrating versus circulating T cells) was not routinely performed, as this stained <1% of total SIINFEKEL-specific T cells.

Live/dead staining (ghost e780 (Corning), 1:500 dilution) was performed in PBS and surface stains in FACS buffer (1 mM EDTA, 25 mM HEPES, 0.5% HI-FBS in PBS) (1:1) on live cells (as described above). Cells were washed and resuspended in FACS buffer for analysis on a BD LSRFortessa four-laser, 18-color flow cytometer running BD FACSDiva v.8.0 software. Results were analyzed in FlowJo v.10.4.2. Single lymphocytes were gated first on FSC- A versus SSC-A and then F-SC-A versus F-H. Then, live CD8+ T cells were gated on positive CD8α and negative ghost e780 staining. Antigen-specific CD8+ T cells were further gated on CD44 and tetramer positivity. Expression of additional markers was analyzed specifically in this antigen-specific CD8+ T cell population in all flow cytometric experiments on T cells presented in this manuscript.

**Antigen-specific in vivo killing assay.** Splenocytes were prepared for in vivo transfer as described previously. Briefly, spleens were collected from female C57BL/6 mice, red blood cells lysed using ACK Lysing Buffer (Thermo Fisher) and cells resuspended in PBS in a round bottom 96-well plate at 1×10⁶ cells per ml. Half of the wells were pulsed with SIINFEKEL peptide (Anaspec) at 1 μg ml⁻¹, followed by labeling with the membrane dye CTV (Thermo Fisher) at 20 μM. The remaining wells were labeled with 2 μM CTV. Peptide-loaded ‘target’ and unloaded control splenocytes were then mixed 1:1 and 200 μl retro-orbitally injected (2×10⁷ cells) into experimental animals 8 and 14 days post-transplantation of DLNs. DLNs and spleens were collected 6 hours later and processed for flow cytometry as described above. Target and control splenocytes were identified by live/dead staining and CTV labeling intensity and percent target killing determined relative to the control population. Targets killed per antigen-specific T cell was determined by dividing the total number of target killed (control minus target splencytos) by the total number of SIINFEKEL tetramer+ CD8+ T cells. This metric was meaningful at 14 days when target killing was incomplete in both loα and hiα animals but precluded at 8 days by effectively complete target killing in hiα animals.

**Peptide stimulation for cytokine stimulation.** Samples were prepared as described above and before surface staining were washed with antibodies for Ly-6G (BioLegend, 1A8, 1:200 dilution), EpCam (BioLegend, G8.8, 1:80 dilution) and F4/80 (BioLegend, BM8, 1:80 dilution) in FACS buffer for 30 min at 4°C and depleted using Dynabeads Goat Anti-Mouse IgG kit (Thermo Fisher) following manufacturer recommendations. T cells were then stimulated in T-cell medium (RPMI-1640 with 10% HI-FBS, 20 mM HEPES, 1 mM sodium pyruvate, 2 mM l-glutamine, 50 μM β-mercaptoethanol, 1× non-essential amino acids and 0.5x penicillin/streptomycin) with 1:1,000 diluted GolgiPlug (BD) and 2 μM Momensin Solution (BioLegend) and 1 μM SIINFEKEL peptide (Anaspec) for 3h at 37°C. Cells were washed and stained for surface and intracellular markers as described above.

**Bone-marrow–derived dendritic cell isolation and OT-1 co-culture.** Bone marrow from C57BL/6 mouse femurs and tibias was isolated, red blood cells lysed and cells plated at 1.5×10⁶ cells per ml and cultured in T-cell medium (described above) plus 600 ng ml⁻¹ recombinant human Flt-3-Lg (hum/hum, BioXcell) and 5 μg ml⁻¹ recombinant mouse GM-CSF (BioLegend). After 1 week, BM-DCs were switched to fresh medium and activated with 20 μg ml⁻¹ of mouse STING ligand DMXAA (InvivoGen). The following day, BM-DCs were plated in 96-well plates of 10,000 cells per well in fresh medium (without DMXAA) and cultured with lysed organoids. Organoids were first dissociated to single cells in TrypLE and counted, then 1×10⁶ cells per 200 μl were plated at 10 μl per 10 min at 37°C. Lysed cells were pelleted at 1,000g for 15 min and resuspended in BM-DC medium at appropriate dilutions. The next day, loaded BM-DCs were washed and cultured with 50,000 naive OT-1s per well. CD8+ T cells were purified from spleen and lymph nodes of OT-1 mice using the CDS8+ T Cell Isolation Kit, mouse (Miltenyi Biotec) following manufacturer specifications. Cells were collected at 72 h for staining and flow cytometric analysis. For cytokine stains, cells were treated with 1:1,000 diluted GolgiPlug and 2 μM Momensin Solution for 3 h before collection.

