Frequency determination of breast tumor-reactive CD4 and CD8 T cells in humans: unveiling the antitumor immune response

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ABSTRACT

As cancer immunotherapy gains importance, the determination of a patient’s ability to react to his/her tumor is unquestionably relevant. Though the presence of T cells that recognize specific tumor antigens is well established, the total frequency of tumor-reactive T cells in humans is difficult to assess, especially due to the lack of broad analysis techniques. Here, we describe a strategy that allows this determination, in both CD4 and CD8 compartments, using T cell proliferation induced by tumor cell-lysate pulsed dendritic cells as the readout. All 12 healthy donor tested had circulating CD4 and CD8 tumor cell-reactive T cells. The detection of these T cells, not only in the naïve but also in the memory compartment, can be seen as an evidence of tumor immunosurveillance in humans. As expected, breast cancer patients had higher frequencies of blood tumor-reactive T cells, but with differences among breast cancer subtypes. Interestingly, the frequency of blood tumor-reactive T cells in patients did not correlate to the frequency of infiltrating tumor-reactive T cells, highlighting the danger of implying a local tumor response from blood obtained data. In conclusion, these data add T cell evidence to immunosurveillance in humans, confirm that immune parameters in blood may be misleading and describe a tool to follow the tumor-specific immune response in patients and, thus, to design better immunotherapeutic approaches.

Introduction

Cancer is a disease of one’s own cells that acquire characteristics that allow tumor progression, due to the accumulation of mutations and protein expression changes. These protein modifications give rise to a number of different antigens that have the potential to be recognized by T cells, a phenomenon that is central to the development of specific immune responses. Today, the ability of the immune system to recognize tumor cells through the presence of T cells that are specific for different tumor antigens is well acknowledged, and is a fundamental concept used in the development of new immunotherapeutic approaches. Indeed, a number of studies have shown, in humans, the existence of both CD4 and CD8 T cells specific for mutated and non-mutated tumor-associated antigens. Not surprisingly, since T cell receptor (TCR) generation is random and independent of presence or exposure to antigens, tumor-specific T cells are found also in healthy individuals.

The presence of tumor-specific T cells has been shown to affect patient prognosis and the response to therapy, even for non-immunological treatments. Thus, tracking the T cell response against the tumor during the course of the disease and therapy is becoming an urgent need, since it can be a powerful tool to improve personalized approaches to treatment. However, although the presence of T cells that recognize certain tumor antigens can be measured using multimers of major histocompatibility complex (MHC) molecules, the construction of multimers for all possible antigens in a tumor is not possible. Broader methods usually used consist of tumor-specific T cell cytotoxicity evaluation or cytokine production (like IFN-γ ELISPOT) as readouts of specificity. Although valid specially for the CD8 compartment, these strategies restrict the evaluation to T cells that have specific cytokine-secretion patterns or that are able to kill tumor cells.

Thus, considering these challenges to assess the total frequency of tumor-reactive T cells in humans, we developed a strategy to determine the frequency of tumor-reactive CD4 and CD8 T cells. Sallusto’s group has described a technique that allows the quantification of antigen-specific T lymphocytes, by generating T cell libraries through non-specifically expanding and challenging T cells with antigen-presenting cells (APC) loaded with the antigens of interest. Here, we include modifications that would allow this quantification in the cancer setting, where antigen-specific T cells may have low antigen affinity due to their resemblance to self-antigens, and/or may be exhausted, thus, decreasing their ability to proliferate. Accordingly, we chose monocyte-derived...
dendritic cells (moDC) as APC, due to their unique ability to prime naïve T cells and even to overcome tolerance-induced anergy. Also, to prevent the analytical bias that the initial nonspecific proliferation could generate in exhausted T cells, we adapted the method in order to allow the frequency determination from nonexpanded T cells. Finally, we tracked T cell proliferation by CFSE dilution, rather than H\(^3\)-thymidine incorporation, to allow the visualization and sorting of the antigen-specific T cells. Using this strategy, we were able to estimate the total frequency of blood CD4 and CD8 T cells reactive for the whole antigen pool expressed by breast tumor cells, in both healthy individuals and breast cancer patients, and in these, also among tumor-infiltrating lymphocytes. The use of this method opens the possibility to track the tumor-specific T cell response in patients, providing a valuable tool for measuring the tumor immune response, before, during and after treatment.

