Evolutionary dynamics of neoantigens in growing tumors

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Cancers accumulate mutations that lead to neoantigens, novel peptides that elicit an immune response, and consequently undergo evolutionary selection. Here we establish how negative selection shapes the clonality of neoantigens in a growing cancer by constructing a mathematical model of neoantigen evolution. The model predicts that, without immune escape, tumor neoantigens are either clonal or at low frequency; hypermutated tumors can only establish after the evolution of immune escape. Moreover, the site frequency spectrum of somatic variants under negative selection appears more neutral as the strength of negative selection increases, which is consistent with classical neutral theory. These predictions are corroborated by the analysis of neoantigen frequencies and immune escape in exome and RNA sequencing data from 879 colon, stomach and endometrial cancers.
Fig. 1 | Tumor growth model predicts two distinct types of immune phenotypes and the necessity of immune escape. a. Schematic representation of the model. Left: tumor growth for four generations. The filled circles represent cells colored by immunogenicity. Related cells are connected with lines. Middle: cell division/mutation process. Right: prior distribution of newly generated neoantigenicities. For details, see Methods. b. Growth curve of 6 simulated tumors at $s = -0.8$. The line color shows the antigen score of the tumor population over time (measured in arbitrary units (a.u.) of simulation time). c. CCF of the most common antigenic mutation of $n = 100$ tumors at the final time point. d. Distribution of the antigenicity values of all neoantigens generated (gray) and only neoantigens present in >10 surviving cells (blue). Thin lines: individual simulations; thick dashed line: ensemble mean. Inset: two-sided Mann-Whitney U-test. e. Distribution of maximum tumor size reached by hypermutated tumors at $s = -0.8$. Inset: growth curve of a single tumor colored by antigenic score as in b. Blue line: number of nonimmunogenic cells. f. Neoantigen scores in $n = 100$ tumors at $s = -0.8$, without (left) and with (right) clonal immune escape. g,h, Number of detectable neoantigens (read depth approximately 50x) in $n = 50$ simulated tumors as a function of negative selection strength. Middle: mean clonal neoantigen burden. Bottom: clonality of immune escape. Only non-hypermutated (g) and hypermutated (h) tumors that reached a detectable size are shown. The violin widths represent raw data density.

strength of negative selection $s$, against the cumulative antigenicity of neoantigens in the lineage; $s$ can be interpreted as the effectiveness of immune predation against an antigen. Thus, $s = 0$ indicates no selection pressure (neutral evolution), and $s > 0$ strong negative selection (following Kessler et al.13). Tumor growth was simulated until the tumor reached a predefined population size (analogous to a clinically detectable size) or until a sufficiently long time elapsed without the tumor reaching detectable size.

We first examined the temporal neoantigen burden in simulated tumors. We defined the ‘antigen score’ of a tumor as the proportion of tumor cells carrying a cumulative antigenicity $\geq T_c$. Tumors simulated with identical parameters were separated into two distinct groups due to the stochasticity of neoantigen accrual: ‘antigen-hot’ and ‘antigen-cold’. Antigen-hot tumors had an antigen score close to 1, corresponding to every tumor cell in the population being highly antigenic, whereas in antigen-cold tumors the majority of cells lacked immunogenic mutations (Fig. 1b,c). The proportion of antigen-hot tumors depended on the negative selection strength (Extended Data Fig. 1a): increased negative selection for neoantigens decreased the probability of observing antigen-hot tumors. In antigen-cold tumors, the proportion of neoantigen-carrying cells also decreased with increasing strength of negative selection.

In the simulations, the antigenicity of newly accrued neoantigens was sampled from a ‘prior’ prespecified distribution.
Regardless of the shape of the prior distribution, surviving lineages always showed enrichment for low-antigenicity alterations with an exponential-like distribution of final antigenicity values (Fig. 1d and Extended Data Fig. 1b).

We next simulated hypermutated tumors that generated a high number of mutations per cell division, causing lineages to rapidly accrue antigenicity. Consequently, most lineages rapidly became neoantigen-hot and were eradicated by negative selection (Fig. 1c). In rare tumors that survived to detectable size, high-frequency neoantigens were absent (Extended Data Fig. 2a,b and Supplementary Note).

Overall, we observed that negative selection prevented subclonal neoantigens rising to high frequency in a tumor; this effect was exacerbated at higher mutation rates.

We compared the dynamics observed in our model to the dynamics of neoantigen accrual in a constant size population (Supplementary Note). Models of negative selection with constant population size\(^{22,35,36}\) can lead to a broad range of evolutionary dynamics when the mutation rate and strength of negative selection are varied. In contrast, in this study we observed that allowing the population size to vary led to broadly consistent dynamics across the parameter space (Extended Data Fig. 2). We considered three scenarios: (1) high \(s\), low \(\mu\). When negative selection was strong and mutations rare, selection operated efficiently in a constant population rendering it devoid of neoantigenic mutations but was attenuated in a variable-sized population due to population expansion decreasing the efficiency of selection, as reported previously for positive selection\(^{19,20}\); (2) low \(s\), high \(\mu\). Due to weak selection, only lineages with multiple mutations experienced nonnegligible selection. As in the previous case, population growth attenuated the influence of selection relative to the constant size population model; (3) high \(s\), high \(\mu\). In constant size populations, the population could not go extinct and dynamics were determined by the relative strength of negative selection between lineages all accruing neoantigenic mutations. The additive effect of any single mutation on fitness was proportionally diminished as the mutation burden increased due to a Muller’s ratchet-like effect\(^{20}\), leading to weakly selected dynamics. In a variable size population, the dynamics were markedly different: populations where all lineages were strongly negatively selected went extinct; surviving populations consisted of the ‘lucky’ lineages that had not accrued neoantigens (Extended Data Fig. 2a,d). These extinction-driven dynamics persisted in the growing population even in the special case of extremely high \(\mu\) and low \(s\), while the constant size population became effectively neutral.

**Modeling shows that immune escape leads to antigen-warm tumors.** We next simulated immune escape alterations acquired by one cell that render its descendants less susceptible to immune predation\(^{14,26}\). Specifically, we set the death rate of immune-escaped cells to the baseline nonimmunogenic death rate irrespective of the cell’s burden of antigenic mutations.

If the founder cell of the tumor contained an escape mutation (clonal escape), tumors with a continuum of antigenicity scores emerged (Fig. 1f). We termed these tumors ‘antigen-warm’ since they contained strong high-frequency and/or several subclonal neoantigens.

We then simulated tumors that could acquire immune escape at a random time (probabilistic escape) and evaluated the detectable neoantigen load in the emerging tumors (Methods). When the mutation rate was low, tumors that reached detectable size had rarely evolved an immune escape and the strength of negative selection imposed on growth was inversely correlated with the subclonal neoantigen burden observed in the final tumor (Fig. 1g). When the mutation rate was high, lineages rapidly accrued neoantigens and were driven to extinction by negative selection (Fig. 1c). Tumors only grew to detectable frequency if the founder lineage stochastically acquired immune escape to ‘rescue’ them. Consequently, at high mutation rates, detectable tumors were exclusively immune-escaped and had a high burden of high-frequency neoantigens (Fig. 1b).