**OT-1 T-cell activation and organoid co-culture.** Spleen and lymph nodes from OT-1 mice were collected in PBS, red blood cells lysed, cells resuspended in T-cell medium (described above) + 10 mg ml⁻¹ IL-2 (PeproTech) and 1 μM SIINFEKEL peptide (Anaspec), counted and plated at 1×10⁶ cells per ml. Stimulation was performed for 24 h at 37°C. CD8+ T cells were then purified using the CD8+ T Cell Isolation kit, mouse (Miltenyi Biotec) and expanded in T-cell medium+ 20 μl HI-2 with daily splitting. T cells were used for ACT or co-culture assays at day 3 or 4.

**Co-cultures and OT-1s were plated at 0:1 and 5:1 effector-to-target ratios at 2,500 organoid single cells in 10 μl minimal medium and 30 μl Matrigel. Co-cultures were plated in triplicate at 20 μl per dish, grown in minimal medium and imaged (control minimal fluorescent area of organoids within images was quantified in ImageJ v2.1.0/1.53c by setting left and right thresholds of grayscale images to 22 and 255 on B&W setting, respectively and analyzing particles with size threshold set to >20 pixel² and circularity set to 0.1–0.9 with holes included.

**Immunohistochemistry and automated quantification.** Tissues were fixed in zinc formalin, washed in 70% ethanol and paraffin embedded. Antibody staining was performed in citrate buffer pH 6 in a pressure cooker at 125°C for 5 min. Blocking was performed with BLOXALL Endogenous Peroxidase and Alkaline Phosphatase Blocking Solution (Vector) followed by Normal Horse Serum (2.5%) (Vector) Slides were stained overnight, incubated with Alkaline Phosphatase (AP) anti-rabbit IgG (Vector) and developed with Vector Black substrate (Vector). Sections were then subsequently incubated with AP anti-rat IgG (Vector) and HRP anti-rabbit IgG (Vector) and developed sequentially with Vector Red (Vector) and Vina Green (Biocare Medical). Slides were counterstained with Harris Acidified Hematoxylin and then dehydrated. Aqueous wash steps following counterstain were shortened from 1 min to 30 s to minimize loss of Vina Green stain.

**In vivo SIINFEKEL tetramer staining.** Tissue was stained in situ with SIINFEKEL tetramer as previously described. Tissues were additionally stained with CD8α AF647 (YTS156.7, BioLegend) (1:100 dilution) and anti-human β2-microglobulin (β2M) PE (2M2, BioLegend) (1:50). Anti-β2M staining is specific to human β2M in the SIINFEKEL tetramer and serves to amplify signal. Images were taken at x30 on an Olympus FV1200 Laser Scanning Confocal Microscope and analyzed in ImageJ v2.1.0/1.53c.

**In vivo antibody and vaccine dosing.** All antibody dosing was performed via intraperitoneal injection in PBS. Anti-CD4 (GK1.5, BioXcell) and anti-CD8 (2.43, BioXcell) depleting antibodies were administered at 200 μg every 4d. Anti-CD4 (2F9A12, BioXcell) was administered at 200 μg three times a week. Anti-CTLA (9H10, BioXcell) was administered at an initial dose of 200 μg, with all subsequent doses at 100 μg, three times a week. Anti-CD40 (FGK4.5, BioXcell) was administered once at the beginning of treatment at 100 μg. Anti-CTLA antibodies (anti-CD8α, anti-CD8β) and apamin were produced as previously described. Briefly, class B Cpg 1826 oligonucleotide with a Gq spacer (5’-diacil lipid-GGTCATGAGCTCCTGCA CGT-3’) was conjugated via the 5’ end to an 18 carbon diacyl tail. Antigen peptide OVA250–270/CCTGCA CGT-3’) was conjugated via the 5’ end to an 18 carbon diacyl tail. Antigen peptide OVA250–270 (CGCLEQLESIINFEKELTWTS) and nonspecific mutant peptide OVA310–320 (CGCLEQLESIINFEKELTWTS) and nonspecific mutant gp100-209, (optimized 527F, EGP long) were conjugated via an N-terminal cysteine residue to 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(maleimidyl polyethylene glycol-2000) (Avanti Polar Lipids). Mice were vaccinated subcutaneously at the base of the tail with 1.24 nmol amphi-Cpg and 25 μg of amphi-peptide, with a half dose given to each side. Vaccination was performed once weekly starting 14 days post-transplant of 10⁶ organoids.

**Colonoscopy imaging.** Tumor progression was monitored longitudinally using a Karl Storz colonoscopy system with white light, RFP and GFP fluorescence. This consists of Image 1 H3-Z Spies HD Camera System (part TH100), Image 1 HUB CCU (parts TC200, TC300), 175-Watt D-Light Cold Light Source (part 201323701-1), AIDA HD capture system and fluorescent filters in the RFP and GFP channels.
(all from Karl Storz). The endoscope used for imaging was the Hopkins Telescope (Karl Storz, part 64301AA) with operating sheath (Karl Storz, part 64301AA). To consistently measure tumor area, biopsy forces (Richard Wolf) were fed through the operating sheath and positioned consistently given two landmks: widthwise grooves that appear as concentric semi-circles in the field of view and a lengthwise groove at the forceps tip. Images were captured upon gentle contact of forceps with tumor. Tumor area in the field of view and length of the lengthwise forceps groove were calculated using ImageJ v.2.1.0.1/3c. Tumor area was normalized to groove length.

