T Cells Expressing Receptor Recombination/Revision Machinery Are Detected in the Tumor Microenvironment and Expanded in Genomically Over-unstable Models

Gaia Morello1, Valeria Cancila1, Massimo La Rosa2, Giovanni Germano3,4, Daniele Lecis5, Vito Amodio3,4, Federica Zanardi5, Fabio Iannelli5, Daniele Greco1, Laura La Paglia2, Antonino Fiannaca7, Alfonso M. Urso2, Giulia Graziano2, Francesco Ferrari2,8, Serenella M. Pupa9, Sabina Sangaletti10, Claudia Chiiodoni5, Giancarlo Pruneri10, Alberto Bardelli3,4, Mario P. Colombo5, and Claudio Tripodo1

Abstract

Tumors undergo dynamic immunoediting as part of a process that balances immunologic sensing of emerging neoantigens and evasion from immune responses. Tumor-infiltrating lymphocytes (TIL) comprise heterogeneous subsets of peripheral T cells characterized by diverse functional differentiation states and dependence on T-cell receptor (TCR) specificity gained through recombination events during their development. We hypothesized that within the tumor microenvironment (TME), an antigenic milieu and immunologic interface, tumor-infiltrating peripheral T cells could reexpress key elements of the TCR recombination machinery, namely, Rag1 and Rag2 recombinases and Tdt polymerase, as a potential mechanism involved in the revision of TCR specificity. Using two syngeneic invasive breast cancer transplantable models, 4T1 and TS/A, we observed that Rag1, Rag2, and Dnmt in situ mRNA expression characterized rare tumor-infiltrating T cells. In situ expression of the transcripts was increased in coisogenic Mlh1-deficient tumors, characterized by genomic overinstability, and was also modulated by PD-1 immune-checkpoint blockade. Through immunolocalization and mRNA hybridization analyses, we detected the presence of rare TDT”RAG1/2” cells populating primary tumors and draining lymph nodes in human invasive breast cancer. Analysis of harmonized single-cell RNA-sequencing data sets of human cancers identified a very small fraction of tumor-associated T cells, characterized by the expression of recombination/revision machinery transcripts, which on pseudo-temporal ordering corresponded to differentiated effector T cells. We offer thought-provoking evidence of a TIL microniche marked by rare transcripts involved in TCR shaping.

Introduction

The induction of tumor-associated immune responses is indicative of different phases of immunoediting, which dynamically span from elimination to equilibrium and escape (1, 2). These phases may be asynchronous for different tumor cell subsets, such as cancer stem cells, due to subcompartments within tissues that have different immune privileges (3). The induction, escape, and reawakening of immune responses delineate a dynamic model biased toward tumor overgrowth but potentially exploitable for tumor control. The analysis of the presence, density, distribution, and functional activation state of tumor-associated immune cells is predominantly a static “snapshot.” Most of the clinical studies exploring the influence of tumor-associated immune responses focus on the opposed activity of effector T-cell and suppressive/regulatory elements (4). Among the driving forces of tumor immunoediting, the generation of neoantigens related with the mutational activity of malignant clones is dominant (2, 5, 6). Neoantigen emergence in an immunocompetent setting implies the induction of control mechanisms over effector T-cell proliferation and cytotoxic activity to enable tumor growth. The frequency of neoantigen generation has been correlated with the disease prognosis in different tumor settings (7, 8). Among mechanisms controlling antitumor T-cell responses, the expression of immune checkpoints on the surface of tumor cells, restraining T-cell activation and inducing effector function exhaustion, has been exploited for therapeutic purposes (9). Other mechanisms coincide to tumor-immune escape. These may be either intrinsic to the tumor cell, such as the modulation of MHC-I expression (10), or engendered by the tumor microenvironment (TME) as the accrual/induction of immunosuppressive accessory elements, namely, tumor-associated macrophages, myeloid-derived suppressor cells, cancer-associated fibroblasts, and mesenchymal stromal cells (11). Most of these mechanisms operate at the site of tumor development, while others may occur systemically, such as the modification of the hematopoietic/lymphopoietic output (12). Other still unexplored events within the TME or at other immunologic sites may influence antitumor-immune responses.

The induction of peripheral, mature T-cell tolerance toward specific antigens in potentially autoimmune settings can be achieved by...
reinduction of recombination activating gene (RAG1 and RAG2) machinery in T cells and revision of T-cell receptor (TCR) antigen specificity through gene editing (13). During the physiologic development of αβ T lymphocytes, the RAG1/2 complex is activated in two steps: the first is at the assembly of TCRβ genes in pro-T cells and the second occurs at TCRα gene assembly (14). The RAG1/2 complex mediates DNA double-strand cleavage at specific signaling regions of the genes undergoing rearrangement (15). dsDNA breaks introduced by RAG activity induce DNA repair by nonhomologous end-joining, involving primarily the induction and recruitment of the terminal deoxynucleotidyl transferase (TdT) codely the DNA gene, which increases diversity by adding random nucleotides at the joints (16). Quenching of RAG1/2 expression is promoted by TCR complex signaling (17) and the DNA damage response via ATM signaling (18). The expression of the recombines complex in peripheral T cells finalized to TCR specificity revision and tolerance induction has been demonstrated to be associated with viral superantigen stimulation (19, 20) and in microenvironments involving delivery of costimulatory signals, such as ICOS and CD28 (21).

In this study, we hypothesized that tumors could represent an antigenic environment that induces the emergence of recombines machinery-expressing (RAG1/2) peripheral tumor-infiltrating T cells, as a mechanism of peripheral tolerance or epitope spreading (22). We studied the in situ mRNA expression of Rag1U2 and Dnnt in two independent syngeneic models of breast cancer, the 4T1 and TS/A models, and found that these elements are expressed within the TME. We show the expression of Rag1/2 in a fraction of T cells, and through the adoption of Mlh1-defective tumors, we demonstrated a significant increase in Rag1/2 and Dnnt expression in a genetically over-unstable setting. We also describe an increase in the fraction of Rag1/2- and Dnnt-expressing TILs following PD-1 immune-checkpoint blockade. In human breast cancer tissues, we confirmed the presence of a very small fraction of TdT-expressing elements, also detected in draining lymph nodes (LN), and highlighted their concomitant expression of RAG1/2 mRNA and pHy2AX, a DNA damage marker. Finally, analyzing single-cell RNA-sequencing (scRNA-seq) data sets of different human cancers, we confirmed the presence of peripheral T cells expressing RAG1/2 and/or DNNT in the TME. Our results offer new perspectives on peripheral recombination events in tumor-infiltrating lymphocytes.