Taken together, these results suggest that there is a nonlinear relationship between the levels of immune surveillance in the microenvironment and the magnitude of immunoediting seen in tumors of detectable size. Moving from low to moderate negative selection, the dynamics increasingly depart from strictly neutral dynamics as expected; correspondingly, the clonal and subclonal neoantigen burden is progressively decreased. At strong negative selection, detectable tumors are those that have stochastically acquired immune escape and consequently show a high proportion of neoantigen-warm and neoantigen-hot cases and evolve effectively neutrally. We also note that the mutation rate is a determinant of the strength of negative selection experienced by a lineage: at high mutation rates, a lineage is likely to accrue multiple negatively selected variants and thus experience stronger negative selection.

**Immune-infiltrated cancers are antigen-hot and escaped.** To compare model predictions to experimentally measured neoantigen landscapes, we analyzed neoantigens in 363 colorectal cancers (CRCs), 146 stomach adenocarcinomas (STADs) and 370 uterine corpus endometrial carcinomas (UCECs) from The Cancer Genome Atlas (TCGA) (Fig. 2a). We focused on these cancer types because of the prevalence of mutator phenotypes, namely cancers with polymerase \(\varepsilon\) mutation (POLE, very high mutation rate), mismatch repair (MMR) deficiency (high mutation rate, often responding well to immunotherapy\(^{17,37}\)) and microsatellite-stable (MSS) tumors (lower mutation rate). Therefore, they provide a good model to explore the effect of different tumor-immune dynamics.

TCGA samples filtered for high sequencing depth and purity were first human leukocyte antigen (HLA)-typed in silico\(^{26}\) and their neoantigens called and filtered\(^{19}\) using the NeoPredPipe pipeline\(^{39}\) (see Methods). We also evaluated T-cell infiltration from paired RNA-seq data\(^{40}\) as a measure analogous to the negative selection strength \(s\) experienced by neoantigens.

Most tumors (90%) had clonal neoantigens (Supplementary Table 1) and so were defined as antigen-hot. We observed that the mutation antigenicity distribution of tumors (see Methods) was...
Figure 1. Density distribution of T-cell score.

(a) Number of subclonal antigenic mutations.

(b) Proportion of samples escaped.

(c) Immune escape.
enriched for low-binding neoantigens irrespective of the level of T-cell infiltration, but still contained a tail of high-scoring neoantigens (Fig. 2b). Subclonal neoantigen burden varied significantly between cancers: cancers with low or medium T-cell infiltration (putative small or moderate $s$) had proportionally fewer subclonal neoantigens than high T-cell infiltrate tumors (high $s$) (Fig. 2c), suggesting a critical role of immune escape in early evolution. Interestingly, this trend was absent in STAD tumors, suggesting a more homogeneous evolution due to either widespread or rare immune escape.

Therefore, we sought evidence of immune escape in the cancers: namely alterations in antigen presentation and overexpression of immune checkpoint genes (Methods). Overall, 57% of all cancers showed evidence of at least one escape mechanism, with increased prevalence of escape in MMR (71%) and POLE (98%) cases and significantly different patterns of immune escape (Fig. 2d and Extended Data Fig. 3a), in agreement with previous studies. STAD cancers in particular had a high proportion of immune-escaped cancers, potentially a result of strong early immune predation. Further work is needed to confirm that these differences between mutational subtypes arose from differential selective pressures for immune escape.

Consistent with the predictions and previous studies, tumors with immune escape had a higher neoantigen burden and most highly antigenic tumors (neoantigen burden >100) were immune-escaped (Fig. 2e). Increased immune infiltration level was strongly associated with immune escape even in non-hypermutated (MSS) samples (Fig. 2f). We expected neoantigen-associated mutations to be more underrepresented among high cancer cell fraction (CCF) subclonal mutations since selection had the longest time to act on these mutations. Therefore, we compared the number of neoantigens at high CCF (present in 30–60% of cells) between MMR cases with and without immune escape and found greater depletion in nongeneic cancers (Fig. 2g), consistent with immunoediting shaping the clonal structure of hypermutated tumors without immune escape. These phenomena were also observed in a meta-cohort that combined the structure of hypermutated tumors without immune escape. These phenomena were also observed in a meta-cohort that combined the three cancer types (Extended Data Fig. 3).

Together, these data suggest that these cancer types usually evolve in the face of stringent immune-selective pressures (analogous to the moderate/high $s$ regime in simulated tumors), and consequently immune escape is frequently selected for at the onset of tumor growth, permitting the development of tumors with a high and clonal neoantigen load.

Subclonal immune escape shapes local neoantigen evolution. Next, we explored the evidence for subclonal immune escape in a previously published multiregion-sequenced colorectal tumor dataset. Overall, loss of heterozygosity (LOH) at HLA loci, called with the LOHHLA tool, was found in 5 of 10 (50%) carcinomas and 1 of 6 (17%) adenomas (Fig. 3a,b). HLA LOH was subclonal in at least one allele in 4 of the 6 cases (Fig. 3a,b).

Simulations of subclonal immune escape in our model predicted that subclones should become proportionally enriched for neoantigens after escape (Fig. 3c), which is consistent with previous observations. In our primary tumor data, a significantly higher proportion of predicted neoantigens were associated with the lost allele in escaped clones than in clones without LOH (Fig. 3d). These results confirm that heterogeneous immune-mediated negative selection pressures can shape individual subclones inside a tumor.

To study how subclonal immune escape mechanisms can influence the efficiency of therapy, we extended our simulations to model immunotherapy. We introduced two different types of escape stochastically during tumor growth, active and passive, which notionally represented reversible escape mechanisms affecting interactions with the microenvironment (for example, expression of programmed death-ligand 1 (PD-L1)) and irreversible cell-intrinsic escape (for example, genomic loss of an HLA allele) respectively (Methods). After the tumor population grew to a detectable size, we simulated immunotherapy by canceling the effect of active immune escape and increasing the negative selection pressure $s$ against neoantigens. The clonal population(s) with active escape rapidly shrank, but clones with passive-type escape continued to grow (Fig. 3e). Neoantigens were progressively pruned from the expanding clone, typically leading to an immune-cold tumor. Thus, the immune landscape of a tumor post-immunotherapy is predicted to be distinct from the original tumor (consistent with observations), with potential implications for the choice of the next line of therapy.

