Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade

Nicholas McGranahan,1,2,3* Andrew J. S. Furness,3,4* Rachel Rosenthal,2,8 Sofie Ramskov,2 Rikke Lyngaa,4 Sunil Kumar Saini,5 Mariam Jamal-Hanjani,3 Gareth A. Wilson,1,8 Nicolai J. Birkbak,1,8 Crispin T. Hiley,1,3 Thomas B. K. Watkins,1,9 Seema Shafi,2 Nirupa Murugaesu,5 Richard Mitter,5 Ayse U. Akarca,4,6 Joseph Linares,4,6 Teresa Marafioti,6,8 Jake Y. Henry,5,8,9 Eliezer M. Van Allen,7,8,9 Diana Miao,7,8 Bastian Schilling,10,11 Dirk Schadendorf,10,11 Levi A. Garraway,7,8,9 Vladimir Makarov,12 Naiyer A. Rizvi,13 Alexandra Snyder,14,15 Matthew D. Hellmann,14,15 Taha Merghoub,14,16 Jedd D. Wolchok,14,15,16 Sachet A. Shukla,7,8 Catherine J. Wu,7,8,17,18 Karl S. Peggs,5,4 Timothy A. Chan,13 Sine R. Hadrup,5 Sergio A. Quezada,5,4† Charles Swanton1,3†

1The Francis Crick Institute, London WC2A 3LY, UK. 2Center for Mathematics and Physics in the Life Sciences and Experimental Biology (CoMPLEX), UCL, London WC1E 6BT, UK. 3Cancer Research UK Lung Cancer Center of Excellence, UCL Cancer Institute, London WC1E 6BT, UK. 4Cancer Immunology Unit, University College London Cancer Institute, University College London WC1E 6BT, UK. 5Section for Immunology and Vaccinology, National Veterinary Institute, Technical University of Denmark, Copenhagen, Denmark. 6Department of Cellular Pathology, University College London, London, UK. 7Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA 02215, USA. 8Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA. 9Center for Cancer Precision Medicine, Dana-Farber Cancer Institute, Boston, MA 02215, USA. 10Department of Dermatology, University Hospital, University Duisburg—Essen, 45147 Essen, Germany. 11German Cancer Consortium (DKTK), 69121 Heidelberg, Germany. 12Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA. 13Hematology/Oncology Division, 177 Fort Washington Avenue, Columbia University, New York University, New York 10032, USA. 14Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA. 15Weill Cornell Medical College, New York, NY, 10065, USA. 16Ludwig Collaborative Laboratory, Memorial Sloan Kettering Cancer Center, New York, NY, 10065, USA. 17Department of Medicine, Harvard Medical School, Boston, MA 02115, USA. 18Department of Internal Medicine, Brigham and Woman’s Hospital, Boston, MA, USA.

*These authors contributed equally to this work.
†Corresponding authors. E-mail: s.quezada@ucl.ac.uk (S.A.Q.); charles.swanton@crick.ac.uk (C.S.)

As tumors grow they acquire mutations, some of which create neoantigens that influence the response of patients to immune checkpoint inhibitors. We explored the impact of neoantigen intra-tumor heterogeneity (ITH) on anti-tumor immunity. Through integrated analysis of ITH and neoantigen burden, we demonstrate a relationship between clonal neoantigen burden and overall survival in primary lung adenocarcinomas (n = 139). CD8+ tumor-infiltrating lymphocytes reactive to clonal neoantigens were identified in early-stage non-small cell lung cancer (NSCLC) and expressed high levels of PD-1. Sensitivity to PD-1 and CTLA-4 blockade in patients with advanced NSCLC (n = 31) and melanoma (n = 135) was enhanced in tumors enriched for clonal neoantigens. T cells recognizing clonal neoantigens were detectable in patients with durable clinical benefit. Cytotoxic chemotherapy-induced subclonal neoantigens, contributing to an increased mutational load, were enriched in certain poor responders. These data suggest that neoantigen heterogeneity may influence immune surveillance and support therapeutic developments targeting clonal neoantigens.