Materials and Methods

Cell lines

The TS/A breast cancer cell line was established from a moderately differentiated mammary adenocarcinoma that arose spontaneously in a BALB/c mouse and were kindly provided by Federica Cavallo (Molecular Biotechnology Center, University of Torino, Italy; ref. 23). TS/A cells were cultured for around 490 days in DMEM-high glucose, 10% FBS plus glutamine, penicillin, and streptomycin (Sigma-Aldrich). CT26 cells were purchased from the ATCC and cultured for around 150 days in RPMI-1640 10% FBS, plus glutamine, penicillin, and streptomycin (Sigma-Aldrich). 4T1 cells were purchased from ATCC and cultured in DMEM ( Gibco) plus 10% FCS (Euroclone) for around 60 days. All cell lines were regularly tested for Mycoplasma.

Generation of Mlh1-ko (knockout) TS/A, 4T1, and CT26 cells

To knockdown the DNA mismatch-repair gene mutL homolog 1 (Mlh1) in TS/A mouse cells, we used the genome editing one vector system (lentiCRISPR-v2; Addgene, #52961) as previously reported (23). Briefly, single-guide (sg)RNAs were designed using the CRISPR tool http://crispr.mit.edu/ to minimize potential off-target effects. For transient expression of the CRISPR-Cas9 system, we transfected cells with lentiCRISPR-v2 vector plasmid (same sgRNAs) as previously described (24). Transfection was carried out using Lipofectamine 3000 (Life Technologies) and Opti-MEM (Invitrogen), according to the manufacturer’s instructions. After 48 hours, cells were incubated with puromycin (15μg/mL; Sigma-Aldrich) for 2 days, and subsequently single-cell dilutions were performed in 96-well plates (5 cells/ml). The absence of Mlh1 was confirmed by Western blot (Supplementary Fig. S1A). The same CRISPR-Cas9 methodology was adopted for the generation of Mlh1<sup>Δ-ΔT2m<sup>Δ</sup>/CT26 tumor cells (25). 4T1 cells were engineered to knock out the Mlh1 gene by CRISPR-Cas9 technology. The 5'-CACCGAGTTCAAGTGTTGTTAAGGA-3' sequence targeting mouse Mlh1 gene was cloned in pSpCas9(BB)-2A-GFP (PX458; Addgene, plasmid ID: 48138, a gift from Feng Zhang) as already described (26, 27). Cells were transfected with Lipofectamine 3000 (Life Technologies) with a plasmid targeting Mlh1 and control vector, and then seeded as single cells in 96-well plates (5 cells/ml). Single clones were expanded for 1 month and tested by Western blot to verify the depletion of Mlh1 expression (Supplementary Fig. S1B).

Murine breast cancer syngeneic models

All animal procedures were approved by the Ethical Commission of the University of Turin, the IRCCS Istituto Nazionale Tumori in Milan, and the Italian Ministry of Health, and they were performed in accordance with institutional guidelines and international law and policies. Tumor size limits were observed in accordance with institutional guidelines. Tumor growth was evaluated by caliper measurement twice a week. Tumor size was calculated using the formula: \[ V = \frac{(d_1^2 \times d_2)}{2} \] (minor tumor axis; \(d_1\); major tumor axis) and reported as tumor mass volume (mm³, mean ± SEM of individual tumor volume). For 4T1 in vivo experiments, 1 x 10⁵ cells were resuspended in PBS and injected in the mammary fat pad of 8-week-old BALB/c mice. For the TS/A in vivo experiments, 1 x 10⁵ cells per mouse were resuspended in a solution of PBS and Matrigel (1:1; Corning) and injected in the mammary fat pad of 6-week-old BALB/c mice. 5 x 10⁵ cells CT26 cells were injected subcutaneously in PBS in 6-week-old BALB/c mice. Animals were examined by veterinary personnel during the duration of the experiments. Mice were monitored at least three times a week for signs of illness and reduced and/or impaired motility. After 30 (TS/A, CT26) or 28 (4T1) days of the inoculum, mice were sacrificed, and tumors were collected for histopathologic, in situ hybridization (ISH), and immunohistochemical analysis. No statistical methods were used to pre-determine sample size.

Anti-PD-1 treatment of TS/A and CT26 tumors

The anti-mouse PD-1 (clone 290-14, Bio X Cell) was injected intraperitoneally (250 μg/mouse). The first dose was administered when TS/A tumor volume was 200 to 300 mm³ and then every 3 days, for three times in total. The same schedule was followed for the CT26 model, with the exception of the first dose being administered 6 days after injection when tumor volume was palpable, for four times in total.

Human tissue samples

Formalin-fixed and paraffin-embedded (FFPE) tissue samples of human breast cancer [96 cases of invasive ductal triple-negative breast cancer (TNBC)], melanoma (15 cases of invasive cutaneous melanoma), tumor-draining LNs (6 from invasive ductal breast cancer and 13...
Double-target in situ mRNA hybridization and quantitative immunolocalization analyses

Tumors dissected from mice were washed in PBS and fixed in 10% neutral buffered formalin overnight, washed in water, and paraffin-embedded. Thymi from 12-week-old C57BL/6 mice were used as controls. IHC and immunofluorescence (IF) staining was performed as previously reported (28). Four-micrometer-thick human and mouse tissue sections were deparaffinized, rehydrated, and unmasked using Novocastra Epitope Retrieval Solutions. Images of original tumor sections were subjected to sequential rounds of single-marker immunostaining, and the binding of the primary antibodies was revealed by the use of secondary antibodies conjugated with different fluorophores or enzymes. The following primary antibodies were used for IHC and IF on mouse and human tissues: rabbit anti-human CD3 (clone 4B11, 1:50, pH 9, Novocastra), rabbit anti-mouse CD8 (clone D4W2Z, 1:400, pH 9, Cell Signaling Technology), mouse anti-human CD7 (clone CD7-272, 1:50 pH 9, Novocastra), rabbit anti-human CD4 (clone EPR6865, 1:500, pH 9, Abcam), rabbit anti-mouse CD4 (clone D7D2Z, 1:50, pH 9, Cell Signaling Technology), mouse anti-human CD8 (clone 4B11, 1:50, pH 9, Novocastra), rabbit anti-human TdT (clone SEN82, 1:50, pH 9, Novocastra), rabbit anti-human Tdt (1:100, pH 9, Cell Marque), mouse anti-human CD44v6 (clone VFF18, 1:500, pH 9 = 6, Abcam), rabbit anti-human ALDH1A1 (clone 1:500, pH = 6, Genetex), mouse anti-human p53 (clone DO-7, 1:50, pH = 6, Novocastra), mouse anti-human MLH1 (clone ES05, 1:50, pH = 6, Novocastra). IHC staining was developed using the Novolink Polymer Detection Systems (Novocastra) or IgG (H&L)-specific secondary antibodies (Life Technologies, 1:500) and DAB (3,3’-Diaminobenzidine, Novocastra) as substrate chromogen. Double IHC staining was performed by applying SignalStainBoost IHC Detection mouse (cod. #31926, Cell Signaling Technology) or rabbit (cod. #18653, Cell Signaling Technology) alkaline phosphatase-conjugated produced in horse and Vulcan Fast Red as substrate chromogen. Anti-mouse and anti-rabbit (Alexa Fluor 488 and 568 conjugate) secondary antibodies were used for IF.