Negative selection leads to a neutral VAF distribution. We sought to explore how negative selection shapes the distribution of subclonal mutation frequencies within an individual cancer. We considered the VAF distribution in simulated tumors with moderate and high negative selection. Evidence for positive selection in the VAF distribution is provided by an overabundance of passenger mutations at high frequency that are within the expanding clone, whereas under pervasive negative selection, antigenic clones are continually depleted and so rarely grow to a large size (rarely reach high VAF). Thus, most high-VAF mutations are neutral passengers that evolve according to neutral dynamics and thus exhibit a characteristic $1/f$ dependence (leading to a $1/f$ dependence of the cumulative VAF distribution; Fig. 4a) (ref.). As negative selection strength increases, the phenomenon is exacerbated: antigenic subclones are more rapidly depleted and so more neutral-like VAF distributions are observed (Fig. 4b). We note that pervasive negative selection is part of the original neutral theory and our observations are consistent with this classical theory.

The VAF distribution computed of solely neoantigens shows depletion relative to the neutral expectation (red lines in Fig. 4a,b), which is consistent with population genetics theory of constant size models (Extended Data Fig. 2c,f). The magnitude of deviation from the neutral curve depends on the strength of negative selection, which means that, in theory, negative selection could be detected from neoantigen VAF distributions (Extended Data Fig. 4). However, in practice, the few persisting neoantigens are at very low VAFs and so are problematic to measure accurately, severely hindering the power to quantify negative selection strength directly from neoantigen VAF distributions.

We performed in silico sequencing on simulated tumors and explored the effect of read depth and false positive neoantigen identification on the identifiability of negative selection in individual tumors (see Methods). The simulations predicted that very-high-depth sequencing was required to robustly call negative selection from VAF distributions and the efficacy strongly depended on the strength of selection against neoantigens (Fig. 4c,d). Erroneously labeling neoantigens also had substantial impact on the power but could be mitigated by very-high-depth sequencing. Detection was mostly limited by the tumors retaining too few neoantigens to reliably evaluate their VAF distribution, a phenomenon further exacerbated when concentrating on strongly immunogenic mutations alone (Extended Data Fig. 5a–d). As the basal death rate was increased, negative selection against new neoantigens strongly prevented lineage expansion, leading to a more stringent depletion observable in the VAF distribution. Further increase in the basal death rate meant that neoantigen accrual became progressively more likely to cause clone extinction, and consequently tumors that grew to detectable size became progressively enriched for immune escape and neutral dynamics (Extended Data Fig. 6). Stronger negative selection and higher neoantigen accumulation rate (increased $p_e$, Extended Data Fig. 7) both amplified the signal of negative selection in the VAF distribution (Extended Data Fig. 8).

To overcome the technical issues of limited sequence depth and low antigen numbers, we pooled mutations from groups of identically simulated and comparable TCGA tumors (Methods) and
Fig. 3 | Subclonal immune escape shapes neoantigen landscape and tumor growth after therapy. a. Immune escape through LOH at an HLA locus in the multiregion-sequenced colorectal cohort. LOH events are divided up according to whether the alteration is detected in all (clonal) or not all (subclonal) biopsies. b. HLA LOH in individual biopsies in tumors with at least one subclonal loss event. The unfilled boxes represent homozygous HLA alleles. c. The number of antigenic mutations detected in two distinct (with and without immune escape) subclones of n = 25 simulated tumors. Antigenic mutations are detected at simulated read depth of 100x. Visual elements of the boxplot correspond to the following summary statistics: center line; median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range. d. The proportion of all neoantigens binding to the HLA allele lost in the LOH event in colorectal tumors that show subclonal HLA LOH (n = 6 subclones in 4 tumors). One-sided Wilcoxon signed-rank tests are reported on c and d. e. Growth curve of simulated tumors following anti-PD-L1-type immunotherapy. The tumors had previously developed active immune escape, but also harbored a small subclone with a different escape mechanism. The black dashed lines show the number of cells in this subclone over time (measured in arbitrary units of simulation time). The inset shows growth around the point when the subclone takes over, on a logarithmic scale.
Fig. 4 | Negative selection leads to characteristic depletion of neoantigens and effectively neutral overall VAF distributions. a, b. Cumulative number of mutations as a function of the inverse of the frequency for all mutations (gray, left axis) and neoantigen-associated mutations (red, right axis) harbored in at least 30 cells in a tumor with $s = -0.8$ (a) and a tumor with $s = -1.2$ (b). c. Power to detect negative selection from the VAF distribution as a function of sequencing read depth (x axis) and false neoantigen rate (y axis). Power is the proportion of 100 simulated tumors with significant difference (two-sided Kolmogorov-Smirnov test, $\alpha = 0.1$) between the distribution of all mutations and neoantigen-associated mutations. d. Power (in $n = 100$ tumors) to identify negative selection as a function of selection strength (x axis) and the stringency of the two-sided Kolmogorov-Smirnov test used for detection ($\alpha = 0.1$, $\alpha = 0.05$ and $\alpha = 0.01$, shown in black, maroon and red, respectively). e. Cumulative VAF distribution as a function of the inverse of the frequency for all (in gray) and neoantigen-associated mutations (in red) detected with a sequencing depth of 800× in antigen-cold tumors from a simulated set of $n = 100$. The y axis shows the proportion of mutations. A mutation antigenicity threshold of 0.2 was used in all cases in a–e. f. Cumulative VAF distribution of mutations detected in any low- and medium-immune-infiltrated TCGA MSS cancers without immune escape. The distribution is shown for all mutations (gray), exonic mutations (blue), exonic mutations in essential genes (purple), antigenic mutations (pink) and neoantigen-associated mutations in essential genes (red).
considered their combined VAF distribution (Fig. 4e) in a similar manner to how cohort-wide positive selection by dN/dS analysis is evaluated\cite{23,24}. In the pooled TCGA cohort, we investigated essential genes\cite{49} that are expected to be constitutively expressed and under selection\cite{24,50}. In cancers with a medium T-cell score and no evidence of immune escape, there was a depletion of all neoantigens
and neoantigens in essential genes compared to other genomic regions (Fig. 4f). In contrast, there was no neoantigen depletion in cancers with a low T-cell score and no evidence of immune escape. Neoantigens in CRC and UCEC cancers individually, as well as frameshift and nonsense mutations in essential genes, showed similar trends (Extended Data Fig. 5e,f), suggesting a more stringent selection in moderately infiltrated tumors upon essential genes.

Proportional neoantigen burden measures negative selection. Depletion of neoantigens relative to the overall nonsynonymous mutation burden is a well-established signature of immunoeediting.\textsuperscript{54} We investigated the relationship between the degree of neoantigen depletion and strength of negative selection experienced by neoantigens.

First, we simulated tumors with a known neoantigen production rate \( \rho = 0.075 \) per nonsynonymous mutation (Supplementary Note) to evaluate how the proportion of immunogenic to nonsynonymous mutations changed with negative selection strength. As expected, stronger negative selection led to proportionally fewer observed neoantigens in the final tumor (Fig. 5a). We also measured the effective mutation rate (the ratio of the per cell division mutation and survival rate) derived from the linear slope of the neutral VAF curve\textsuperscript{9} as a function of increasing negative selection for neoantigens (Supplementary Note). Stronger negative selection caused higher effective mutation rates in antigenic tumors (Fig. 5b) as a consequence of increased death rate. We suggest that the higher cell death rate inferred in hypermutated tumors\textsuperscript{12} is likely to be, at least in part, a direct consequence of immunoeediting.