**TCR sequencing.** Using a BD FACS Aria flow cytometer, live SIINFEKL tetramer-positive CD8+ T cells were directly sorted into 50 μl lysis buffer with proteinase K, from the Arcturus PicoPure DNA Extraction kit (Thermo Fisher), in low binding microcentrifuge tubes (Biotix) and genomic DNA extraction was performed following manufacturer instructions. Mouse TCR-β sequencing was performed by Adaptive Biotechnologies. Analysis was performed in R v.4.0.2 and Simpson diversity calculated using the ‘vegan’ v.2.5.7 package. To account for differences in total numbers of T cells surveyed in samples between groups, unique productive TCR sequences were randomly downsamleld to match between groups. Downsampled data are presented in Extended Data Fig. 3k, although downsampling did not impact observed trends.

**Statistics and reproducibility.** Statistical analyses and figure generation were performed in R v.4.0.2 using built in functions and ggplot2 v.3.3.3, beeswarm v.0.3.1, and violinplot v.0.2.9. For statistical assessment of differences in proportionality, Fisher’s exact test was performed. For continuous data, two-tailed Wilcoxon rank-sum test was performed, apart from the organoid and OT-1 co-culture results, which were analyzed with two-tailed Student’s t-test. Multiple comparison corrections were performed using Holm’s method. No statistical method was used to predetermine sample size. Of animals transplanted with loSIIN organoids, only those that formed tumors were taken for flow cytometric analysis. No other data were excluded from analyses. Preclinical trials were randomized and investigators were blinded to allocation during dosing, colonoscopy imaging and tumor quantification. Preclinical studies were performed across three independent cohorts with the aim of validating consistency and reaching ten or more animals per treatment arm. All in vivo and co-culture experiments were repeated at least twice. No experiments presented in this manuscript failed to replicate.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

MS data generated in this study have been deposited on MassIVE under accession code MSV000087648. TCR-β sequencing data generated in this study have been deposited on immuneACCESS [https://clients.adapтивebiotech.com/pub/westcott-2021-nc]. TCGA COADREAD data analyzed in this study are available for download on the National Cancer Institute Genomics Data Commons. All other data supporting the findings of this study are available from the corresponding author on reasonable request. Source data are provided with this paper.

**Code availability**

Analyses were performed using open-source software and in-house scripts in R v.4.0.2 and Python v.2.7.13, which are available from the corresponding author on reasonable request. Quantification of CD4, CD8 and FOXP3 staining by IHC was performed using a custom CNN developed with Aiforia’s cloud-based image analysis platform. This is a commercial platform with proprietary technology and therefore did not generate any code. An interactive example of algorithm functionality can be provided free of charge upon request at https://www.aiforia.com.