The mouse Dnmt probe hybridization (Dnmt; Cod. 561961) was performed using RNAscope 2.5 HD Detection Reagent-BROWN (Advanced Cell Diagnostic) in accordance with the manufacturer’s protocol. Mouse and human RAG1 and RAG2 probe hybridization (Mm-Rag1, Cod. 436831; Mm-Rag2-C2, Cod. 545131; Hs-Rag1, Cod. 545751; Hs-Rag2-C2, Cod. 549591) was performed using RNAscope 2.5 HD Duplex Reagent Kit (Advanced Cell Diagnostic) in accordance with the manufacturer’s protocol, adopting an extended 1-hour incubation in Amp 5 and 30-minute incubation in Amp 6 for Hs-RAG1/ RAG2 hybridization.

Slides were analyzed under a Zeiss Axiocam A1 microscope equipped with four fluorescence channels widefield IF. Microphotographs were collected using a Zeiss Axiocam 503 Color digital camera with the Zen 2.0 Software (Zeiss). Slide digitalization was performed using an Aperio CS2 digital scanner (Leica Biosystems) with the ImageScope software (Aperio ImageScope version 12.3.2.8013, Leica Biosystems).

Quantitative analyses of IHC stainings were performed by calculating the average percentage of positive signals in five nonoverlapping fields at medium-power magnification (>200) using the Nuclear Hub software. Images were acquired using a Leica DM2000 LED microscope equipped with a 10× objective, with a resolution of 512 x 512 pixels, and the slides were scanned at 20× magnification. A custom-designed mask was used to select the tumor regions, and the images were analyzed using the Nuclear Hub software. The percentage of positive cells was calculated for each tumor area, and the mean value was used for statistical analysis. The average percentages of positive cells were compared using the Wilcoxon rank sum test, and the significance level was set at p < 0.05.

Statistical analysis of human TNBC clinical-pathologic data

In order to have the most balanced classes in terms of size, TNM staging variables were transformed in binary categorical variables by grouping observations considering the following categories: T = 1, T > 1 for size of the original (primary) tumor; N = 0, N > 0 for nodal disease status; M = 0, M = 1 for distant metastasis occurrence; tumor_grade = 2, tumor_grade > 2 for grade of the cancer cells. Observations were grouped as “low” or “high” Ki-67 by applying the prognostic threshold of 30% to the % of Ki-67 (continuous) variable, resulting in the Ki67 ≤ 30% threshold (binary) categorical variable. Expression of p53 was semiquantitatively scored as 0 (absent), 1+ (weak/focal staining), 2+ (moderate/multifocal), 3+ (strong/diffuse). MLH1 nuclear staining was scored as positive or negative.

The Wilcoxon rank sum test was used to test differences for average % TdT with respect to all binary categorical variables. No significant associations were detected between average % TdT and clinicopathologic features. Fisher exact test was applied to test for significant associations between all possible pairs of binary categorical variables. As reported in the paper, significant positive associations between Ki67+ 30% threshold and T (P < 0.001), N (P = 0.0082), M (P = 0.0003), and relapse (P = 0.0059) were found. All statistical analyses were performed with the R package "stats" version 4.0.0, R version 3.6.2 (https://www.r-project.org).

scRNA-seq analyses from data sets

Selected human scRNA-seq data sets relative to tumor-associated immune cells in different solid tumor histotypes (Supplementary Table S1) were transcribed per million (TPM)–normalized, log2-transformed using Scaler R package version 1.14.29), and then integrated using the Harmony approach (30) and principal component analysis (PCA) embedding (31), adjusting for data set–specific effects.
including both technical (batch effects) and biological (tissue sources) heterogeneity (as described in the next section). Cell typing was performed using two different strategies (as described in the next section). For data sets Sade-Feldman and colleagues (32) and Zhang and colleagues (ref. 33; Supplementary Table S1), cell types were available from the authors, whereas for the remaining ones, they were inferred using the SCINA algorithm (34). For signature genes, we used the LM22 expression matrix provided by the CIBERSORT team (35, 36) and additional T-cell marker genes manually curated (Supplementary Table S2).

For visualization, 2D Uniform Manifold Approximation and Projection (UMAP) plots labeled with cell types were produced using the scater R package version 1.14 (ref. 29; as described in the next section). Cluster analysis was performed using the Leiden clustering algorithm (37), and cell partitions, representing well-separated cell groups, were extracted using a statistical test included in the PAGA algorithm (ref. 38; as described in the next section). Both clustering and partitioning were performed using the Monocle3 R package (39). Finally, trajectory analysis and pseudotime computation was applied using the Monocle3 R package. Clustering, partitioning, trajectory analysis, and pseudotime calculation have been carried out considering only the T cells (as described in the next section).

Differential gene-expression analysis was performed using MAST R package (40). Differentially expressed genes were filtered considering a log2(FC) > 0.6 and false discovery rate (FDR) < 0.05. GO enrichment analysis was applied to differentially expressed genes using the GO service powered by the Panther database (http://geneontology.org/). KEGG pathway enrichment was carried out using the clusterProfile R package (41). Differentially expressed genes in RAG1/RAG2/DNTT+ T cells versus other T cells were considered. Visualization of genes of interest in pathways was done using pathview R package (42).

For the GSE120575 data set (Supplementary Table S1; ref. 32), the 2D UMAP visualization was obtained following the same pipeline. The cells were labeled as reported in the original publication: CD8_B (exhaustion) and CD8_G (activation/memory/survival; Supplementary Table S3).