Next, we examined the proportional neoantigen burden in TCGA CRC, STAD and UCEC cancers stratified by cancer type and predicted immune escape status. We observed no difference in overall proportional neoantigen burden according to cancer type (Extended Data Fig. 9a) and so combined all data into a single metacohort. We detected no significant difference in overall proportional burden between MSS and MMR, and immune-escaped or unescaped cancers (Extended Data Fig. 9b,c). The observed uniformity in overall proportional burden across the cohort was consistent with the lack of neoantigen depletion signal reported in Van den Eynden et al.\textsuperscript{51}. Most mutations considered in these analyses were clonal and so were likely accrued before tumor expansion and acquisition of immune escape. To better delineate the decrease in negative selection expected after immune escape, we computed the subclonal proportional neoantigen burden for mutations with a CCF < 0.6. Comparison of the total and subclonal proportional burden (considering all tumors with >30 subclonal mutations) showed a lower subclonal proportional burden in unescaped cancers; however, no shift was detected in cancers with immune escape (Fig. 5c), which is consistent with immune-mediated negative selection operating in unescaped cancers. When cancer types were considered independently, UCEC and CRC cancers showed a similar pattern, but no subclonal depletion was evident in STAD cancers (Extended Data Fig. 9d).

To examine the potential confounding effect of different mutational processes, we generated synthetic cohorts analogous to real tumors (Methods). Comparing the synthetic cohorts matching the overall mutation composition of CRCs showed no significant difference in proportional burden, suggesting that MMR-specific mutational processes (for example, signature 6 from Alexandrov et al.\textsuperscript{12}) are not strongly biased for neoantigen generation (Extended Data Fig. 9e). A synthetic matched cohort of Fig. 5c confirmed that the observed difference in subclonal proportional neoantigen burden was also independent of mutational processes (Extended Data Fig. 9f). Burden normalized to this synthetic cohort provided weak evidence of lower than random subclonal neoantigen burden (Extended Data Fig. 9g). These observations imply the presence of active immune surveillance when escape has not occurred and highlight the high interpatient variability in evolutionary dynamics.

Discussion
In this study, we investigated the evolutionary dynamics of neoantigens and immune escape in growing tumors using a mathematical model of tumor evolution. Our analysis shows how negative selection by the immune system (immunoediting) sculpts the clonal architecture of the tumor: the hallmark of negative selection is the lack of neoantigens at intermediate subclonal frequency within a tumor; conversely, the presence of numerous neoantigens at intermediate frequency is a hallmark of immune escape. Moreover, strong negative selection for neoantigens inevitably provides a strong selective pressure for the evolution of immune escape. Consequently, the observation that many cancers are both (neo)antigenic and have immune escape points to a critical role for immune escape in the genesis of malignancy. Further work directly measuring the immune repertoire at the time invasion first occurs is required.

Our simulations show that under negative selection, the overall VAF distribution of a tumor will be effectively neutral since it will be dominated by neutral passenger mutations that can spread through the tumor unimpeded by immune predation. In constant size models, neutral mutations linked to disadvantageous alterations show a pattern of background selection\textsuperscript{26–28}; however, in growing populations selection can only be observed on the selected mutations directly. The VAF distribution observable in cancer genome sequencing data becomes more neutral-like as the strength of negative selection increases and negatively selected clones are pushed to harder-to-detect frequencies leaving only neutrally evolving lineages at high VAF. Furthermore, our analysis suggests that most tumors with high mutational burden—where in theory VAF distributions and thus evolutionary dynamics should be easier to resolve—are most likely to be immune-escaped and so only exhibit effectively neutral dynamics. Consequently, we suggest that the lack of an immune-related selection signal (for example, as identified by Van den Eynden et al.\textsuperscript{51}) could be due to unclassified immune escape or false positive neoantigen calls that together mean the mutations studied are likely to be overall only very weakly negatively selected. Pooling data across cancers increases power to resolve VAF distributions and detect negative selection, and could be combined with dN/dS methods to evaluate selection of gene sets, such as natural HLA binders\textsuperscript{12,55} and major histocompatibility complex class II-presented peptides\textsuperscript{56}.

Our modeling offers insight into the challenges of predicting the immunotherapy response using tumor mutational burden (TMB) alone. Strong negative selection (effective immune surveillance) leads to a high rate of cell death, a corresponding increase in the effective mutation rate of tumors and the net result of high TMB with severe neoantigen depletion. Thus, despite having high TMB, such tumors would be unlikely to respond to immune checkpoint blockade. Assessment of neoantigens should be more predictive: tumors with clonal or numerous subclonal neoantigens are very likely to have evolved immune escape—particularly if the patient’s immune system is highly predatory—and to respond to therapies reactivating immune predation. This is consistent with previous studies suggesting that clonal antigens predict sensitivity to immune checkpoint blockade\textsuperscript{47–49}. We illustrate that immune therapies targeted against a specific neoantigen or immune mechanism are vulnerable to intratumor heterogeneity since subclones where this target is altered or lost (for example, neoantigen-depleted or HLA haplotype-mutated) will experience net positive selection when the therapy is applied\textsuperscript{47–49}. Relatively, a subclone that escapes immune blockade therapy and reforms the tumor is predicted to have a different immune landscape due to the action of immune predation during clone emergence, with potential implications for additional lines of therapy.

In summary, our mathematical framework provides insights into the evolutionary dynamics of negatively selected neoantigens in growing tumors and the detectability of these dynamics in genomic data.
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Methods
Mathematical model of tumor growth and mutation accumulation. We created a minimal stochastic branching process model to represent tumor growth and accumulation of mutations under selection pressure from the environment. The model described the proliferation, death and mutation accumulation of tumor cells; environmental factors (for example, the level of T-cell infiltration) were described implicitly through parameters that quantified the strength of selection against tumor cells.