Recent studies have highlighted the relevance of tumor neoantigens in the recognition of cancer cells by the immune system (1–4), prompting a renewed interest in personalized vaccines and cell therapies targeting cancer mutations (5, 6). However, while genomic data are revealing the extent of genetic heterogeneity within single tumors (7), the influence of ITH upon the neoantigen landscape and sensitivity to immune modulation is unclear.

To explore neoantigen heterogeneity and its influence on anti-tumor immunity in early-stage NSCLC, we applied a bioinformatics pipeline to seven primary NSCLCs subjected to multi-region sequence analysis (table SI) (8, 9). In total, 2860 putative neoantigens were predicted across the cohort, with a median of 326 neoantigens predicted per tumor (range 80-741) (Fig. 1A). Neoantigen heterogeneity varied considerably, with an average 44% of neoantigens found heterogeneously, in a subset of tumor regions (range 8-78%).

To address the clinical relevance of neoantigen burden and, specifically, the importance of clonal (present in all tumor cells) versus subclonal (present only in a subset) neoantigens, we subjected a predominantly early-stage cohort of 106 stage I/II, 43 stage III/IV and 1 unknown stage lung adenocarcinoma (LUAD) and 92 stage I/II and 32 stage III/IV lung squamous cell carcinoma (LUSC) cases from TCGA to neoantigen and clonality analysis (10–12) (Fig. 1B). In this setting, to determine clonality from sequencing of a single sample, the cancer cell fraction, describing the proportion of cancer cells harboring a mutation, was deter-
mined for each neoantigen (I3).

A high neoantigen burden, defined as the upper quartile of neoantigen load, was associated with significantly longer overall survival in LUAD (P = 0.025) (Fig. 1, C and D, and fig. S1A) and a trend for homogeneous tumors (neoantigen ITH≤1%) to have longer overall survival times compared to heterogeneous tumors was also observed (P = 0.061) (fig. S1B). Although tumors with a high burden of neoantigens were found to be significantly more homogeneous than those with a low burden of neoantigens (P < 0.0001, Wilcoxon rank-sum test) (fig. S1C), a combination of neoantigen ITH and neoantigen burden (as outlined in the schematic in Fig. 1C) was more significant than simply considering either metric alone and was observed across multiple different neoantigen ITH thresholds (without ITH threshold, P = 0.025; ITH threshold = 0, P = 0.019; ITH threshold = 0.01, P = 0.0096; ITH threshold = 0.05, P = 0.021) (Fig. 1, C and D), remaining significant in multivariate analysis when including tumor stage (table S2).

Despite a comparable range of predicted neoantigens in LUSC, no statistically significant association between overall survival and neoantigen load was observed in this subtype, even when incorporating neoantigen ITH (fig. S2, A to D). To investigate the reason for this disparity, we explored whether any immune-regulatory genes were differentially expressed between these two cancer types. HLA class I genes, including HLA-A, HLA-B, HLA-C, HLA-E, HLA-F and HLA-G, as well as beta-2 microglobulin (B2M), a component of the MHC class I molecule, were expressed at a significantly lower level in LUSC compared to LUAD (fig. S3A and table S3A), and this difference was observed across all levels of neoantigen burden (fig. S3B and table S3B). HLA class I genes were also down-regulated compared to matched normal samples in LUSC (table S3C). These data suggest the presence of a high number of clonal neoantigens in homogeneous LUAD may favor effective immune-surveillance, whereas, in LUSC immune-escape may be more prevalent through HLA down-regulation.

We next evaluated whether immune-related genes were differentially expressed between homogeneous LUAD tumors (≤1% neoantigen ITH) with a high clonal neoantigen burden (≥ upper quartile clonal neoantigens) compared to heterogeneous (>1% neoantigen ITH) or low clonal neoantigen burden tumors (<upper quartile clonal neoantigens). Eight genes were found to be differentially expressed between these two groups (table S4A). Programmed cell death ligand-1 (PD-L1) and the pro-inflammatory cytokine IL-6 were the most significantly differentially expressed genes, up-regulated in the homogeneous and high clonal neoantigen group. When we specifically compared tumors in the upper quartile of clonal neoantigen burden with tumors in the lower quartile, we identified an additional 25 differentially expressed genes (table S4B and fig. S4A). CD8A, CD8B and genes associated with antigen presentation (TAP-1, TAP-2, STAT-1), T cell migration (CXCL-10, CXCL-9) and effector T cell function (IFN-γ, granymes B, H and A) were up-regulated in the high clonal neoantigen cohort and found to cluster together, indicating co-expression (fig. S4B). PD-1 and lymphocyte activation gene 3 (LAG-3), negative regulators of T cell function (I4), were also identified in this cluster as were the ligands PD-L1 and PD-L2.