Results

The use of tumor-lysate loaded moDC allows the quantification of tumor-reactive T cells

The broad analysis of tumor-reactive T cells in cancer patients is still methodologically challenging. Tumor-specific T cells can be evaluated by multimers, but this is restricted to single epitopes of specific antigens, or by T cell cytotoxicity and cytokine production, which restrict the evaluation to T cells with specific phenotypes. Thus, to calculate the frequency of tumor-reactive T cells, considering all antigens expressed by the tumor and regardless of their function, a strategy based on antigen-induced T cell proliferation was developed and used in this work. Since dendritic cells are the most potent antigen presenting cell, moDC were generated in vitro from blood monocytes, loaded with tumor lysates (from tumor cell lines or ex vivo tumor samples) and cocultured with purified autologous CFSE-labelled T cells (Figure 1a). Before coculture, moDC were irradiated and stained with CFSE, to avoid the confusion of proliferating T cells with the added APC. The irradiation would prevent the added cells to proliferate, while their CFSE staining would avoid the confusion of non-labeled added cells with proliferating CFSE\(^{low}\) cells. For each lysate, at least eight wells were seeded, and were independently cocultured. After 5 days of coculture, cells from each well were independently harvested and the number of CFSE\(^{low}\)CD25\(^{+}\) proliferating T cells was determined by flow cytometry.

As expected, when stimulated with unloaded autologous moDC, T cells did not proliferate (Figure 1b), but when tumor-lysate loaded moDC were added to the T cell wells, some wells, but not all, showed T cell proliferation,
determined by the appearance of T cells with CFSE dilution and CD25 expression (Figure 1c). It is important to note that the frequency of CFSE\(^{low}\)CD25\(^+\) T cells in positive wells was small, suggesting that these wells contained few or even a single responding T cell clone. This is a requirement of the method, and indicates that it will only be useful where the antigen-specific T cell frequency is low, since the Poisson distribution is based on the frequency of negative wells. Negative wells were those where the frequency of CFSE\(^{low}\)CD25\(^+\) T cells was lower than a threshold value. The threshold was calculated as the mean plus 1.96 times the standard deviation of control wells challenged with unloaded moDC, which would establish a 95% confidence interval, considering that the data follow a normal distribution.

As a control, autologous moDC were loaded with allogeneic PBMC lysates and the frequencies of responding CD4 and CD8 T cells were determined. As expected, the frequencies of responding cells were low (Figure 1d).

**Patients and healthy individuals have tumor-reactive T cells**

To evaluate the frequency of tumor-reactive T cells in the blood of healthy individuals and breast cancer patients, we used lysates from three model cell lines of different breast cancer subtypes: MCF-7, a luminal breast cancer cell line; SK-BR-3, a breast cancer cell line with Her2 overexpression; and MDA-MB-231, a triple-negative breast cancer cell line. Lysates from these cell lines can be easily generated and stored and, if the tumor-reactive frequencies found with their use were comparable to those found using patients’ samples, it would preclude the necessity of tumor samples from the patients for this analysis.