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Extended Data Fig. 1 | Lower burden and expression of predicted neoantigens in MSS versus MSI-H CRC. (a) Total expressed neoantigens by patient including hypermutant MSS cases (in purple). N = 62 MSI-H, 68 MSI-L, and 275 (including 9 hypermutant) MSS patients. All other plots exclude hypermutant MSS cases. (b) Mean expression of all neoantigens, regardless of clonality, by patient. N = 62 MSI-H, 68 MSI-L, and 266 MSS patients. (c–d) Analysis of patients with available ABSOLUTE purity for estimation of clonality (adjVAF). N = 50 MSI-H, 58 MSI-L, and 236 non-hypermutant MSS patients. (c) Empirical cumulative distribution function of mean neoantigen clonality (adjVAF) by patient. Significance was assessed by two-sided Kolmogorov-Smirnov test. (d) Total expressed clonal neoantigens with predicted HLA-I binding IC50 ≤ 500 nM by patient. (e) Mean allele-specific expression of clonal SNV-derived neoantigens by patient, excluding neoantigens with zero gene level expression but including those with zero allele-specific expression. N = 41 MSI-H, 53 MSI-L, and 219 MSS patients. (f–g) Abundance distributions of HLA-I ligandomes by MS in PDOs from MSS CRC patients CRC_01 (f) and CRC_04 (g) with epitope abundance above the median in gray, below the median in light blue, and neoantigens in red. Data from Newey, A, et al., 2019. Significance in (b), (d), and (e) was assessed by two-tailed Wilcoxon Rank Sum test with Holm’s correction for multiple comparisons. Source data for panels (a–e) can be found in Source Data Fig. 1a–e.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Development of in vivo lentiviral and organoid models of CRC with neoantigen expression. (a) Lentiviruses used to initiate colon tumors in Apc<sup>flox/flox</sup> and Apc<sup>flox/flox; Rag2</sup>−/− mice. (b) Efficiency of tumor formation 16 weeks post-injection. N = independent animals. Significance assessed by two-tailed Wilcoxon Rank Sum with Holm’s correction for multiple comparisons. (c) Antigen expression in LucOS-induced tumors in Rag2<sup>−/−</sup> (left) and wild-type (right) mice at 12 weeks (colonoscopy above, bioluminescence below). (d) Efficiency of tumor induction with LucOS lentivirus at 20,000 and 100,000 transduction units (TU)/μl. N = 26 independent animals. (e) Antigen expression (bioluminescence). N = 26 independent animals. Significance assessed by two-tailed Wilcoxon Rank Sum. (f) Antigen expression in LucOS-induced tumors with continuous T-cell depletion at 5 weeks (left) and 7 weeks after T-cell depletion (right), and colonoscopy (above). (g) Antigen expression versus relative tumor size (percent of colon occluded) following withdrawal of depleting antibodies. N = 4 independent animals. (h) Correlation of antigen expression and tumor burden in Rag2<sup>−/−</sup> (dark pink) and αCD4/8 (light pink)-treated mice 12 weeks post-injection with LucOS. N = 17 independent animals. Significance measured by Spearman’s rank-order correlation. (i) no<sub>SIIN</sub>, hi<sub>SIIN</sub>, and lo<sub>SIIN</sub> organoids grown in the absence of WNT. Scale bars = 1 mm. Representative of N = 3 independent cultures. (j) Top 10 mutated genes in MSK-IMPACT colon adenocarcinoma (cBioPortal). (k) Lentiviral constructs used to generate organoids expressing only EGFP (no<sub>SIIN</sub>GFP) and SIINFEKL expression variants. (l) Linear regression with Pearson correlation of SIINFEKL abundance (TMT-MS) versus mScarlet<sup>hi</sup> MFI (flow cytometry). TMT-MS was performed on three independent preparations of each line. (m) H&E and IHC of no<sub>SIIN</sub> primary colon tumor 42 days post-transplant. Representative of N = 9 independent animals. Scale bar = 100 μM. (n-o) Images of dim<sub>SIIN</sub> (n) and mid<sub>SIIN</sub> (o) tumors that formed in N = 2/9 and 1/9 transplanted animals, respectively. (p) Lentiviral constructs used to generate organoids expressing SIYRYYGL, ITYTWTRL, and VGFNFRTL at high and low levels. (q) ITYTWTRL and VGFNFRTL tetramer-specific CD8<sup>+</sup> T cells infiltrating 42-day lo<sub>ITY</sub> and lo<sub>VGF</sub> tumors by flow cytometry. Representative of N = 10 lo<sub>ITY</sub> and 9 lo<sub>VGF</sub> transplanted animals.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Low neoantigen expression drives reduced T cell function and diversity. (a-m) Flow cytometry of CD44+/CD8+ antigen-specific T cells from lesions and DLNs post-transplant of hi\textsuperscript{HI} (red) and lo\textsuperscript{LO} (blue) organoids. (a) Total antigen-specific T cells and (b) percent Ki67 positive in DLNs at 8 days. N = 10 hi\textsuperscript{HI} and 9 lo\textsuperscript{LO}-transplanted animals. (c-d) TCF1 and GZMB expression in antigen-specific T cells in lesions at 8 (c) and 14 (d) days. Representative of N = 5-9 animals per line and timepoint. (e) Percent of antigen-specific T cells double-positive for TNFα and IFNγ, and (f) and representative expression of TCF1 and GZMB in this subset within DLNs at 8 days. N = 10 hi\textsuperscript{HI} and 9 lo\textsuperscript{LO}-transplanted animals. (g) Inhibitory receptor expression on TCF1+/GZMB+ antigen-specific T cells from