These data suggest that a high clonal neoantigen burden in LUAD is associated with an inflamed tumor microenvironment enriched with activated effector T cells, potentially regulated by inhibitory immune checkpoint molecules and their ligands. We therefore attempted to identify and characterize T cells reactive to neoantigens in patients with early-stage NSCLC. We focused on two tumors, L011 and L012, with a comparable number of predicted neoantigens and a similar smoking history, but divergent levels of neoantigen ITH (8% vs. 74% heterogeneous predicted neoantigens) (Fig. 2, A to C). 288 and 354 putative neoantigen-loaded, HLA-matched multimers derived from L011 and L012 respectively, were used to screen CD8+ T cells expanded from individual tumor regions and adjacent normal lung tissue, using a previously described high throughput method (Fig. 2, D and E) (I5).

CD8+ T cells reactive to mutant MTFR2D326Y (FAFQEYDSF) were identified in L011, while in L012, two distinct CD8+ T cell responses to mutant CHTF18L369V (LLDIYAPK) and MYADM8R30W (SPMVGSPW) were observed (Fig. 2, D and E, and fig. S5, A and B). MTFR2D326Y, CHTF18L369V and MYADM8R30W all represent clonal neoantigens suggesting that immune-reactivity against clonal neoantigens can be detected in both homogeneous and heterogeneous NSCLC. High HLA binding affinity was predicted for MTFR2D326Y and CHTF18L369V in both wild-type and mutant forms but only the mutant peptide was found to elicit a T-cell response. Higher binding affinity to mutant versus wild-type form was predicted for MYADM8R30W however in this case reactivity toward wild-type peptide was also observed (fig. S5C). The mutation in the MYADM8R30W peptide lies in the anchor residue, primarily affecting HLA binding and not T cell recognition. While the data suggest that T cells in this patient can recognize both mutant and wild-type peptides when stabilized within a MHC-multimer system, the very low predicted affinity of the wild-type peptide to HLA would be expected to prevent adequate presentation in vivo.

MHC multimers identifying neoantigen-reactive T (NAR-T) cells were next used to characterize NAR-T cells in unexpanded samples (Fig. 3, A to D). MTFR2D326Y-reactive CD8+ T cells, identified in unexpanded L011, were analyzed by multi-color flow cytometry. Relative expression of co-inhibitory
immune checkpoint molecules and effector cytokines between tumor-infiltrating CD4\(^+\)FoxP3\(^+\) (regulatory T cell), CD4\(^+\)FoxP3\(^-\) (CD4\(^+\) helper T cell), CD8\(^+\) monomer negative and CD8\(^+\) monomer-reactive (MTFR2\(^{D326V}\)) T cell subsets was assessed. MTFR2\(^{D326V}\) -reactive CD8\(^+\) T cells expressed high levels of co-inhibitory receptors PD-1 and LAG-3 (Fig. 3C), consistent with our bioinformatics findings (fig. S4). Almost all NAR-T cells (97\%) expressed high levels of PD-1 compared to 49\% of tumor-negative tumor-infiltrating CD8\(^+\) T cells. CTLA-4 expression was largely confined to CD4\(^+\)FoxP3\(^+\) regulatory T cells, consistent with pre-clinical findings (16). PD-1\(^+\) MTFR2\(^{D326V}\) -reactive CD8\(^+\) T cells co-expressed high levels of granzyme B (GzmB) (74.8\%) (Fig. 3D). Characterization of CHTF18\(^{L269V}\) and MYADM\(^{R30W}\) -reactive CD8\(^+\) T cells mirrored findings in L011, with high expression of PD-1 observed in 97\% and 99.6\% of CHTF18\(^{L269V}\) and MYADM\(^{R30W}\) -reactive CD8\(^+\) T cells respectively (fig. S5, D and E).