We were able to find CD4 and CD8 T cells reactive for at least one of the three cell lines in the blood of all healthy donors (Figure 2). The frequency of T cells reactive for the cell lines in the blood of luminal breast cancer patients was similar to that found in healthy donors. Unexpectedly, Her2-subtype tumor-bearing patients were those with the highest frequency of tumor cell lines-reactive T cells, while triple-negative breast cancer patients had the lowest. Half of the luminal patients showed no CD8 T cells reactive for MCF-7, the cell line described as having the same subtype of the patients’ tumor. Interestingly, there was no difference between the frequency of tumor-reactive T cells against the cell line corresponding to the patients’ tumor subtype and the other cell lines. Overall healthy individuals and breast cancer patients tend to have higher frequencies of CD8 than CD4 tumor-reactive T cells in the blood. However, this higher CD8 T cell frequency was statistically significant only for luminal patients challenged with the triple-negative tumor cell line (Figure 2c).

We also challenged T cells (from healthy donors and from patients) with autologous moDC loaded with tumor lysates obtained from different ex vivo breast cancer samples. Healthy individuals showed frequencies of tumor-reactive T cells when challenged by tumor lysates obtained from ex vivo samples similar to those obtained against cell lines’ lysates (Figure 3a). Intriguingly, luminal breast cancer patients, when challenged with lysates coming from ex vivo samples from different patients, seemed to have more reactive CD8 T cells, than they had against the cell lines (Figure 3b). The same tendency to observe higher frequencies of tumor-reactive T cells when the challenging antigens were obtained from ex vivo samples was present in patients with Her2-overexpressing tumors (Figure 3c), but, in this case, the tendency occurred also for CD4 T cells. These phenomena may point to essential differences between cell lines and tumors in vivo, and provide an argument against the use of cell lines to evaluate T cell reactivity to tumors.

Interestingly, luminal breast cancer patients showed significantly higher frequencies of tumor-reactive CD8 T cells than tumor-reactive CD4 T cells (Figure 3b). This predominance of tumor-reactive CD8 T cells was not clearly observed in healthy individuals neither in patients bearing the other cancer subtypes, which seem to have equivalent frequencies of both CD4 and CD8 tumor-reactive T cells.

Overall, tumor patients seem to have higher frequency of tumor-reactive T cells in the blood than healthy donors, except for the triple-negative patients (Figure 3d). Luminal breast cancer patients had a strong tendency to have more CD8 T cells reactive for ex vivo tumor lysates than healthy donors (ex vivo tumor-reactive CD8 T cells in healthy donors: 25.3 ± 36.4, n = 20, vs ex vivo tumor-reactive CD8 T cells in luminal breast cancer: 103.0 ± 196.1, n = 14; p = 0.077). This tendency was weaker but present for both CD4 and CD8 T cells of Her2-subtype tumor-bearing patients, where some patients had a frequency of T cells reactive for Her2 ex vivo lysates of at least twice as large than found in healthy donors challenged with the same type of lysates.

**In healthy donors, tumor-reactive T cells were found in both naïve and memory compartments**

The presence of tumor-reactive T cells in healthy donors is expected, since the TCR repertoire is generated before and independently from antigen exposure. However, in the absence of prior tumor exposure, these T cells should be in the naïve compartment. To address this question, we evaluated whether tumor-specific T cells found in the blood of healthy donors were naïve or memory cells. For this, CD3\(^+\) T cells were separated by fluorescence-activated cell sorting into CD45RA\(^+\)CCR7\(^+\) cells, corresponding to the naïve population, and the remaining, which should contain all memory subpopulations. Both naïve and memory cells were independently cocultured with autologous moDC loaded with lysates from the SK-BR-3 tumor cell line. Since, in healthy donors, the frequency of reactive CD4 and CD8 T cells was more homogeneous for the SK-BR-3 cell line, the lysate from this cell line was used to detect naïve T cells, which were present in all donors, among CD4 cells, but only in some, for CD8 cells (Figure 4a). However, tumor-reactive memory T cells were present in the blood of all individuals tested against at least one of the three model tumor cell lines used (Figure 4b). Thus, although it might be unexpected, healthy individuals presented tumor-reactive memory T cells. This could be explained by cross-reactivity, but also by a previous encounter with the tumor antigen in a lesion that did not progress into
a tumor. Regardless of their priming, these memory T cells would be capacitated to perform an active immunosurveillance against the development of tumors that express the antigens they recognize.