tumors at 14 days. Representative of N = 6-7 animals per line. (h) Median percent of TCF1+/GZMB− antigen-specific T cells from tumors expressing 0 through 4 inhibitory receptors (PD-1, TIM3, LAG3, and 2B4) at 8 days. N = 9 hi\textsuperscript{HI} and 9 lo\textsuperscript{LO}-transplanted animals. Bars = standard deviation. (i-j) Percent of SIINFEKL-loaded “target” splenocytes killed in DLNs and spleens from killing assay at 8 (i) and 14 (j) days post-transplant of hi\textsuperscript{HI} and lo\textsuperscript{LO} organoids. N = 6-7 animals per line and timepoint. (k-l) Frequency of most common clonotypes (k) and Simpson diversity score (l) from TCRβ chain sequencing of antigen-specific T cells from hi\textsuperscript{HI} (down-sampled) and lo\textsuperscript{LO} lesions at 8 days. N = 4 independent animals per line. (m-n) Total antigen-specific T cells isolated from lesions at 14 days across all lines (m) and versus mScarlet\textsuperscript{HI} MFI (n). (o-p) Percent of antigen-specific T cells from lesions at 8 days double-negative for TCF1 and GZMB across lines (o) and versus mScarlet\textsuperscript{HI} MFI (p). Dashed lines connect medians. Significance assessed by Spearman’s rank correlation. N = 5-9 independent animals per line in (m-p). Significance in (a-b), (e), (i-j), (l-m) and (o) assessed by two-tailed Wilcoxon Rank Sum. Holm’s correction applied in (m) and (o). Source data for panels (a, h-j, m-p) can be found in Source Data Fig. 3a, i, n-r.
Extended Data Fig. 4 | T cells in tumors with low neoantigen expression lose effector function over time. (a-b) Percent of antigen-specific T cells from DLNs and tumors at 42 days negative for TCF1 and positive for TIM3 (a), and positive for TCF1 and negative for TIM3 (b) by flow cytometry. N = 4-5 independent animals per line. (c-d) Percent of antigen-specific T cells from DLNs and tumors double-positive for TNFα and IFNγ at 42 days (N = 4-5 independent animals per line) (c) and both 8 and 42 days (d) (N = 4-9 independent animals per line). Red = hiSIIN, blue = loSIIN. Significance in (a-d) was assessed by Wilcoxon Rank Sum. Source data for panels (a-d) can be found in Source Data Fig. 4d-e.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Neoantigen expression is limiting for cross priming by canonical and non-canonical antigen-presenting cells. (a) Flow cytometry gating strategy to determine percentage of CD11c+/CD103+ DC1s in BM-DC culture. (b) Schematic of BM-DC isolation, activation, and co-culture with naïve OT-1s. (c) Histograms of CD44, Ki67, GZMB, TNFα, and IFNγ expression on OT-1s representative of N = 4 co-cultures in the 400,000 lysed organoid cells condition. (d-f) Flow cytometric analysis of antigen-specific T cells from DLNs and lesions 8 days post-co-transplant of hiSIIN (red) and loSIIN (blue) organoids at separate sites. Percent TCF1+/GZMB- (d), TCF1+/GZMB+ (e), and TCF1-/GZMB- (f). N = 12 animals. Significance assessed by two-tailed Wilcoxon Rank Sum. (g) Brightfield and fluorescent images of colons and tumors 6 weeks post co-transplant of loSIIN and hiVGF or hiITY, representative of N = 9 animals each. (h-j) Flow cytometric analysis of antigen-specific (CD44+/SIINFEKL+) CD8s in colon and DLNs 6 weeks post-transplant of hiSIIN in Batf3-/− mice. (h) Total SIINFEKL+ CD8s, with progressive tumors in gray (N = 4 animals) and rejected lesions in red (N = 4 animals). (i) Flow plot of SIINFEKL+ CD8s infiltrating rejected lesion, and (j) PD-1 and GZMB expression on CD44+/SIINFEKL+ CD8s (red) versus CD44− CD8s (gray) from rejected lesion representative of N = 4 animals. (k) Flow plots of H-2Kb/H-2Db expression on hiSIIN organoids post electroporation with Cas9 complexes targeting H2-k1 and B2m, or untargeted control (pre-sorting). Organoids were pre-treated with IFNγ. N = 1 experiment. Source data for panels (d-f) can be found in Source Data Fig. 5j.
Extended Data Fig. 6 | Design of preclinical trials to test therapies that rescue priming in low neoantigen expressing tumors. (a) Schematic of vaccination and immunotherapy preclinical trial design and dosing schedule. (b–c) Flow plots of peripheral blood antigen-specific (CD44+ SIINFEKL tetramer+) CD8+ T cells from non-specific peptide-based vaccination (b) and no vaccination control (c) mice, representative of N = 7 and 8 independent animals, respectively. (d–e) Change in tumor size after 14 days of treatment, as determined by colonoscopy. ACT = adoptive cell transfer of OT-1s. N = 17 (d) and 10 (e) independent animals. Significance assessed by Wilcoxon Rank Sum of percent change in tumor size of treatment group versus no treatment, with Holm’s correction. (f) Fraction of mice with any metastases (liver, lung, or omentum), including only mice with progressive primary disease. N = independent animals. Significance assessed by 2×2 Fisher’s exact test of number of mice with metastases across all αCD40 treatment arms (with and without ICB) versus all other arms (no treatment and ICB single agent arms). Source data for panels (d–e) can be found in Source Data Fig. 6e–j, m.
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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