We made use of a rejection kinetic Monte Carlo algorithm\(^1\) to permit efficient simulation of large populations of cells. Tumor evolution was initiated by a single transformed cell that produced two surviving offspring at birth rate \(b\) per unit time. Cells in clone \(i\) died at rate \(d_i\) per unit time, where the death rate increased with the neoantigen burden of the clone. Each time a cell divided, it acquired new unique mutations at an overall rate \(\mu\) (Poisson distribution), which were assigned as neoantigens at rate \(p\), or as passengers (evolutionarily neutral) at rate \(1 - p\). Each antigenic mutation was assigned an antigenicity value (denoted \(A_i\) for the \(i\)th antigen in a given cell) sampled from an exponential distribution with the rate parameter set to 5 to produce a skewed distribution wherein \(>99\%\) of antigenicity values fall between 0 and 1 and most neoantigens are only negligibly immunogenic (Fig. 1a). Neoantigens caused the death rate \(d_i\) of the lineage to increase from a basal rate of \(d = 0.1\) to a higher value determined by the strength of negative selection against each newly acquired neoantigen, controlled by the parameter \(s\). The overall effect on the birth/death rate of cells was determined by the cumulative antigenicity of neoantigens harbored in the lineage, \(\sum A_i\). The death rate of a subclone was computed as:

\[
d_i = \left(1 + s \sum A_i\right)\left(d - 0.1\right) + 1
\]

(1) We defined the selective (dis)advantage of a subclone by its effective proliferation rate (the difference of its birth and death rate) compared to a nonimmunogenic clone:

\[
1 + s \sum A_i = \text{fitness} = \frac{b - d_i}{b - d}
\]

(2) where \(A_i\) denotes the \(i\)th neoantigen in lineage \(i\), \(s = 0\) stands for neutral evolution with no neoantigen-associated selection and negative selection is represented by \(s < 0\).

This antigenicity-dependent increase in the clone death rate represented an aggregate of the many stochastic factors that lead to the negative selection of neoantigens, including: (1) sufficient presentation of neoantigens on the cell surface; (2) recognition of neoantigens by T cells; (3) antigen-mediated recruitment of further T cells; and (4) T-cell killing efficiency. We chose to integrate all variability into a single probabilistic rate to be able to observe general qualities of the tumor-immune interaction without the need for precise parameterization. For details on the steps of in silico simulations, see the Supplementary Note and the code at https://zenodo.org/record/3601322#.XvKCGJXKiU.

We also modeled the acquisition of immune escape during tumor growth. Known immune escape mechanisms include mutations affecting the antigen-presenting machinery and expression of immune checkpoint molecules\(^{4,14}\). Immune escape was modeled as a heritable property of a cell (representing, for example, copy number alteration of the PD-L1 or HLA gene). Immune escape occurred as a result of a mutation with probability \(p\) per non synonymous mutation or through manual introduction of the escape alteration at a predetermined clone size to achieve clonal or subclonal immune escape. We considered two different types of escape mechanism: (1) active escape, which shields the clone from negative selection (decreasing the clone death probability to \(d_i\)) but does not decrease the neoantigen burden of the cell (corresponding to escape mechanisms such as PD-L1 overexpression); and (2) passive escape, which renders a portion of neoantigenic mutations neutral (by rendering their antigenicity \(A_i\) to 0; representing, for example, loss of an HLA allele that predicts a subset of neoantigenic peptides being presented).

We also incorporated therapeutic intervention in our model by time-dependently changing model parameters. The most commonly used agents in immunotherapy target and inhibit immune checkpoint pathways, helping the immune system to overcome the immune escape achieved by checkpoint overexpression and reactivate immune predation of neocarcinogen cancer cells. We simulated this effect by rendering active type immune escape ineffective (the death rate of escaped cells is increased by antigenic load) and simultaneously increasing the negative selection strength experienced by each neoantigen.

We chose model parameters to represent a wide range of possible tumor-immune environments and correspond to phenotypic properties of real cancers (Extended Data Fig. 7). The following parameters were used in all simulations: \(b = 1\); \(d_i = 0.1\); \(s = 1\) (not hypermutated) and \(s = 10\) (hypermutated); \(-2 \leq s \leq 0\) (as indicated in the figures or in the caption); \(p = 0.0075\) and \(p = 10^{-6}\) (probability of immune escape, where applicable). For the analyses where cells and mutations were classified as antigenic or not, the cell and mutation antigenicity thresholds \(T_c = 0.5\) and \(T_m = 0.2\) were used, unless stated otherwise. For further discussion on the simplifications applied in the model and the choices of simulation parameters and how they influence results, see the Supplementary Note and Extended Data Figs. 6–8.

Simulation of VAF/CCF distributions and power calculation. To evaluate the mutation spectrum of simulated tumors, mutations harbored in at least 10 cells out of 10\(^5\) (0.01%) were collected at the end of each simulation and the number of carrier cells reported. Real sequencing data naturally introduce uncertainty about mutated allele frequency due to limited sequencing depth and several sources of sampling bias\(^{23}\). To account for imperfect measurements, CCF values were either computed by taking the raw frequency values or via a simulated sequencing step introducing noise to these frequencies with the indicated read depth. For a given read depth \(D\), each frequency value was substituted by a new frequency sampled from a binomial distribution with parameters \(D\) and \(f = \text{Binom}(D, f) / D\). We filtered for mutations with \(f\) above 0 to discard mutations that are not picked up due to limited detection power.

In addition to sequencing limitations, neoantigen identification from DNA sequencing alone has a high rate of false positive calls\(^{26}\); therefore, the VAF distribution of neoantigens is expected to be ‘contaminated’ with a large proportion of neutrally evolving passenger mutations. To simulate this effect when evaluating the power of detecting selection, we randomly sampled nonantigenic mutations of simulated tumors (varied between 5 and 500% of the number of true neoantigens; Fig. 4c) that were falsely labeled as neoantigens and included in the neoantigen-based VAF distribution.

We computed the power to detect selection by comparing the distribution of all detected mutations to that of the neoantigens labeled subset using a two-sample Kolmogorov–Smirnov test and identified any samples as under selection where the \(P\) value of the test was below 0.1 (Fig. 4c) or a predefined value (Fig. 4d).

TCGA sample acquisition and processing. All samples from the TCGA colon adenocarcinoma and rectum adenocarcinoma (merged together as CRC), SWI and UCEC domains were retrieved through the National Cancer Institute Genomics Data Commons portal\(^{12}\) between 15 June 2018 and 13 November 2019. Only patients with matched germline (from blood samples) and primary tumor information available were considered. For each sample, purity (fraction of tumor cells in the sample) and overall ploidy were evaluated using ASCAT (version 2.5)\(^21\) on Affymetrix SNP array data. Samples with purity below 0.4 and ploidy above 3.6 were excluded from the analysis, leaving 363 CRC, 146 STAD and 370 UCEC samples for which HLA typing and neoantigen calls were performed (Supplementary Table 1 and Fig. 2a).

To analyze immune escape, the cohort was narrowed down to patients for whom gene expression data was available in the Genomics Data Commons; at least 1 pair of their HLA-A/B/C alleles were heterozygous and distinct enough to allow for LOH calls (n(CRC) = 341, n(STAD) = 118, n(UCEC) = 362).

For each patient considered, the following information was downloaded: blood-derived normal BAM files; primary tumor BAM files; unfiltered variant call (VCF) files processed with Mutect2 (GATK build 2016-02-25); SNP array files; gene expression HIFseq counts (where available); clinical tumor information available were considered MMR; those with MANTIS<0.5 and MSI-L/MSS were labeled MSS. If the two sources of information contradicted each other, neither of the categories was assigned. Samples with at least 1,000 mutations inferred to originate from the characteristic POLE signature (signature 10 in Alexandrov et al.\(^{18}\)) were labeled as POLE tumors regardless of their MMR status.