The frequency of tumor-reactive T cells in the blood was not positively correlated to the frequency of tumor-reactive infiltrating T cells in the patients

To evaluate the presence and frequency of autologous tumor-reactive T cells infiltrating the tumor, we isolated tumor-infiltrating T lymphocytes (T-TIL) from tumors, obtaining similar number of T-TIL per milligram of tumor, regardless of the tumor subtype (Figure 5a). These T-TIL were cocultured with moDC loaded with autologous tumor lysates, and the frequency of tumor-reactive T-TIL was calculated and compared to the frequency of autologous tumor-reactive T cells in the blood of the same patient. Surprisingly, no correlation was found between the frequencies of tumor-reactive T cells in the blood and of T-TIL (Figure 5b).

Furthermore, the majority of the patients, regardless of their tumor subtype, showed a higher frequency of tumor-reactive CD4 T cells infiltrating the tumor than in the blood,
a difference that did not occur for tumor-reactive CD8 T cells (Figure 5c). Actually, 11 out of 14 patients had more tumor-reactive CD8 T cells than CD4 T cells in the blood, the inverse (4 out of 12) was observed among T-TIL. Interestingly, all three patients that had more tumor-reactive CD4 T cells than tumor-reactive CD8 T cells in the blood had Her2-subtype tumors. This CD4 predominance among T-TIL might be related to a higher susceptibility of CD8 T cells to the immunosuppressive factors present in the tumor. Indeed, this possible immunosuppressive environment would be coherent with the tumor-reactive T cell frequency among T-TIL. This was never higher than 300 T cells per million, a very low frequency, even if consider that both activated antigen-specific T cells and antigen-unrelated memory cells migrate
to non-lymphoid tissues,\textsuperscript{15} but that could be explained, if we consider that such environment might prevent specific T cells to proliferate and, thus, be detected.

Nevertheless, we did observe an enrichment of tumor-reactive T cells within tumors. Patients bearing luminal breast cancers (Figure 5d) showed significantly higher frequency of tumor-reactive CD4 T cell among T-TIL, than among blood CD4 T cells (tumor-reactive CD4 T-TIL: 80.4 ± 68.8 vs blood tumor-reactive CD4 T cells: 18.0 ± 32.1; \( p = 0.04; n = 6 \)). Though this is in agreement with the reported greater frequency of antitumor than antivaccine T cells inside melanomas,\textsuperscript{16} in our data, it was dependent on the tumor type, since in patients with Her2 subtype tumors, this tumor-specific enrichment was not observed (Figure 5e). Triple-negative tumor-bearing patients appear to have enrichment for both CD4 and CD8 T cells inside the tumor, a difference, though, that was not statistically significant (Figure 5f).

\section*{Discussion}

Few are the reports in the literature that broadly evaluate the frequency of T cells capable of recognizing a tumor and, to the best of our knowledge, none of these refer to breast tumors. The most abundant data refer to the frequency of melanoma-specific CD8 cytotoxic T lymphocytes (CTLs). It is known that the frequency of tumor antigen-specific T cells in melanoma is higher than in other tumors, most likely due to its high mutational burden.\textsuperscript{17} Studies using different techniques have shown that the frequency of melanoma-reactive CD8 T cells in the blood of patients can range from 15 to 1000 T cells per million, more frequently...
around 200 CTLs per million. Our data show that the frequency of tumor-reactive CD8 T cells in breast cancer patients’ blood reaches up to 800 per million but, more frequently is around five to ten times lower than the average of 200 per million found in melanoma patients. The literature lacks data on the frequency of tumor-reactive CD4 T cells, but our data show that these cells are present in breast cancer patients’ blood, at lower frequencies than tumor-reactive CD8 T cells in luminal subtype tumors patients, but at similar frequencies in Her2 and triple-negative breast cancer patients. It is true that this technique, which relies on the T cell proliferation to detect specificity, will fail to detect non-proliferating cells, a phenomenon that might affect especially poorly proliferating cells, like regulatory T cells, but, nevertheless it allowed us to broadly recognize and quantify tumor-reactive CD4 and CD8 T cells in breast cancer patients, a condition where this had not been reported. Still, CFSE dilution can detect very few rounds of T cell proliferation that might be enough to spot even such poorly proliferating cells.