Bioinformatics pipeline
Preprocessing

Because some of the scRNA-seq data sets are only available as TPM counts, we first computed TPM normalization for those data sets having raw read counts. Second, TPM expression values were log2-transformed. R package Scater version 1.14 (29) was used to perform both computational steps. We then assigned each cell a biological type using two different strategies. First, we used the cell types found in the data set–related metadata (“Reference” column in Supplementary Table S1). When the cell type was not available, we inferred it by means of an in silico predictor—the SCINA algorithm (34). SCINA is able to assign a cell type to each cell in a data set by using a set of signature genes of reference cell types. SCINA fits a bimodal statistical model, where the higher mode corresponds to the cell types related to the signature genes, whereas the lower mode represents the remaining cells. SCINA can also assign the label “undetermined” to those cells not identified according to the available reference cell types. For signature genes, we used CIBERSORT LM22 expression matrix (43, 44). We grouped LM22 cell types into seven groups: T cells, B cells, dendritic cells (DC), mast cells, natural killer (NK) cells, macrophages, and granulocytes. For each group, we extracted marker genes by the means of the pairwise Welch t tests as implemented in the “findMarkers” function of scran R package version 1.14.6 (45). We manually integrated some known marker genes for T cells (Supplementary Table S2). We considered marker genes as those upregulated with a P value < 0.05. Lastly, we concatenated all the data sets in order to obtain a single scRNA-seq data set for the following analysis.

Analysis

scRNA-seq data sets are usually analyzed by projecting cells into a low dimensional space. PCA (31) is one of the most used embedding algorithms for scRNA-seq data. By computing the top principal components (PC), we capture the largest source of variation among genes, allowing to preserve and better represent biological variation and its underlying structure, while at the same time reducing the dimensionality of the original data. In order to analyze all the single-cell data sets at the same time, we adopted an integration approach called Harmony (30). Harmony allows embedding of all the cells into a shared reduced dimensional space, obtained for example via PCA, and tuning of their coordinates with regard to data set-specific effects, considering both technical (batch effects) and biological (tissue sources) heterogeneity. In the preprocessing step, we concatenated all the available scRNA-seq data sets (Supplementary Table S1) and computed PCA over the integrated data set. We then adjusted the PC coordinates of the whole data set using Harmony. PCA was run under the scater package using default parameters. We considered the top 50 PCs. Harmony version 1.0 is available as an R library at https://github.com/immunogenomics/harmony, and it was run with default parameters. In order to visualize the scRNA-seq data set, we used the UMAP method (43), a nonlinear dimensionality reduction algorithm. UMAP is able to discover a low dimensional representation of input data, preserving at the same time neighborhood relationships of the original high-dimensional space. 2D UMAP visualization was labeled with cell types. We used the UMAP implementation provided by the scater R package with default parameters and the number of nearest neighbors set to 10.

For the Mus musculus scRNA-seq OT-I CD8+ T-cell data set (Supplementary Table S1, Accession E-MTAB-6051), normalization, PCA embedding (40 PCs), and UMAP (nearest neighbors = 10) projections were performed using Scany v1.5.0 (44) and Python version 3.7.6 (https://www.python.org/downloads/release/python-376/) in order to reproduce the original preprocessing and plot layouts. In this case, cells were color-coding combined “Factor Value(stimulus)” and “Factor Value(time)” attributes found in the experiment design file (TSV format) available metadata.

After 2D projection, we performed cluster analysis. We applied the Leiden clustering algorithm (37), a graph-based clustering algorithm that guarantees to find well-connected communities in the input data. Apart from clustering, we also extracted cell partitions, representing well-separated cell groups, using a statistical test included in the PAGA algorithm (38). For both clustering and partitioning, we used the Monocle3 R package (46). Clustering and partitioning are obtained using a single Monocle3 function, cluster_cells, with default parameters and the number of nearest neighbors for building the initial graph set to 40. Finally, we performed trajectory analysis and pseudotime computation using the Monocle3 package. For trajectory inference, we considered the discovered partitions and one trajectory for each partition. The root for computing the pseudotime in each partition has been identified according to what suggested by Monocle3 tutorial (https://cole-trapnell-lab.github.io/monocle3/docs/trjectories/#learn-graph). In particular, we applied a function that first groups the cells according to which trajectory graph node they are nearest to and then it calculates what fraction of the cells at each node come from the earliest time point. The algorithm then picks the node that is most heavily occupied by early cells and returns that as the root.
partitioning, trajectory analysis, and pseudotime calculation have been carried out considering only the T cells.

**Statistical analyses**

Statistical analyses on data from quantitative ISH/IHC assays (mRNA signal counts, positive cell frequencies) were performed using GraphPad Prism (version 8.2.1) software. To determine statistical significance unpaired, two-tailed Student t test was used. All data are presented as mean ± SEM. Statistical analyses of human clinical–pathologic data were performed with the R package “stats” version 4.1.0, R version 3.6.2 (https://www.r-project.org).

**Results**

**Rag1/2 and Dntt mRNAs mark a tumor-infiltrating T-cell microneph in syngeneic breast cancer**

To investigate in situ the expression of Rag1/2 recombinases and Tdt polymerase within the TME of 4T1 and TS/A syngeneic mouse breast cancer lesions, we adopted an mRNA hybridization approach, overcoming limitations of antibody-mediated detection. In situ mRNA hybridization was first performed for control purposes on normal thymi from 12-week-old wild-type (WT) C57Bl/6 mice. In the control thymi, the in situ mRNA for Rag1, Rag2, and Dntt marked cortical thymocytes, with Rag2 showing a lower density of signals compared with Rag1, consistent with its limiting expression in regulating recombinase machinery (Fig. 1A and B). Analysis of 4T1 and TS/A orthotopic tumor lesions showed a variable presence of Rag1/2 and Dntt signals within tumor infiltrates (Fig. 1C and D; Supplementary Fig. S2A and S2B), suggesting the occurrence of recombinase machinery element transcription in the TME. In situ mRNA hybridization for Rag1, Rag2, and Dntt on control mouse mammary glands of age-matched BALB/c mice revealed no signals (Supplementary Fig. S2C and S2D). Prompted by the detection of Rag1/2 and Dntt mRNA signals in the tumor foci, we tested whether the expression of recombinase genes was associated with tumor-infiltrating T cells. To this aim, Rag1/2 in situ mRNA hybridization slides were immunostained for the T-cell markers CD3 and CD8. Whole slide scans of in situ Rag1/2 mRNA and CD3 and CD8 IHC were then comparatively analyzed through a dedicated software tool, which highlighted the presence of CD3+ and CD8+ T cells expressing Rag1/2 in the two syngeneic breast cancer models (Fig. 1E–H; Supplementary Fig. S2E–S2H). Some cells expressing Rag2 also showed expression of the Ifng transcript, indicating an effector state (Supplementary Fig. S3A). These findings supported the hypothesis that key components of the recombinase recombinase/revision machinery could be expressed in tumor-infiltrating T lymphocytes.