Multiregion-sequenced dataset processing. The multiregion-sequenced colorectal dataset was accessed from Cross et al.\(^{38}\) (raw data available from the European Genome-phenome Archive (https://ega-archive.org) under accession no. EGAS00001003068). BAM files with marked duplicates were used for HLA calling and HLA variant detection. As in the original work, variants were called using Platypus (version 0.3.2)\(^6\), annotated by ANNOVAR (version 20180416)\(^3\) and filtered to only contain somatic single-nucleotide variations that were present in at least 1 tumor sample and in either 0 reads in the normal sample (for normal coverage ≤30 reads) or in 1 read at most (for normal coverage above 30 reads).

HLA haplotyping and calling immune escape. HLA-A, HLA-B and HLA-C haplotyping was performed on blood-derived normal BAM files using POLYSOLVER (downloaded on 6 January 2018)\(^14\). Since POLYSOLVER uses the individual’s ethnicity to compute the likelihood of each allele haplotype, we supplied ethnicity data, where available from the clinical TCGA information, and ran haplotyping with race ‘unknown’ otherwise.
Using exome and RNA-seq data, we tested for the presence of three types of immune escape mechanisms: (1) somatic mutations in either one of the HLA alleles or in the B2M gene; (2) loss of an HLA haplotype through LOH in the corresponding genomic locus; and (3) PD-L1 or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) overexpression.

Mutations in HLA alleles were called using the previously called HLA haplotypes and the corresponding functionality of POLYSOLVER. Variant calling was run using default settings and HLA was considered mutated if at least one allele had a nonsynonymous somatic mutation located in an exon or at a splice site. Mutations in B2M were called if the sample contained a nonsynonymous somatic mutation located inside one of the exons of the B2M gene, as annotated by ANNOVAR and confirmed using Variant Effect Predictor (release 87). LOH at the HLA locus was assessed using the software LOHHLA (downloaded on 14 June 2018), using blood-derived normal and tumor BAM files. Tumor purity was assessed by pooling mutations detected from ASCAT (for the TCGA data) and from Sequenza (version 3.0.0) (for the multiregion–sequenced colorectal tumors). A sample was considered to have allelic imbalance at an HLA locus if the corresponding P value was below 0.01 and LOH if, in addition, the copy number prediction of that allele was below 0.5, with the confidence interval strictly below 0.7. Immune checkpoint overexpression was assessed using RNA-seq data. Normal expression values (in transcripts per million mapped reads (TPM) of PD-L1 and CTLA-4 were established for each cohort from the TCGA based on RNA-seq counts of the two proteins in solid tissue normal samples. Checkpoint overexpression was called if either PD-L1 or CTLA-4 expression in the tumor was higher than two s.d. above the mean of normal expression. Immune checkpoint overexpression could not be inferred for the multiregion–sequenced dataset since only genomic data were available.

We note that the extent of the impact of these escape alterations is not always known—especially for mutations altering antigen-presenting proteins—but we argue that nonetheless they represent a level of impairment in the tumor-immune interaction.

Immune infiltration levels were computed from RNA-seq data based on the method of Grasso et al.13: a signature of 12 genes (CCL2, CCL3, CCL4, CXCL9, CXCL10, CDRA, HLA-DOB, HLA-DMA, GZMK, ICOS and IRF1) was extracted and a continuous T-cell score derived as their log(TPM) average. The continuous score was then divided into three equally sized intervals (based on all cancers) to provide low, medium and high T-cell score levels.

Neoantigen prediction. Neoantigens were predicted from variant call tables and HLA types using the NeoPredPipe®, a neoantigen prediction and evaluation pipeline, designed for parallel analysis of single-region and multiregion samples. We only evaluated single-nucleotide variants leading to a single amino acid change; novel peptides of nine and ten amino acids were considered. The pipeline was run with default analysis settings and preserving intermediate files (option –p), using hg38 and hg19 ANNOVAR® reference files for annotation of the TCGA and multiregion CRC samples, respectively. The analysis outputted a table of novel peptides binding the patient’s MHC complex I molecules (considering all six alleles independently) and their respective recognition potential calculated from their MHC-binding affinity and similarity to pathogenic peptides, as described in Lulka et al.14. To evaluate the recognizability (R) part of the recognition potential, we used the parameter values provided by Lulka et al.14. Unless stated otherwise, we labeled a peptide as a neoantigen if its recognition potential was ≥10−5 (with regard to any of the patient’s HLA types) to focus on antigens with the highest predicted probability of eliciting an immune response: both similar to known pathogens and similar or stronger MHC binders than their wild-type counterpart. A mutation was considered (neo)antigenic if there was at least a single peptide produced from the mutated base that got labeled as a neoantigen.

To evaluate the antigenicity distribution of tumors, we used the predicted percentile rank of neoantigens that ranks a putative antigen against a large set of random substrates to the same HLA molecule and thus eliminates bias introduced by the structural properties of HLA alleles15, which might be present in plain binding affinity values considered in the recognition potential. We inverted this value to obtain a normalized binding score that correlates with the importance ranking of peptides, where values above approximately 1.3 represented strong putative antigens.

Computation of VAF and CCF values. For each mutation, we calculated the VAF as the number of mutant reads spanning the position, divided by the number of total reads of the position. The proportion of cancer cells carrying a particular mutation (CCF) was calculated from the VAF of the mutation, sample purity (tumor content) and copy number (CN) of the mutation’s genomic locus as: (VAF × purity) / CN; CCF values above 1 (arising from sequencing noise and copy-number LOH events) were assumed to be 1. We only considered a mutation as subclonal if it had CCF < 0.6, to account for the possibility of ‘bleeding’ of clonal mutations into the subclonal frequency range because of the limited sequence depth of the TCGA samples.

To pool together the VAF distributions of a cohort of samples (Fig. 4f), we first filtered the set of TCGA cancers: cancers with any evidence of immune escape (including allelic imbalance of the HLA locus); MMR or POLE cancers and cancers with purity <50% were discarded. The remaining cancers were divided into low and medium immune infiltration groups. (All highly T-cell score cancers were immune-escaped and previously discarded.) Total and neoantigen-associated cumulative VAF distributions were computed from all mutations detected at subclonal frequencies in the two groups. In a similar manner, TCGA MSS cancers with purity >70% (to ensure more accurate VAF and ploidy calls) were combined into a cohort to study mutations in essential genes (Extended Data Fig. 5f). Essential genes, and antigenic mutations located in essential genes were identified using the list of shared genes in Blomen et al.18.

Synthetic cohorts. To evaluate the antigen-producing capacity of different mutational processes, we generated synthetic tumor cohorts matching the mutation number and trimutation composition of real cancers. We measured the average combination (as measured by 6-channel composition16) of the real cohort (for example, TCGA CRCs: Extended Data Fig. 9d) and randomly sampled a matching number of exonic mutations at a probability specified by the respective channel intensities. Six HLA haplotypes were also randomly sampled from the complete list of alleles in the real cohort. Sampling was repeated independently 100 times to generate a synthetic cohort.