This same limitation may contribute, however, to point to differences in the immunosuppressive environment of tumors and the sensitivity of T cells to it. In patients with luminal-type tumors, the frequency of CD8 T-TIL was clearly lower than that of CD4 T-TIL, in an inverse correlation to what we found in blood, but the same was not true in patients with Her2 and triple-negative subtype tumors.Could this point to a different immune contexture of these tumor subtypes, and/or to differences between CD4 and CD8 cells to that contexture? In any case, the restriction of detecting only proliferating T cells might even be seen as positive, since these would be the cells more readily responding to the antigenic challenges, and, thus, melding the disease evolution in the patients.

Tumor samples from cancer patients can be difficult to obtain due to both localization and size of the tumor. Thus, it would be desired not to need tumor cells from the patient to evaluate the tumor-reactive T cell repertoire. Thus, we compared the frequency of tumor-reactive T cells found using cell lines, which could be a good alternative to tumor samples, versus ex vivo tumor samples obtained from breast cancer patients, and found, however, significant differences among them. These phenomena may point to essential differences between cell lines and tumors in vivo, since fresh tumor cells seem to share more pathways which each other than they share with tumor cell lines. Though these data are not definitive, they indicate that, when aiming to study a patient’s immune repertoire, and not having an autologous tumor sample for that it would be better to use another ex vivo sample rather than using tumor cell lines as source of tumor antigens.

Interestingly, we could find tumor-reactive memory CD4 and CD8 T cells in the healthy donors tested. Contrastingly, in the literature, no memory T cells for CMV were reported, using similar analysis methods, in CMV-seronegative donors, which gives more credibility to our finding. This presence of tumor-reactive memory T cells in healthy individuals can be seen as another evidence for the immune surveillance against cancer. This theory predicts that the immune system is constantly searching and eliminating cells that become malignant and, if true, this elimination should be traceable, in the form of memory cells, as we, indeed, found. One can argue that these tumor-reactive T cells might have been induced by cross-reactivity with non-tumor antigens, and, thus, had no relation with tumor surveillance. Although we cannot discard this hypothesis, all attempts to measure the frequency of cross-reactive T cells showed that these are rare, and regardless of the inducing antigen, memory T cells acquire the ability to migrate into non-lymphoid tissues. Therefore, these memory T cells would impart to the system the ability to patrol the whole body against tumors, hence, providing a mechanistic support for its already demonstrated ability to edit tumors.

It is also important to note that, although in most experiments we used allogeneic lysates, the proliferation observed was not due to an allogeneic effect, since the frequency of reactive T cells against allogeneic PBMC was very low. This reinforces the hypothesis that the observed frequencies were due to T cells that were specific for tumor-associated antigens present in the lysates of tumor cells, but absent in normal PBMC.

Among the different breast cancer subtypes, the triple-negative is considered to be the most immunogenic, due to its higher frequency of mutations, higher expression of cancer testis antigens and higher immune infiltration. Surprisingly, however, triple-negative breast cancer patients showed the lowest frequencies of tumor-reactive T cells in the blood. The presence of tumor-reactive T cells in the blood, however, may not reflect with precision the tumor microenvironment. Our data point to this lack of correlation between the frequency of tumor-reactive T cells found in the blood and infiltrating the tumor of breast cancer patients and, while in the blood of triple-negative breast cancer patients they were less frequent, infiltrating the tumor they were detected in similar frequencies as in Her2 patients. Thus, although mouse models have shown systemic and coordinated antitumor immunity after immunotherapy, we show, here, that in humans the circulating and local tumor-reactive T cell frequencies may differ significantly. Therefore, data of blood tumor immunity should be carefully considered before being extrapolated as representative to the conditions inside the tumor.