**T cells expressing Rag1/2 and Dntt increase in Mlh1-ko tumors and are modulated by anti-PD-1**

We subsequently investigated the hypothesis that the expression of Rag1/2 and/or Dntt within the TME depended on recognition of tumor-associated neoepitopes. To modulate the neoantigen burden in the syngeneic tumor models, we adopted Mlh1-deficient tumors obtained by Cas9-mediated Mlh1 inactivation in both 4T1 and TS/A clones. Mlh1-defective orthotopic tumor implants of both 4T1 and TS/A showed a significant increase in the frequency of pytH2AX (a DNA damage response marker) expression in the nuclei compared with WT tumors (Fig. 2A and B; Supplementary Fig. S4A), supporting the ongoing accumulation of DNA damaging events. Consistent with the increase of DNA damage–induced neoantigens in Mlh1-deficient tumors, the density of tumor-infiltrating CD3+ T lymphocytes was significantly higher compared with WT tumors (Fig. 2C and D; Supplementary Fig. S4B), as assessed by quantitative IHC. Such an increase in TILs was paralleled by an overall significant increase in the amount of Rag1/2 and Dntt in situ mRNA expression signals (Fig. 2E–H; Supplementary Fig. S4C and S4D). Based on this, we quantitatively evaluated the small fraction of Rag1/2- and Dntt-expressing CD3+ and CD8+ TILs in WT and Mlh1-defective 4T1 and TS/A tumors and found that their frequency, particularly among CD8+ T cells, significantly increased in the Mlh1-defective setting (Fig. 2I–P; Supplementary Fig. S4E, S4G, S4H, and S4J–S4N). These results suggest that the induction of Rag1/2 and Dntt within the TME is positively influenced by the increase in genomic instability.

We also evaluated whether the in situ expression of recombinase/revision machinery elements could be influenced by the interference with immune checkpoints restraining T-cell activation, prototypically PD-1. To this end, we analyzed the in situ mRNA expression of Rag1/2 and Dntt, along with the total amount of infiltrating T cells, including Rag1/2- and Dntt-expressing CD3+ and CD8+ T-cell fractions, in both WT and Mlh1-deficient tumors either untreated or treated with anti–PD-1. A significant increase in DNA damage accumulation, overall CD3+ T-cell infiltration, and total amount of Rag1/2 and Dntt hybridization signals was observed in both WT and Mlh1-deficient tumors following anti–PD-1 treatment (Supplementary Fig. S4A–S4D; Supplementary Fig. S5A and S5B). Consistently, the fractions of Rag1/2- and Dntt-expressing CD3+ and CD8+ T cells significantly increased in anti–PD-1-treated tumors (Supplementary Fig. S4E–S4N; Supplementary Fig. S5C–S5F), and such an increase was generally more pronounced in WT treatment-naive and treated tumors compared with the Mlh1-deficient counterpart. The observed variations in Rag1/2- and Dntt-expressing TILs, however, did not reflect univocally on the outcome of anti–PD-1 treatment on tumor growth control because the Mlh1-deficient tumors, not the WT tumors, were significantly smaller in size following anti–PD-1 treatment (Supplementary Fig. S5G). As a further control, we investigated the frequency of Rag1/2- and Dntt-expressing TILs in Mlh1-deficient CT26 tumors lacking β2-microglobulin expression and either treated or not with anti–PD-1 (Supplementary Figs. S6 and S7). In this setting, the frequency of Rag1/2+ and Dntt+ CD3+ TILs was slightly, but significantly, induced upon treatment (Supplementary Fig. S7A, S7B, S7G, and S7H), but it was not paralleled by an increase in Rag1/2+ or Dntt+ CD8+ T cells (Supplementary Fig. S7C, S7D, S7L, and S7J). In these same tumors, a trend toward the induction of Rag1/2+ and Dntt+ CD4+ T cells was observed (Supplementary Fig. S7E, S7F, S7K, and S7L), in line with the demonstration of immune-checkpoint inhibitor activity in mismatch-repair–defective tumors, also defective in β2-microglobulin, being primarily mediated by tumor-infiltrating CD4+ T cells (44). Altogether these results indicated that the magnitude of recombination/revision machinery element induction was associated with conditions promoting TCR activation; however, they did not resolve the conundrum intrinsic to the significance of the observed events, which may underlie an attempt toward peripheral tolerance induction, epitope spreading and broadening of antitumor immunity, or the indication of a transcriptional state of responding effectors.

**RAG1/2 expression is associated with TDT in human breast cancer foci and draining LNs**

Prompted by the detection of Rag1/2 and Dntt expression within the TME of syngeneic mouse breast cancer models, we investigated human cancer samples. To this purpose, we first analyzed tumor tissues by IHC for TDT. We quantitatively analyzed a series of 90 TNBC primary tumor samples. We focused on TNBC samples because of their reported high frequency of TILs and their candidate adoption of...
**Figure 1.**