Given the potential ability of clonal neoantigens to promote priming and infiltration by neoantigen reactive-T cells expressing high levels of PD-1, we explored whether response to PD-1 blockade in patients with advanced NSCLC may be influenced by neoantigen ITH. Exome sequencing data from a recent study in which 34 patients were treated with pembrolizumab, an antibody targeting PD-1, was obtained (table S5) (2) and the clonal architecture of each tumor estimated (possible for 31/34 tumors).

Neoantigen burden was related to clinical response to pembrolizumab, with a high neoantigen repertoire associated with improved outcome, as previously reported (Fig. 4A). However, consistent with the importance of clonal neoantigens, the clinical efficacy of PD-1 blockade also appeared related to the clonal architecture of each tumor (Fig. 4A), with tumors derived from patients with no durable benefit (defined as in (2)) exhibiting significantly higher neoantigen ITH than tumors from patients with a durable clinical benefit (P = 0.006, Wilcoxon rank sum test). Almost every tumor (12/13) exhibiting a low neoantigen subclonal fraction (<5\% subclonal) and high mutation burden (≥70, median clonal neoantigens of the cohort), demonstrated durable clinical benefit, following anti-PD-1 therapy. Conversely, only two out of eighteen tumors with a high subclonal neoantigen fraction (>5\%) or low clonal neoantigen burden benefited from pembrolizumab (Y2087 and SB10944). For example, despite a large neoantigen burden, ZA6505 exhibited progressive disease, relapsing after 2 months. ZA6505 was one of the most heterogeneous tumors within the cohort, with over 80\% of mutations classified as subclonal.

Tumors with both a high clonal neoantigen burden and low neoantigen ITH were associated with significantly longer progression free survival and this relationship remained robust to the choice of ITH threshold, with lower hazard ratios observed compared to using neoantigen burden alone (Fig. 4B). The majority of clonal neoantigens could be attributed to smoking induced mutations (Fig. 4A). Greater PD-L1 expression was observed in tumors harboring a large clonal neoantigen burden and low neoantigen heterogeneity compared to the remaining tumors (P = 0.0017, \(\chi^2\) test) (Fig. 4A and fig. S6).

Next, we obtained data from 64 melanoma patients treated with either ipilimumab or tremelimumab, antibodies against CTLA-4 (4). Clonal architecture analysis was possible for 57/64 tumors and significantly improved overall survival was observed in tumors exhibiting a low neoantigen ITH and a high clonal neoantigen burden. This relationship was observed using multiple different ITH thresholds similar to the NSCLC cohort (Fig. 4C; ITH threshold = 0.01, P = 0.008; ITH threshold = 0.02, P = 0.011, ITH threshold = 0.05, P = 0.083). Notably, the relationship with survival outcome was not statistically significant without an ITH threshold (P = 0.083) (Fig. 4C).

To address whether radiation or cytotoxic exposure might stimulate production of subclonal neoantigens that could contribute to total neoantigen burden but not the efficacy of checkpoint blockade, sequencing data from a more heavily pre-treated melanoma cohort, comprising 110 tumors, was obtained (17). For the subset of tumors where clonal analysis was possible (78/110 tumors, a smaller and less adequately powered cohort compared to the published analysis), total neoantigen burden was not significantly associated with efficacy of immune checkpoint inhibition [classified as in (17)], although a trend was observed (P = 0.24, Wilcoxon rank sum test) (fig. S7A). However, an enrichment for tumors exhibiting high neoantigen heterogeneity or low clonal neoantigen burden (both stratified according to the median of the cohort) reached borderline significance in patients with minimal or no benefit compared to patients exhibiting a clinical benefit (P = 0.06, Fisher’s exact test). Neoantigen burden was not found to be significantly associated with overall survival in this cohort (fig. S7B). Two of the most heterogeneous tumors (Pat58 and Pat151) with minimal or no benefit were amongst those treated with the alkylating agent DTIC prior to anti-CTLA therapy and for both >98\% of subclonal mutations were attributable to mutational Signature II, a signature associated with prior exposure to alkylating agents (18, 19). One patient with stable disease, Pat80, who was also treated with DTIC prior to anti-CTLA-4, also harbored an increase in Signature II, and progressed by 6 months [classified as no durable benefit according to (2)]. These data suggest that therapy may induce subclonal mutations that fail to drive an efficient anti-tumor response, although further data are needed to confirm this observation.