Statistical analysis. Details of the statistical analysis performed are summarized in the Life Science Reporting Summary. All data processing and statistical tests were performed in R v.3.5.0 using built-in functions. The tests and functions used were as follows: Figs. 1c, d and 2b–d: two-sided Student’s t-test/mann–whitney U-test/Wilcoxon rank-sum test (wilcox.test, default settings); Fig. 2f and Extended Data Fig. 3a,b,d: chi-squared test (chisq.test); Fig. 2g and Extended Data Fig. 3e: one-sided Mann–Whitney U-test (wilcox.test with option alternative=’greater’); Fig. 3.c,d: one-sided, paired Wilcoxon signed-rank test (wilcox.test with options paired=TRUE and alternative=’greater’); Fig. 4c,d and Extended Data Fig. 5b,c: Kolmogorov–Smirnov test (ks.test) between the raw VAF distribution of neoantigens and all mutations. The two distributions were deemed to have a significance level of P < 0.1 or as indicated in Fig. 4c,d and Extended Data Fig. 5b,c; Fig. 5c and Extended Data Fig. 9d,e: paired Wilcoxon signed-rank test (wilcox.test, option paired=TRUE); Extended Data Fig. 9g: Student’s t-test against a mean of 1 (t.test, mu=1).

All violin plots were generated with an automatic smoothing bandwidth value of geom_violin. Individual observations for the TCGA samples are shown above the violin plots generated with geom_dotted.

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

Data availability
The datasets analyzed during the current study are available from the National Cancer Institute Genomics Data Commons Data Portal (https://portal.gdc.cancer.gov) colon adenocarcinoma, rectum adenocarcinoma, STAD and UCEC domains and from the European Genome-phenome Archive (https://ega-archive.org/) under accession no. EGAS00001030066.

Code availability
The Julia v0.5+ (https://julialang.org/) code implementing simulations of the tumor growth model is available from Zenodo (https://doi.org/10.5281/zenodo.3601322).

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**Author contributions**

E.L., A.R.A.A., A.S. and T.A.G. conceptualized the study. A.R.A.A., A.S. and T.A.G. acquired the funding for the study. E.L., A.S. and T.A.G. led the investigation, analyzed the data and wrote the original manuscript. E.L., M.J.W., W.C.H.C., B.W., R.O.S., C.G., J.H., L.Z., M.R-T. and C.P.B. contributed to the mathematical model, computational framework and bioinformatics analysis. All authors reviewed and approved the final manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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Extended Data Fig. 1 | Population-level statistics under varying levels of selection. **a**, Distribution of neoantigen scores of tumour cell populations when reaching 100,000 cells, for increasing selection strengths (left column, top to bottom) and cell-antigenicity threshold (Tc) used for analysis (right column). n=100 tumours were simulated for each parameter combination. **b**, The distribution of antigenicity values of all neoantigens present in at least 10 cells in the final tumour population simulated with exponential, uniform and normal prior neoantigen distributions. The thick line shows the mean density of 50 simulated distributions at no, moderate and high selection pressure (s=0 (yellow), s=-0.8 (teal), s=-1.6 (blue), respectively), the shaded regions represent ±1 standard deviation around this mean. Priors are shown with a grey dashed line.
Extended Data Fig. 2 | Dynamics of the growing population compared to a constant size population model. Tumours simulated using our tumour growth model (growing population, (a)–(c)) and a death-birth Moran process (constant size population, (d)–(f)) at different selection strength and mutation rate regimes, as indicated in each row and detailed in the main text. Simulations were run for a final population of 10,000 cells in the growing population model and for 50,000 steps in the constant size model. (a) & (d) The mean antigenicity of the tumour for 6 individual simulations. Tumours that reached detectable size are shown in blue, eradicated tumours (cell count reaches 0) are in red, the constant size tumours are invariably in grey. (b) & (e) Distribution of CCF values of the most common neoantigen computed from 20 tumours. For the growing population, only tumours that did not go extinct are shown, and consequently no graph is included for the last two rows. (c) & (f) Cumulative VAF distribution of all mutations (grey) and neoantigens (red). The thick line shows the mean of 20 simulated cumulative distributions, the shaded regions represent ±1 standard deviation around this mean. Note that in the first row, there are no neoantigens in the studied frequency range (no red line), while in the last row the grey and red curves overlap.
Extended Data Fig. 3 | Immune escape and antigenicity in TCGA CRC, STAD and UCEC samples. 

**a.** Prevalence of the individual immune escape mechanisms considered in the combined cohort of CRC, STAD and UCEC samples. P-values shown on top of each bar indicate the result of chi-squared test for that mechanism, corrected for multiple comparisons using the Holm-Bonferroni method. An additional test comparing the presence/absence of any immune checkpoint escape is also indicated above the checkpoint columns. 