In situ detection of Rag1/2 and Dntt in T cell–infiltrating 4T1 tumors. **A** and **B**, Representative microphotographs of mRNA in situ hybridization for Rag1/Rag2 and Dntt in control 12-week-old C57BL/6 mice thymi (n = 2 mice). Original magnification, ×200 and ×400 (insets). Scale bar, 100 µm. **C** and **D**, mRNA in situ hybridization for Rag1/Rag2 and Dntt within 4T1 tumor lesions (n = 5 mice/group). Original magnification ×400 and ×630 (insets). Scale bar, 50 µm. **E–H**, Comparative images of mRNA in situ hybridization for Rag1/Rag2 and IHC for CD3 (**E** and **F**) or CD8 (**G** and **H**) in 4T1 tumors (n = 5 mice/group). Original magnification, ×400 and ×630 (insets). Scale bar, 50 µm.
Figure 2.
T cells expressing Rag1/2 and Dntt increase in Mlh1-ko 4T1 tumors. A–H, Representative input (left) and output (right) images and quantitative analyses of pγH2AX (A and B), CD3 (C and D), Rag1/Rag2 (E and F), and Dntt (G and H) to assess the differences between WT and Mlh1/C0/C0/C0 mice in the 4T1 model (n = 4 mice/group). Original magnification, ×630. Scale bar, 25 μm. I–L, Comparative images and quantitative analyses of mRNA in situ hybridization for Rag1/Rag2 and IHC for CD3 (I and J) or CD8 (K and L), assessing the percentage of CD3+ or CD8+ cells expressing Rag1/2 in WT and Mlh1/C0/C0/C0 4T1 tumors (n = 5 mice/group). Original magnification, ×630. Scale bar, 25 μm. M–P, Comparative images and quantitative analyses of mRNA in situ hybridization for Dntt and IHC for CD3 (M and N) or CD8 (O and P) showing the percentage of CD3+ or CD8+ T cells expressing Dntt in WT and Mlh1/C0/C0/C0 4T1 tumors (n = 5 mice/group). Original magnification, ×630. Scale bar, 25 μm. Statistical analysis: two-tailed unpaired Student t test (B, D, F, H, J, L, N, and P). Mean ± standard error shown; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
immunotherapy-based treatment (47). Of the tested TNBC cases, clinical–pathologic data were available (Supplementary Table S4). In most of the TNBC cases, rare TDT cells were detected mainly distributed in peri-tumor areas and/or in the proximity of foci of dysplastic lesions, and to a lower extent in infiltrating cancerous foci (Supplementary Fig. S8A). The frequency of TDT cell immune cells, as determined by quantitative analysis of whole slide digital scans, ranged from 0.01% to 0.83% of the total cellularity, with a median frequency of 0.23%. The analysis of associations among clinical–pathologic data, including TDT cell frequencies, revealed significant positive associations between the percentage of Ki-67+ tumor cells scored as high/low according to the prognostic cutoff of 30% (48) and tumor grade (P < 0.001), as well as between nodal disease status and distant metastasis occurrence (P = 0.0003) or relapse (P = 0.0059). No significant associations were detected between the frequency of TDT cells and clinicopathologic features. The TDT cells detected by IHC in invasive ductal breast cancer samples were consistently characterized by RAG1/2 mRNA expression by ISH (Fig. 3A and B). We demonstrated that RAG1/2-expressing cells lining primary lesions coincided with CD8+ T cells (Fig. 3C and D). The frequency of these cells was higher in tumor foci than in control, non-tumoral, human mammary glands, where almost no TDT or RAG1/2+ cells were detected (Fig. 3E and F; Supplementary Fig. S9A and S9B).

Following the hypothesis of reconstruction/revision machinery-expressing lymphoid cells being associated with the tumor-immune milieu, we investigated the presence of RAG1/2+ TDT cells in whole sections of tumor-draining LNs and LNs with nontumor-associated reactive hyperplasia. In tumor-draining LNs, RAG1/2+ cells also expressing TDT were detected loosely clustered in interfollicular or medullary areas and lining dilated sinusoids (Fig. 3G and H), whereas in control reactive LNs, isolated cells were identified (Supplementary Fig. S9C). RAG1/2+/TDT+ cells were marked by CD8 expression (Fig. 3I and J), and their frequency was significantly higher in tumor-draining LNs than in control reactive LNs (Fig. 3K and L).

To assess whether lymphoid cells expressing TDT and RAG1/2 transcripts had signs of DNA cleavage activity, as expected by the expression of RAG transcripts and TDT, double-marker IF for TDT and pH2AX (as a marker of DNA damage) was performed in representative samples with a high frequency of RAG1/2+/TDT+ cells. IF revealed that TDT+ nuclei had evident foci of pH2AX, indicating DNA damage accumulation (Supplementary Fig. S9D).

To verify that the presence of the rare TDT+ cells was not an exclusive feature of invasive ductal breast cancer, we also investigated the occurrence of TDT+ cells in primary melanoma lesions and draining LNs. TDT+ cells were variably detected in the primary melanoma microenvironment and in the draining LNs (Supplementary Fig. S10A and S10B), where they associated with RAG1/2 expression, and T-cell markers including, besides CD8, the pan T-cell marker CD7 and/or CD4 (Supplementary Fig. S10C–S10G). The frequency of TDT+ and RAG1/2+/CD8+ cells was higher in melanoma peri- and intratlesional areas and in the draining LNs than in control nontumor skin samples or reactive LNs, where almost no TDT+ immune cells or RAG1/2+ expression were detected (Supplementary Figs. S10H–S10K and S11A and S11B). These results suggest that the occurrence of receptor reconstruction/revision machinery expression may represent a microniche both at primary tumor sites and in the draining LNs.

**T cells expressing RAG1/2 and DNTT in scRNA-seq data sets of tumor-associated immune cells**

To provide an independent validation of the rare occurrence of tumor-infiltrating T cells expressing one or more of the hallmark transcripts of receptor recombination/revision, we analyzed scRNA-seq data sets of tumor-associated immune or T cells relative to multiple histotypes including breast cancer (49, 50), melanoma (32, 51), hepatocarcinoma (52), lung cancer (53), and colorectal cancer (33). We applied the Harmony algorithm (30), allowing the harmonization of the different data sets and the analysis of tumor-associated T cells taking into account multiple tumor histotypes (Supplementary Table S1). Overall, 46,124 out of 111,564 single cells were identified as T cells based on clustering analysis and cell type prediction (Fig. 4A). According to UMAP (43) analysis (k = 40), three compartments were identified in the T-cell cluster (Fig. 4B). Of the 46,124 T cells, 378 cells (0.8%, a fraction compatible with the observed frequency of TDT cells in human cancers in situ) showed expression of RAG1, RAG2, and DNTT alone or in combination (Fig. 4C). Of these cells, 228 (60%) were identified as CD8+, while 150 (40%) were identified as CD4+. Analysis of the differentially expressed genes between RAG1/2/DNTT-expressing CD8+ and CD4+ T cells showed positive enrichment of genes involved in T-cell activation, TCR signaling, and cell metabolism (Fig. 4D and E; Supplementary Table S5), which were also found by Gene Ontology analysis of enriched biological processes (Supplementary Fig. S12A and S12B; Supplementary Table S5). TCR signaling pathway enrichment in RAG1/2/DNTT-expressing T cells involved key receptor complex downstream effectors, namely, ZAP70, LCK, and LAT, and costimulatory elements including CD28, ICOS, and CTLA4, as shown by KEGG interaction/reaction network (Supplementary Fig. S12C). When RAG1/2/DNTT-expressing T cells from tumoral, peritumoral, and peripheral blood compartments were comparatively analyzed by focusing on the harmonized GSE140228 (52), GSE99254 (53), and GSE108989 (33) data sets (Supplementary Fig. S12D), TCR signaling gene enrichment emerged as higher in tumoral (P = 1.23E–07; FDR = 5.1E–06) than peritumoral (P = 4.19E–06; FDR = 1.8E–04) and peripheral blood (P = 3.1E–04; FDR = 2.7E–03) T cells (Supplementary Fig. S12E–S12G). Analysis of the expression of genes related with T-cell maturation stages, phenotype, and activation across a T-cell functional differentiation pseudotime was performed on all the 46,124 T cells and 378 T cells expressing hallmark T-cell receptor recombination/revision genes. Analysis on the whole T-cell cluster identified effector cytotoxic T cells (CD8B, GNLY, GZMA, IFNG high) as the terminal functional differentiation stage of tumor-associated T cells (Fig. 4F). The ST18 and PTCRA genes, hallmarks of double-negative (DN) stages, were limited to very few cells along the pseudotime, whereas AQP3 and TOX2 double-positive (DP) and DP-to-single-positive (SP) hallmark trends moved toward downmodulation from earlier to more advanced pseudotimes, as did helper T-cell (Th) or regulatory T-cell (Treg) hallmark genes (Fig. 4F). The pseudotemporal ordering of the 378 T cells characterized by TCR recombination/revision expression showed enrichment in intermediate and more advanced pseudotimes, in accordance with the expression of CD8B, GNLY, GZMA, and IFNG effector markers and lack of DN or DP precursor marker genes (Fig. 4G). This analysis further substantiated the existence of a tumor-associated T-cell microniche comprised of peripheral cells with an effector phenotype and signs of TCR recombination/revision transcriptional activity.