The Cancer Genome Atlas (TCGA) colorectal adenocarcinoma [COADREAD] RNA sequencing and clinical data were downloaded from the NCI Genomics Data Commons (GDC). Mass spectrometer-scanning functions and HPLC gradients were controlled by the Xcalibur data system (Thermo Scientific). Flow cytometry data was collected in BD FACSDiva v8.0. ABSOLUTE purity estimates of TCGA COADREAD were taken from a previous publication (Aran, D., et al., Systematic pan-cancer analysis of tumor purity, Nature Communications, 2015), version number not indicated in this publication.

Data analysis

A custom pipeline was constructed for neoantigen prediction employing a number of published algorithms. Predicted neoantigens and their correlation with other variables in the TCGA were analyzed in R v4.0.2. The following algorithms were called in this pipeline (see Methods): OptiType v1.3.1, Samtools v1.10, RazerS 3 v3.5.8, Strelka2 v2.9.2, Scalpel v0.5.4, VCFtools v0.1.13, Ensembl Variant Effect Predictor (VEP) v99, pVACtools v1.5.7, NetMHC-4.0, NetMHCpan-4.0, SMV v1.0, SMMPMBEC v1.0, bam-readcount v0.8.0, and custom scripts in Python v2.7.13 as described in Methods.

Tandem mass spectra were searched with Sequest (Thermo Fisher Scientific, San Jose, CA, USA; version IseNode in Proteome Discoverer 2.5.0.400). Sequest was set up to search a mouse uniprot database [database version July 3, 2020; 55650 entries containing common contaminants and the three proteins mScarlet-S1INFkt, mScarlet-VGFlipRTL and EGFP] assuming no digestion enzyme (unspecific).

Flow cytometry data were analyzed in FlowJo v10.4.2.

Triple IHC staining (CD8, CD4, FOXP3) of histological tumor sections was quantified using a custom convoluted neural network developed with Aiforia’s cloud-based image analysis platform. This is a commercial platform with proprietary technology and therefore did not generate any code. An interactive example of algorithm functionality can be provided free-of-charge upon request at https://www.aiforia.com.

Image: v2.1.0/1.53c was used for the following analyses: generation of composite images from immunofluorescent confocal z stacks (Fig. 2n); calculation of fluorescent area of organoids in co-culture experiments (Fig. 5f-g); quantification of tumor sizes in colonoscopy and gross
stereoscopic images [Fig. 6c,e-g, Extended Data Fig. 6d-e].

Statistical analyses and figure generation were performed in R v4.0.2 using built-in functions and ggplot2 v3.3.3, beeswarm v0.3.1, corrplot v0.88 and RColorBrewer v1.2.2. Simpson diversity of TCRb sequencing was calculated in the package Vegan v2.5.7.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
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TMT-MS data generated in this study (Fig. 2c, Extended Data Fig. 2i, Source Data 2) have been deposited on MassIVE, accession code: MSV000087648. https://massive.ucsd.edu/ProteoSAFe/private-dataset.jsp?task=d137f7a0bb3e4376809237c584e73aacc

TCRB sequencing data generated in this study (Extended Data Fig. 3k-l) have been deposited on immuneACCESS, accession code #????????.

The Cancer Genome Atlas (TCGA) colorectal adenocarcinoma (COADREAD) level 1 sequencing data were downloaded from the NCI Genomics Data Commons (GDC).

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

No sample size power calculations were performed. Most in vivo experiments for flow cytometry analysis were performed first as small pilots of 3-4 animals in each group to identify trends, and then repeated once or twice with 4-5 animals in each group to validate and power statistical analyses. Tumor efficiency experiments were also performed as smaller pilots to start, and repeated to reach n = 10 or more in each group where phenotypes were not absolute (~100% rejection or progression). Where phenotypes were near absolute, smaller numbers of animals were sometimes used as these reached statistical significance. Preclinical treatment studies were performed across 3 independent cohorts with the aim of validating consistency of results and reaching n = 10 or more per treatment group, which was sufficient to power detection of meaningful differences with statistical significance.

Data exclusions

Mice transplanted with loSIN organoids that failed to develop tumors [less than 10% of animals] were excluded from flow cytometric analyses of antigen-specific T cell responses (Figures 3 and 4). This was done to facilitate comparison of progressing loSIN tumors versus rejected hiSIN lesions. In preclinical trials, mice were injected at only one site in the colon (as opposed to two) with loSIN organoids, leading to a slightly higher rate of mice without tumors by 2 weeks (~20%). Only mice with clear tumor formation by colonoscopy at 2 weeks were recruited into treatment arms (Figure 6).

Replication

All in vivo experiments were repeated 2-3 times (as described in Sample size and Data exclusions above) to verify reproducibility. No experiments presented in this manuscript failed to replicate. Organoid co-culture experiments were repeated two (Fig. 5b-e) or three (Fig. 5f-g) times and showed consistent results. TMT-MS and TCRb sequencing were performed once.

Randomization

Randomization was performed in assigning mice to treatment arms in preclinical trials (Fig. 6 and Extended Data Fig. 6). Colonoscopy was performed at 2 weeks and mice without tumors (as confirmed by GFP positivity) excluded. Mice of each sex (approximately equal numbers) were then randomly and evenly [as possible] assigned across treatment arms using the Sample function in R v4.0.2. Randomization was not appropriate for other experiments presented in this manuscript as mice transplanted with each organoid line were age-, litter-, and sex-matched for consistency. In these studies, tumors were characterized (by colonoscopy, flow cytometry, gross-imaging, etc.) without further perturbations requiring randomized assignment.