Finally, we reasoned that T cells recognizing clonal antigens should be detectable in patients deriving favorable re-
sponses to checkpoint blockade. Previous analysis of peripheral blood lymphocytes (PBLs) from CA9903, a LUAD patient with an exceptional response to pembrolizumab, identified a CD8+ T cell population in autologous PBLs recognizing a predicted neoantigen resulting from a HERC1P3278S mutation (ASNASSAAK) (2). Consistent with the relevance of clonal neoantigens, this mutation was found to be present in 100% of cancer cells within the sequenced tumor (Fig. 4D). Similarly, analysis of PBMCs from the patients with CR9309 and CR0095–melanomas that responded to anti-CTLA-4 therapy, resulting in prolonged patient survival–identified CD8+ T cell populations recognizing tumor-specific neoantigens (4). In both cases, the neoantigens linked to a T-cell response were derived from clonal mutations, predicted to be present in 100% of cancer cells (Fig. 4, E and F).

Previous studies have reported that neoantigen burden influences sensitivity to immune checkpoint blockade in NSCLC and melanoma (2, 4, 17). However, the influence of ITH on this relationship has not been investigated. Our results, while limited by access to small and diverse patient cohorts and single-site biopsy data that likely over-estimate the number of clonal mutations, suggest clonal and subclonal neoantigens do not drive equally effective anti-tumor immunity. Indeed, using the described approach, despite screening over 250 peptides against putative subclonal neoantigens, we were only able to detect T cells recognizing clonal neoantigens. Conceivably, a higher neoantigen ITH may result in lower antigen dosage compared to homogeneous tumors with high clonal neoantigen burden, thus reducing the chances of identifying T cells reactive to subclonal neoantigens. Furthermore, in cases where T cells reactive to subclonal neoantigens are generated, these will be unable to target all tumor cells, thereby limiting overall tumor control.

The observation that certain anti-CTLA-4 refractory tumors were enriched for subclonal mutations caused by alkylating agents suggests that mutations induced by therapy may enhance total neoantigen burden but might not elicit an effective anti-tumor response, possibly due to the subclonal nature of the neoantigens resulting from cytotoxic exposure. These results highlight the need to consider both the antitumor effects of alkylating agents as well as the potential risk of inducing subclonal mutations (19).

The identification of cytotoxic tumor-infiltrating T cells recognizing clonal mutations, shared by all tumor cells, might hold promise for adoptive therapy strategies to address the challenges of ITH (20). The extensive clonal mutational repertoire present in smoking-associated NSCLC (8, 21) could render this disease vulnerable to vaccination or T cell therapies targeting multiple clonal neoantigens, in combination with appropriate immune checkpoint modulation.

REFERENCES AND NOTES
13. Materials and methods are available as supplementary materials on Science Online.


ACKNOWLEDGMENTS

C.S. is a senior Cancer Research UK clinical research fellow and is funded by Cancer Research UK (TRACERx), the CRUK Lung Cancer Centre of Excellence, Stand Up 2 Cancer Laura Ziskin prize (SU2C), the Rosetrees Trust, NovoNordisk Foundation (ID 16584), the Prostate Cancer Foundation, the Breast Cancer Research Foundation, the European Research Council (THESEUS) and National Institute for Health Research University College London Hospitals Biomedical Research Centre. S.A.O is funded by a CRUK Career Development Fellowship. A.J.S.F receives support from the Sam Keen Foundation. RR, NM, NB and GW are supported by the National Institute for Health Research University College London Hospitals Biomedical Research Centre. Certain data were received under a material transfer agreement with Memorial Sloan Kettering Cancer Center. Data