Prompted by the observed increase in the frequency of RAG1/2- and Dntt-expressing CD8+ T cells in TS/A tumor infiltrates following PD-1 immune-checkpoint blockade, we focused on the analysis of scRNA-seq data relative to CD8+ T cells from melanoma patients profiled pre or post immune-checkpoint inhibitor treatment and classified as either responders or nonresponders (GSE120575; ref. 32). The 6350 profiled CD8+ T cells, classified as CD8_B (exhaustion
profile) and CD8_G (activation/memory/survival programs) according to the original report (32), included 42 (0.66%) CD8⁺ T cells expressing RAG1/2 and/or DNTT (Fig. 4H), and the relative frequency of which increased from pre- (0.32%) to posttreatment (0.87%) samples, specifically among CD8_B cells in nonresponder patients (1%; Fig. 4I; Supplementary Table S3). These results further suggest a potential link between TCR activation, promoted by immune-checkpoint inhibition, and expression of recombination/revision marker transcripts.

To determine whether the expression of recombination/revision markers in peripheral CD8⁺ effector T cells could be induced by TCR antigenic stimulation, as indicated by the increase in expression in genomically over-unstable tumors, we exploited the scRNA-seq data generated in a study focusing on T-cell cytolytic responses to TCR–antigen interactions of different magnitudes using OT-I CD8⁺ T cells stimulated with ovalbumin-derived peptides (54). Analysis of the single-cell transcriptomes revealed that in a small subset of stimulated OT-I cells, Rag2 (3.7%) and Dntt (2.3%) transcript expression was...
Figure 4.
Analysis of RAG1/2 and DNTT expression in T cells from harmonized scRNA-seq data sets. **A**, UMAP 2D visualization of integrated scRNA-seq data sets of 105 patients (see Supplementary Table S1). Cells are color coded according to their cell type. **B**, UMAP 2D visualization of integrated scRNA-seq data sets. Cells are color coded according to partition group found during the clustering phase (see Materials and Methods). **C**, UMAP 2D visualization of integrated scRNA-seq data sets. Only the cells expressing RAG1, RAG2, and DNTT genes, individually or coexpressed, are color coded. **D** and **E**, Volcano plots of differentially expressed genes for CD4⁺ and CD8⁺ cells from the integrated data sets. In both cases, we considered RAG1/RAG2/DNTT⁺ T cells versus the other T cells. P-value threshold = 10⁻⁵, log₁₀ fold-change (FC) threshold = 1. **F**, Pseudotime chart of a set of marker genes, considering all T cells. **G**, Pseudotime chart of a set of marker genes, considering T cells that expressed at least one gene among RAG1, RAG2, and DNTT. **H**, UMAP 2D visualization of GSE120575 scRNA-seq data set. Cells are color coded according to their type as reported in the original publication: CD8_B (exhaustion) and CD8_G (activation/memory/survival). RAG1/RAG2/DNTT⁺ cells of interest are highlighted. **I**, Combined plot showing the different composition of CD8_B and CD8_G cells in the immunotherapy data set. The histogram, referring to the y values on the left axis, shows the proportion of CD8_B and CD8_G cells with respect to the total number of cells, considering four different conditions: pre/responder, pre/nonresponder, post/responder, and post/nonresponder. The two line charts, referring to the y values on the right axis, show how many, in percentage, RAG1/RAG2/DNTT⁺ CD8_B and CD8_G cells are present in the four above-mentioned conditions.
induced (Supplementary Fig. S13A). The frequency of Rag2 and Dntt expression in OT-1 cells was higher and occurred earlier in the presence of N4 high-potency ligand stimulation (Supplementary Fig. S13B). Although recombinase machinery reexpression could not result in receptor recombination in that experimental setting because of the genetic deficiency of Rag1, this piece of information is of potential relevance as it implies that CD8+ effectors can be driven toward transcriptional activation of recombination/revision genes with TCR engagement by antigens with diverse affinity.

**Discussion**

Tumor-associated T-cell responses demonstrate recognition of antigens associated with tumor cells or other components of the TME (55). In the dynamic reshaping of the antigenic landscape, adaptive T-cell clones undergo expansion and decline with different helper, effector, or regulatory functions and diverse receptor specificities. In the context of the TME, the engendering or proinflammatory conditions also favor the activation of T cells via antigen presentation in a costimulatory signal-rich setting, a process that is considered the basis of epitope spreading (56). Similar conditions may occur upon breaking tolerance toward self-antigens in autoimmunity. Under these circumstances, peripheral T-cell clones may be driven toward revising TCR specificity through the reinduction of recombination/revision machinery (20). The process of TCR revision, which has been reported to be dependent on the quality of the costimulatory and antigenic signals, such as CD40 costimulation and the presence of viral-associated superantigens (57), has been interpreted in the light of a salvage tolerance-promoting mechanism but has also been taken into account as underlying the generation of autoreactive peripheral T cells (58).