Blinding

Investigators were blinded to treatment groups when dosing, performing colonoscopy, quantifying tumor burden from colonoscopy and gross dissection tumor images, and identifying metastases. Blinding was not performed in assessing presence or absence of tumors in loSIN versus hiSIN models (or other organoid models), as fluorescent is different between these lines (GFP versus mScarlet, respectively). Importantly, presence or absence of fluorescent tumors in these models is readily perceived and not subjective. Blinding was not performed in other experiments, such as analyzing flow cytometric data of antigen-specific T cells, as the same gating strategies and analysis methods were applied across all samples.

Reporting for specific materials, systems and methods
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|----------------------------------|---------|
| [ ] n/a                           | [ ] n/a |
| [ ] Antibodies                   | [ ] Involved in the study |
| [ ] Eukaryotic cell lines        | [ ] ChiP-seq |
| [ ] Palaeontology and archaeology| [ ] Flow cytometry |
| [ ] Animals and other organisms  | [ ] MRI-based neuroimaging |
| [ ] Human research participants  |         |
| [ ] Clinical data                |         |
| [ ] Dual use research of concern |         |

### Antibodies

**Antibodies used**

IHC and IF:
- CD8a [EPR21769, Abcam Cat#: ab217344] 1:1000 for IHC
- FOXP3 [FJK-16s, eBioscience Cat#: 14-5773-82] 1:125 for IHC
- CD4 [EPR19516, Abcam Cat#: ab183685] 1:400 for IHC
- CD80 AF647 [YTS156.7.7, BioLegend Cat#: 126611] 1:100 for IF
- Beta-2-microglobulin (B2M) PE (2M2, BioLegend Cat#: 316305) 1:50 for in situ tetramer staining

**In vivo dosing:**
- CD4 [GK1.5, BioXcell Cat#: BE0003-1] [see Methods for in vivo dosing]
- CD8 (2.43, BioXcell Cat#: BP0061) [see Methods for in vivo dosing]
- PD-1 (29F.1A12, BioXcell Cat#: BE0273) [see Methods for in vivo dosing]
- CTLA4 4 (9H10, BioXcell Cat#: BE0131) [see Methods for in vivo dosing]
- CD40 [FGK4.5, BioXcell Cat#: BE0016-2] [see Methods for in vivo dosing]

**Flow cytometry:**
- TNFα PE (MP6-XT22, BioLegend Cat#: 506305) 1:400
- GZMB PE-CF594 (GB11, BD Biosciences Cat#: 562462) 1:250
- IFNγ PerC-Py55 (XM6G2, BioLegend Cat#: 505621) 1:200
- PD-1 BV421 (RMP1-30, BioLegend Cat#: 109121) 1:400
- Ki67 BV510 (B56, BD Biosciences Cat#: 563462) 1:400
- Ki67 AF700 (B56, BD Biosciences Cat#: 563277) 1:200
- TIM3 BV605 (RMT3-23, BioLegend Cat#: 119721) 1:200
- CD44 BV711 (IM7, BioLegend Cat#: 103057) 1:200
- CD44 BV661 (IM7, BD Biosciences Cat#: 741471) 1:400
- CD8a BV395 (53-6-7, BioLegend Cat#: 563786) 1:400
- CD8a BV496 (53-6-7, BD Biosciences Cat#: 750024) 1:200
- IAG3 BV737 (C9B7W, BD Biosciences, Cat#: 741820) 1:100
- SIINFEKL tetramer PE (NIH Tetramer Core custom request) 1:400
- ITF2-WTR tetramer PE (NIH Tetramer Core custom request) 1:400
- VGFN/RNL tetramer PE (NIH Tetramer Core custom request) 1:400
- 2B4 PE-CY7 [eBioscience, Cat#: 25-2441-82] 1:200
- TCF-1 AF647 (C6309, CST Cat#: 67095) 1:250
- Tg11FITC (GG1D7 ebioscience, Cat#: 11-9501-82) 1:100
- CD45 PE-CF594 (30-F11 BD Biosciences, Cat#: 562420) 1:6400
- MHC II FITC (2G9, BD Biosciences, Cat#: 562009) 1:100
- CD11b BV605 (M1/70, BioLegend, Cat#: 101237) 1:200
- Ly6C PerC-Py55 (HK1.4, BioLegend Cat#: 128011) 1:200
- CD64 BV421 (K54-5-7.1, BioLegend, Cat#: 139309) 1:300
- CD11c eFluor 660 (N418, eBioscience Cat#: 50-0114-82) 1:1000
- CD103 BUV395 (M290, BD Biosciences, Cat#: 740038) 1:300
- H-2Kb APC (AF6-88.5.5.3, eBioscience Cat#: 17-9598-82) 1:200
- H-2Db B700 (28-14-8, BD Biosciences Cat#: 745842) 1:200
- PD-L1 PE-CY7 [10F.9G2, BioLegend Cat#: 124313] 1:200