**b–e.** Antigen landscape and immune escape characteristics of a combined cohort from TCGA. Figures correspond to Fig. 2d–g. Two-sided chi-squared test is indicated on top of (b) and (d). Mann-Whitney tests (c: two-sided, e: one-sided) are reported above (c) and (e). No adjustment for multiple comparisons was made. Violin widths in (c) & (e) represent raw data density with binned individual data-points overlaid on top.
Extended Data Fig. 4 | VAF distribution of neoantigens under different selection strengths and mutation-antigenicity thresholds. Cumulative VAF distribution as a function of the inverse of the frequency, for all mutations (grey) and antigenic mutations (red) at increasing selection strengths (top to bottom) and mutation-antigenicity threshold (Tm) applied to label mutations as antigenic (left to right). The thick line shows the mean of 100 simulated cumulative distributions, the shaded regions represent ±1 standard deviation around this mean.
Extended Data Fig. 5 | Detection of negative selection in variant allele frequency distribution. a, Number of detected (true) neoantigens in n=100 simulated tumours for each selection strength between s=0 and s=-3. The mean number detected at each selection value is shown in red. b-d, Power (detection rate) to identify negative selection using two-sided Kolmogorov-Smirnov test (b, c) and number of detected neoantigens (d) as a function of read depth, false positive neoantigens amongst antigenic mutations and selection strength, when only mutations above the mutation-antigenicity threshold (T_m) of 0.35 are analysed as antigenic, instead of 0.2 (c.f. Fig. 4c, d, Extended Data Figs. 4 & 5a). n=100 simulated tumours are used in the computation. e, Cumulative VAF distribution of mutations detected in low and medium immune infiltrated CRC (upper panel) and UCEC (lower panel) MSS cancers without immune escape. VAF distributions of STAD sample could not be established due to low sample and mutation numbers. f, Cumulative VAF distributions of mutations detected in essential genes in all TCGA MSS cancers with good tumour cellularity (above 70%). The curves show synonymous (purple), frameshift and nonsense (green), missense (red) and hemizygous (located in haploid regions of the genome, yellow) mutations found in essential gene exons.
Extended Data Fig. 6 | Immune properties of tumours at different basal death rates. **a**, The number of detectable neoantigen-associated mutations (at simulated sequencing depth of ~50x) in n=50 simulated tumours with increasing base (non-immunogenic) death rate. The bottom panel shows the ratio of tumours with different levels of immune escape. Violin widths represent raw data density. **b**, Cumulative VAF distribution as a function of the inverse of the frequency, for all mutations (grey) and antigenic mutations (red) at increasing base death rate, \( d_b \). The thick line shows the mean of \( n=100 \) simulated cumulative distributions, the shaded regions represent ±1 standard deviation around this mean. At very high base death (last panel), the VAF distribution of neoantigens and neutral mutations overlaps as tumours are exclusively immune-escaped and evolve neutrally.
Extended Data Fig. 7 | Values of $\mu$ and $p_a$ based on TCGA CRCs. a, b, The number of subclonal (CCF < 0.6) missense mutations in MSS (a) and MMR (b) CRC samples; shown together with the subclonal mutation count of ‘normal’ (a) and hyper-mutated (b) simulated tumours sequenced at a depth of 30-60x (sequencing depth sampled randomly in the range). Violin widths represent raw data density with individual data-points (of exact y values) scattered on top. c, The distribution of the proportion of antigenic mutations in a randomised TCGA MSS colon dataset, where patient mutation load and HLA types were extracted from the data and the proportion of antigenic mutations calculated by sampling randomly from missense mutations found in TCGA CRCs. The thick solid black line shows $p_a=0.075$, the values used for simulations presented in main figures. Dashed red lines show $p_a=0.025$ and $p_a=0.15$ used in Extended Data Fig. S8.
Extended Data Fig. 8 | VAF distribution of neoantigens under different selection strengths and antigen-generation probabilities. Cumulative VAF distribution as a function of the inverse of the frequency, for all mutations (grey) and antigenic mutations (red) at increasing selection strengths (top to bottom) and antigen-generation probability, $p_a$ (left to right). The thick line shows the mean of $n=100$ independently simulated cumulative distributions, the shaded regions represent ±1 standard deviation around this mean.
Extended Data Fig. 9 | Proportional burden of TCGA tumours. a, Inter-tumour distribution of the antigenic proportion of missense mutations across CRC, STAD and UCEC cancers with >30 missense mutations. b, Inter-tumour distribution of proportional burden in MSS and MMR cancers of the meta-cohort combining CRC, STAD and UCEC cancers with >30 missense mutations. c, Inter-tumour distribution of proportional burden in escaped and non-escaped cancers of the meta-cohort. Two-sided Wilcoxon test p-values are reported on (a–c). d, Proportional burden computed for all (red) and subclonal (salmon) mutations in immune-escaped and non-escaped samples of each TCGA cohort. Lines connect values computed from the same cancer. e, Inter-tumour distribution of proportional burden in real CRC samples stratified by MSS/MMR status (left) and synthetic samples matching the mutational composition of real samples (right), with two-sided Wilcoxon test reported on top. f, Total and subclonal proportional antigen burden computed on a matched synthetic cohort of n=100 tumours (cf. Fig. 5c). The p-value of paired two-sided Wilcoxon test is reported on (d) & (f). g, Normalised proportional subclonal burden computed by dividing subclonal burden of the meta-cohort by average subclonal burden of the synthetic cohort of n=100. P-value of a one-sample two-sided t-test against null-hypothesis of mean=1 is reported above each violin. Violin widths represent raw data density with binned individual data-points overlaid on top in (a–d) & (g) and indicated by the end-points of connecting lines in (e, f). Visual elements of boxplots in (d) correspond to the following summary statistics: centre line, median; box limits, upper and lower quartiles; whiskers, 1.5x inter-quartile range; additional points, outliers outside of 1.5x inter-quartile range.
Reporting Summary

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Statistics

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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- [x] The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement

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- [x] For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  - *Give P values as exact values whenever suitable.*

- [ ] For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

- [ ] For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

- [x] Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

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**

Data from GDC portal was collected using the GDC Data Transfer Tool (https://gdc.cancer.gov/access-data/gdc-data-transfer-tool). Simulated tumour growth data was generated using custom code written in Julia (https://github.com/elakatos/CloneGrowthSimulation, https://julialang.org/, version 0.5.2).

**Data analysis**

The following open-source software was used in the bioinformatic analysis: ASCAT (https://github.com/Crick-CancerGenomics/ascat); Sequenza (https://cran.r-project.org/web/packages/sequenza/vignettes/sequenza.html, version 3.0.0); polysolver (https://software.broadinstitute.org/cancer/cga/polysolver); LOHHLA (https://bitbucket.org/mcgranahanlab/lohhla); ANNOVAR (http://annovar.openbioinformatics.org/en/latest/, version 20180416); Variant Effect Predictor (https://www.ensembl.org/info/docs/tools/vep, release 87); NeoPredPipe (https://github.com/MathOnco/NeoPredPipe). Calls derived from MANTIS (https://github.com/OSU-SRLab/MANTIS, not performed by the authors) were also used. All statistical analysis was carried out in R (https://www.r-project.org/about.html, version 3.5.0).

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/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**

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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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The datasets analysed during the current study are available from the NCI Genomics Data Commons Portal (https://portal.gdc.cancer.gov) COAD, READ, STAD and UCEC domains, and from the European Genome-Phenome Archive (https://ega-archive.org/) at accession code: EGAS00001003066.
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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 calculation was performed. We analysed all the suitable data (as according to exclusion criteria below) available to us, and comment on the statistical power of our analysis to resolve patterns in these data throughout the manuscript. In addition, the manuscript contains simulation-based power analysis commenting on the required data resolution. |
| Data exclusions | TCGA data was filtered according to pre-established criteria to ensure the quality of variant calls and VAF estimation: Only samples with available matched germline information (from blood samples) were considered. Samples with purity below 0.4 and ploidy above 3.6 were excluded before analysis. For immune escape analysis, only samples with gene expression data available in GDC, and at least one heterozygous HLA allele were considered. For comparison of escaped and non-escaped samples, all samples with less than 30 (subclonal) exonic mutations were excluded to avoid noise due to alternative biological pathways and small sample set of mutations. |
| Replication | No primary experiments were carried out in this study. No biological or technical replicates were considered: only a single sample was analysed for each TCGA cancer. |
| Randomization | Randomization was not relevant for comparison of different sample subtypes (immune escape vs no escape; MSI vs MSS) in the bioinformatic analysis. All samples meeting the preset criteria were considered. |
| Blinding | Blinding was not relevant due to only bioinformatic analysis carried out in the study. |

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| - □ Antibodies | - □ Flow cytometry |
| - □ Eukaryotic cell lines | - □ MRI-based neuroimaging |
| - □ Palaeontology | |
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