In the TME, the possibility to reinstate expression of TCR recombinase machinery could favor the generation of localized subsets of responding T cells, or, conversely, T-cell responses as part of tumor-immune escape. We identified the expression of recombinase/revision elements Rag1/2 and Dntt in a subset of tumor-infiltrating cells in syngeneic mouse models of invasive breast cancers and in human invasive breast cancer cases. The extremely low density of these cells, which could be also identified as occasional events in scRNA-seq data sets of tumor-associated T cells makes hard envisaging a relevance in the outcome of the tumor–immune interface. Nonetheless, the evidence of their increase in coisogenic tumors with Mlh1 deletion-triggered genetic instability per se and their detection within tumor-draining LNs suggests their involvement in the sensing of the tumor-associated antigens. Consistently, a significant increase in the frequency of Rag1/2- and Dntt-expressing TILs, more prominently of CD8+ T cells, was observed in the TS/A model following PD-1 checkpoint blockade, a condition associated with unrestrained TCR activation. Such an induction of Rag1/2- and Dntt-expressing CD8+ TILs was, however, not univocally associated with a specific outcome, either positive or negative, of anti–PD-1 treatment on tumor growth control. Yet, its abatement in an anti–PD-1–treated Mlh1- and B2m-deficient CT26 further underlined the importance of antigen-mediated TCR stimulation in the control of the Rag1/2/Dntt+ state. TCR-dependent development of specific T-cell subsets, such as naturally arising Tregs, is favored by the maintenance of low clonal frequencies in the microniche (59). In our setting, the expression of recombination/revision genes might be part of a complex regulation of TCR-mediated competition among peripheral precursor T cells. In our scRNA-seq analyses, a minor fraction of immune cells marked by RAG1/2 and/or DNTT expression were identified in the B-cell cluster (0.43% of the B cells). Immature B-cell precursors have been reported to be mobilized from the bone marrow of mice bearing 4T1 breast cancers, eventually supporting metastatic spreading (60). Besides B cells, other recombination/revision gene-expressing cells, falling into myeloid (0.4% of the myeloid) or undetermined (0.26% of the undetermined) clusters, were detected, which is in line with in situ data showing TdT+ or RAG1/2-expressing cells lacking T-cell phenotypic markers. Whether these events relate to a transient phenotype of a functionally differentiated cell upon recombination program reactivation or to a differentiation plasticity proper of precursor cells is not known. In conclusion, we report evidence of the multifarious landscape of tumor-associated immune responses by describing the potential occurrence of tumor-associated T cells expressing receptor recombination/revision machinery elements. The significance of the rare events that are described in this study deserves further investigation in ad hoc designed experimental settings.

**Authors’ Disclosures**

G. Germano reports grants from FPRC Saxmile 2017 Ministero Salute PTIRC-Intra 2020 (REGENERATION-YIG 2020 project) and NeoPhore, and other support from Fondazione Umberto Veronesi during the conduct of the study; other support from NeoPhore outside the submitted work; and a patent for PCT/GB2017/051062 pending and licensed to NeoPhore/Phoremost. A. Bardelli reports grants from AIRC during the conduct of the study, as well as grants from NeoPhore outside the submitted work. No disclosures were reported by the other authors.

**Authors’ Contributions**

G. Morello: Conceptualization, data curation, formal analysis, investigation, visualization, writing—original draft, writing—review and editing. V. Cancila: Conceptualization, data curation, formal analysis, investigation, visualization, writing—original draft, writing—review and editing. M. La Rosa: Resources, data curation, software, formal analysis, and methodology. G. Germano: Resources, formal analysis, investigation, visualization. D. Lecis: Resources, formal analysis, investigation, visualization. V. Amadio: Resources, formal analysis, investigation, visualization. F. Zanardi: Resources, data curation. F. Iannelli: Resources, data curation. D. Greco: Resources, data curation, software, formal analysis, methodology. L. La Paglia: Resources, data curation. A. Fiannaca: Resources, data curation. A.M. Urso: Resources, data curation. G. Graziano: Resources, data curation. F. Ferrari: Resources, data curation. S.M. Papa: Resources, data curation. S. Sangaletti: Resources, formal analysis, investigation, visualization, writing—original draft. C. Chi sodomini: Resources, investigation, methodology, G. Pruneri: Resources. A. Bardelli: Resources, data curation, supervision. M.P. Colombo: Conceptualization, formal analysis, supervision, investigation, visualization, writing—original draft, writing—review and editing. C. Tripodo: Conceptualization, data curation, formal analysis, supervision, validation, investigation, visualization, writing—original draft, writing—review and editing.

**Acknowledgments**

The authors are grateful to Professor Maurilio Ponsoni (San Raffaele Hospital, Milan), Dr. Stefano Casola and Dr. Federica Pisati (The FIRC Institute of Molecular Oncology, IFOM, Milan), and Dr. Davide Vacca (Tumor Immunology Unit, University of Palermo). The study was supported by Italian Foundation for Cancer Research (AIRC) under 5 per mille 2019 ID. 22759 program—G.L. C. Tripodo; AIRC IG ID. 22145 to C. Tripodo; AIRC Accelerator Award ID. 24296 to C. Tripodo and M.P. Colombo; AIRC 5 per mille ID. 21901 program to A. Bardelli; AIRC IG ID. 21923 to A. Bardelli; AIRC-CRUK-FC AEC; Accelerator Award ID. 22795 to A. Bardelli; and AIRCIGID. 18425 to C. Tripodo and G. Germano was supported by Fondazione Umberto Veronesi and by FPRC 5 per mille 2017 Ministero Salute PTIRC-Intra 2020 (REGENERATION-YIG 2020 project).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 31, 2020; revised January 8, 2021; accepted April 29, 2021; published first May 3, 2021.
References


12. Germano G, Lamba S, Rospo G, Barault L, Magr...


T Cells Expressing Receptor Recombination/Revision Machinery Are Detected in the Tumor Microenvironment and Expanded in Genomically Over-unstable Models

Gaia Morello, Valeria Cancila, Massimo La Rosa, et al.

*Cancer Immunol Res* Published OnlineFirst May 3, 2021.

**Updated version**
Access the most recent version of this article at:
doi:10.1158/2326-6066.CIR-20-0645

**Supplementary Material**
Access the most recent supplemental material at:
http://cancerimmunolres.aacrjournals.org/content/suppl/2021/05/01/2326-6066.CIR-20-0645.DC1

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, use this link
http://cancerimmunolres.aacrjournals.org/content/early/2021/06/08/2326-6066.CIR-20-0645. Click on “Request Permissions” which will take you to the Copyright Clearance Center’s (CCC) Rightslink site.