**Depletion:**
- Ly6G (1A8, BioLegend Cat#: 127601) 1:200
- EpCAM (G8-8, BioLegend Cat#: 118201) 1:80
- F4/80 (BM6, BioLegend Cat#: 123101) 1:80

**Validation**

Abcam antibodies and optimal dilutions used for IHC were validated for our experiments by confirming expected staining patterns of CD8, CD4, and CD4/FOXp3 T cells in positive control spleen and lymphnode tissue, with minimal background in non-lymphoid tissue. Anti-CD4 (GK1.5, BioXcell Cat#: BE0003-1) and anti-CD8 (2.43, BioXcell Cat#: BP0061) at the indicated in vivo doses (see Methods) showed nearly complete depletion of CD4 and CD8 T cells in our models by flow cytometry of peripheral blood, spleens, draining LNs, and colons following two doses. On target depletion of PD-1 on antigen-specific T cells following treatment with anti-PD-1 (29F.1A12, BioXcell Cat#: BE0273) was confirmed by flow cytometry of peripheral blood (using a different anti-PD-1 clone, RMP1-30) of tumor-
Eukaryotic cell lines

Policy information about cell lines

| Cell line source(s) | Organoid lines developed by the authors of this study starting from normal colon crypts from mice. HEK-293 cells were ordered from ATCC. |
|---------------------|----------------------------------------------------------------------------------------------------------------------------------|
| Authentication      | Authentication of genetic events (LSL-Kras and Trp53-flox recombination, Smad4 knockout) were confirmed during the establishment of organoid lines as described in the manuscript. HEK-293 cells were not authenticated. |
| Mycoplasma contamination | Organoid lines and HEK-293 cells used to generate lentivirus have been routinely tested and confirmed negative for mycoplasma contamination. |
| Commonly misidentified lines (See ICTAC register) | None |

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | Approximately equal numbers of male and female C57BL/6 mice between 6 to 12 weeks of age were used for all experiments. Mice were housed in a facility with a 12-hour light/12-hour dark cycle with temperatures within 68-72°F and 30-70% humidity. |
| Wild animals       | This study did not involve wild animals. |
| Field-collected samples | This study did not involve field-collected samples. |
| Ethics oversight   | All mouse work was approved by the Department for Comparative Medicine (DCM) at MIT and the Institutional Animal Care and Use Committee (IACUC) |

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

This is described extensively in the methods under the subheader Tissue preparation and flow cytometry. Briefly, T cells were harvested from tumors or resected lesions at the sites of injection of organoids in the colon, the colon draining lymph nodes (caudal and iliac), and the spleen. Tumor tissue was digested and processed as described in the methods. Lymphnodes were crushed, spun, and resuspended directly for staining. Spleens were mashed through a 70 micron filter and red blood cells lysed before spinning and resuspending for staining.

Instrument

BD LSR Fortessa Flow Cytometer with 355 nm, 405 nm, 488 nm, and 640 nm excitation lasers was used for all T cell flow cytometry. For flow cytometry on loSiN and hiSiN organoids, an instrument with better ability to resolve PE and FITC channels was used: BD LSR Fortessa Flow Cytometer with 355 nm, 405 nm, 488 nm, 561 nm, and 640 nm excitation lasers.

Software

BD FACSDIVA v8.0 was used to collect data. FlowJo v10.4.2 was used to analyze data.

Cell population abundance

Populations were not sorted for downstream manipulation in this study.

Gating strategy

Cells in all flow cytometry experiments were first gated on FSC-A vs SSC-A, then on FSC-A vs FSC-H for single cells within a roughly 45 degree straight line, and then on negative staining for the live/dead dye ghost 780 (Corning). For identifying SiNFEX1 tetramer+ CD8+ T cells, cells were then gated on CD8a positivity, and antigen-specific CD8+ T cells identified as double positive for CD44 vs SiNFEX1 tetramer staining. This population was then further analyzed for expression of TCF1, GZMB, Ki67, PD-1, TIM3, 284, LAG3, and TIGIT, and IFNg and TNFa in stim and cytokine staining experiments. Positive versus
negative gating for these markers was determined using the CD44 negative population in the CD44 vs SIINFEKL tetramer plot. EGFP (FITC channel) vs mScarlet (PE channel) was used to assess fluorescence of loSiN and hiSiN organoids following gating of live single cells as described. CTV labeled splenocytes in the in vivo killing assay were first identified by SSC and FSC and live/dead staining as described above, then all CTV positive cells were gated using CTV vs FSC-H. A histogram of CTV intensity was then plotted, and target splenocytes (higher intensity population) normalized to control splenocytes (lower intensity population).

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.