First release: 3 March 2016

www.sciencemag.org (Page numbers not final at time of first release) 5
from multi-region sequenced NSCLC are available at EBI (accession number EGAS00001000809). Data from the Rizvi cohort (2) are available at dbGaP (accession number phs000980.v1.p1). Data from the Snyder cohort (4) are available at dbGaP (accession number phs001041.v1.p1). Data from the Van Allen cohort (17) are available at dbGaP (accession number phs000452.v2.p1). The results published here are in part based on data generated by TCGA pilot project established by the National Cancer Institute and National Human Genome Research Institute. The data were retrieved through dbGaP authorization (accession no. phs000178.v9.p8). Information about TCGA and the investigators and institutions that constitute the TCGA research network can be found at http://cancergenome.nih.gov/. C.S. is a paid advisor for Janssen, Boehringer Ingelheim, Ventana, Novartis, Roche, Sequenom, Natera, Grail, Apogen Biotechnologies, Epic Biosciences, and the Sarah Cannon Research Institute. D.S. is a paid advisor for Bristol Myers Squibb, Roche, Novartis, Merck, and Amgen. B.S is a paid advisor for Bristol Myers Squibb. T.A. C. is a cofounder of and holds equity in Gritstone Oncology, Inc.; is a paid advisor for Geneocea Inc., OncoSpire, and Cancer Genetics; and receives funding from Bristol Myers Squibb for research on the genomics of immune response. N.A. R. is a cofounder of and holds equity in Gritstone Oncology, Inc. E.V.A. is a paid advisor for Syapse, Roche Ventana, Takeda, and Third Rock Ventures. M.D. H is a paid advisor for Bristol Myers-Squibb, Merck, Genentech, AstraZeneca, and Neon. J.D.W is a paid advisor for Bristol-Myers-Squibb. L.A.G. is a paid scientific advisor for Novartis, Boehringer Ingelheim, Foundation Medicine, and Warp Drive Bio, LLC. C.S, N.M., R.R., S.A.Q. and K.S.P. are co-inventors on patent applications (1516047.6, 1601098.5, 1601098.5, 1601099.3) filed by Cancer Research Technology Ltd. relating to methods for identifying and targeting neo-antigens, methods of predicting prognosis of cancer patients and/or identifying cancer patients who will benefit from treatment involving determining the number of neo-antigens.

SUPPLEMENTARY MATERIALS
www.sciencemag.org/cgi/content/full/science.aaf1490/DC1
Materials and Methods
Figs. S1 to S7
Tables S1 to S5
References (22–38)

15 August 2015; accepted 11 February 2016
Published online 3 March 2016
10.1126/science.aaf1490
Fig. 1. Heterogeneity and prognostic value of neoantigen landscape in primary NSCLC. (A) Total putative neoantigen burden in multi-region sequenced NSCLC tumors. Proportion of clonal neoantigens, identified ubiquitously in every tumor region are shown in blue, while shared subclonal neoantigens, identified as shared in multiple tumor regions, but not all, are shown in yellow, and private subclonal neoantigens, identified in only one tumor region, are in red. (B) Total putative neoantigen burden in TCGA LUAD tumors. Proportion of neoantigens arising from clonal (blue) or subclonal (red) mutations is shown. (C) Schematic illustrating use of different neoantigen ITH thresholds, with bar plot showing separation into the two groups. Without an ITH threshold, samples are simply grouped according to upper quartile of total neoantigen burden. For each ITH threshold, the upper quartile of clonal neoantigens is used to separate tumors with high and low clonal neoantigen burden, and the neoantigen ITH threshold further groups samples. For example, an ITH threshold =0 involves grouping tumors with high clonal neoantigen burden and zero neoantigen heterogeneity separately from those with low clonal neoantigen burden or any neoantigen heterogeneity. (D) Overall survival curves for samples using different ITH thresholds: without an ITH threshold (log-rank, $P = 0.028$, HR = 0.46 [0.23–0.94]); ITH threshold = 0 (log-rank, $P = 0.019$, HR = 0.21 [0.051–0.88]); ITH threshold = 0.01 (log-rank, $P = 0.0096$, HR = 0.33 [0.14–0.79]); ITH threshold = 0.05 (log-rank, $P = 0.021$, HR = 0.45 [0.22–0.90]). The number of patients in each group is listed below the survival curves.
Fig. 2. Prediction and identification of neoantigen-reactive T cell in NSCLC samples. (A) Putative neoantigens predicted for all missense mutations in L011. The MTFR2<sup>D326Y</sup> neoantigen (FAFQEYDSF) is highlighted. (B) Putative neoantigens predicted for all missense mutations in L012. The CHTF18<sup>L769V</sup> neoantigen (LLLDIVAPK) and MYADM<sup>R30W</sup> neoantigen (SPMIVGSPW) are indicated. (C) Evolutionary trees for L011 and L012 based on predicted neoantigens. (D and E) MHC-multimer screening of expanded, region-specific, tumor-infiltrating CD8<sup>+</sup> T lymphocytes and healthy donor (HD) CD8<sup>+</sup> PBMC controls with candidate neoantigens (L011, n = 288 and L012, n = 354) and control HLA-matched viral peptides (L011, n = 10 and L012, n = 9). Frequency of CD8<sup>+</sup> MHC-multimer positive cells out of total CD3<sup>+</CD8</sup> TILs is displayed for (D) and (E), with size of symbol increasing with frequency.
Fig. 3. Identification and characterization of tumor-infiltrating neoantigen-reactive CD8+ T cells in early-stage NSCLC. (A) MHC-multimer analysis of non-expanded, tumor-infiltrating CD8+ T lymphocytes isolated from tumor regions 1-3 and normal lung tissue of patient L011 identifies CD8+ TILs reactive to mutant MTFR2 peptide. (B) MHC-multimer analysis of non-expanded, tumor-infiltrating CD8+ T lymphocytes isolated from tumor regions 1-3 and normal lung tissue of patient L012 identifies two distinct populations of CD8+ TILs reactive to mutant CHTF18 and MYADM peptide. The frequency of CD8+ MHC-multimer positive cells out of total CD3+CD8+ TILs is displayed for (A) and (B). (C) Multi-parametric flow cytometric analysis of tumor-infiltrating T lymphocyte subsets isolated from L011 region 3. Phenotypic data are representative of all tumor regions. Relative expression of iCTLA-4 (intracellular CTLA-4), surface PD-1 and surface LAG-3 by CD4+FoxP3+ (regulatory T cell), CD4+FoxP3- (CD4 helper T cell), CD8+ multimer negative and CD8+ multimer-reactive (CD8+ MTFR2+) T cells is displayed, plotted against iKi67 (intracellular Ki67). (D) Co-expression of PD-1 and iGzmB (intracellular granzyme B) by tumor-infiltrating T lymphocyte subsets isolated from L011 region 3.
Fig. 4. Neoantigen clonal architecture and clinical benefit of immune checkpoint blockade. (A) Samples are grouped according to clinical benefit, with durable clinical benefit on left and no durable benefit on right [defined as in (2)]. Bar plot depicts clonal neoantigens in blue and subclonal neoantigens in red. Mutational signatures identified within each sample, subtype and expression of PD-L1 are shown below. (B) Progression free survival in NSCLC (2) cohort treated with anti-PD1 either without an ITH threshold (HR = 0.29 [0.12 – 0.69], log-rank P = 0.0032), or an ITH threshold of 0.01(HR = 0.20 [0.07 – 0.60], log-rank P = 0.0017), 0.02(HR = 0.25 [0.09 – 0.67], log-rank P = 0.0034), or 0.05(HR = 0.17 [0.07 – 0.44], log-rank P = 0.000061). (C) Overall survival in melanoma (4) cohort treated with anti-CTLA-4 either without an ITH threshold (HR = 0.51 [0.23 – 1.11], P = 0.083), or an ITH threshold of 0.01(HR = 0.29 [0.11 – 0.77], log-rank P = 0.008), 0.02(HR = 0.34 [0.14 – 0.81], log-rank P = 0.011), or 0.05(HR = 0.51 [0.23 – 1.11], P = 0.083). Notably, an ITH threshold of 0.05 results in the same survival curve as no ITH threshold as no tumors with a high neoantigen burden exhibit > 0.05 neoantigen ITH. (D to F) Clonal architecture of CA9903 (D), CR9306 (E), and CR0095 (F), with mutations yielding neoantigens that elicit a T-cell response highlighted. Blue dots represent clonal mutations, with subclonal mutations depicted as red dots. Density plots are shown above.