In conclusion, the method showed here seems to be a robust and powerful strategy to evaluate the T cell response against cancer. The data obtained concerning breast cancer unveiled some intriguing aspects of the tumor-reactive T cell repertoire in humans – as the presence of tumor-reactive memory T cells in healthy donors and the lack of correlation between the frequencies of tumor-reactive T cells in the blood and infiltrating the tumors. Noteworthy, this work opens a range of new perspectives to the field of tumor immunology, since the strategy here described allows the quantification of the total frequency of tumor-reactive T cells in the blood as well as tissue-resident and tumor-infiltrating. Also, it opens the possibility to track the T cell response of a patient over time, allowing the design and improvement of personalized therapeutic approaches.

Materials and methods

Patient’s sample collection

This work was approved by the ethics committee of the Institute of Biomedical Sciences of the University of Sao Paulo
statistically significant (41905115.0.0000.5467) and all methods were performed in accordance with the relevant guidelines and regulations. Peripheral blood and leukapheresis chambers were collected after participants gave written informed consent. Women with ductal invasive carcinomas treated at the Perola Byington Hospital, Sao Paulo, Brazil, were included after signing an informed consent form (Table 1). Blood was collected right before resection of the primary tumor, and fresh tumor sample, when available, were obtained during surgery.

### Table 1. Age, sex and tumor subtype of patients included in the study.

| Age | Sex | Tumor subtype | Blood | Tumor |
|-----|-----|---------------|-------|-------|
| 39  | F   | Luminal       | Yes   | Yes   |
| 64  | F   | Luminal       | Yes   | Yes   |
| 49  | F   | Luminal       | Yes   | Yes   |
| 51  | F   | Luminal       | Yes   | Yes   |
| 55  | F   | Luminal       | Yes   | No    |
| 47  | F   | Luminal       | Yes   | No    |
| 60  | F   | Luminal       | Yes   | No    |
| 44  | F   | Luminal       | Yes   | No    |
| 50  | F   | Luminal       | Yes   | Yes   |
| 58  | F   | Her2          | Yes   | Yes   |
| 48  | F   | Her2          | Yes   | Yes   |
| 45  | F   | Her2          | Yes   | No    |
| 59  | F   | Her2          | Yes   | No    |
| 50  | F   | Her2          | Yes   | No    |
| 44  | F   | Triple-negative| Yes | Yes   |
| 45  | F   | Triple-negative| Yes | Yes   |
| 59  | F   | Triple-negative| Yes | Yes   |

Peripheral blood mononuclear cells (PBMC) were obtained by separation over Ficoll-Paque gradient (GE Healthcare Cat# 17–1440-03). PBMC (5 x 10⁶) were seeded in 12-well culture plates and incubated for 2 h at 37°C and 5% CO₂. After incubation, nonadherent cells were removed and kept in culture during DC differentiation, while the adherent cells were cultured in AIM-V (Thermo Fisher Scientific Cat# 12055–091) supplemented with GM-CSF (50 ng/ml; Peprotech Cat# 300–03) and IL-4 (50 ng/ml; Peprotech Cat# 200–04). After five days, the cells received a maturation stimulus with TNF-α (50 ng/ml; Peprotech Cat# 300–01A), and were harvested using PBS + EDTA (2 mM), 48 h after activation.

### PBMC isolation and monocyte-derived dendritic cell (moDC) generation

T cells were purified using the nonadherent cell fraction by negative selection with the Pan T cell isolation kit (Miltenyi Biotec Cat# 130–096-535), following the manufacturer’s protocol. When isolated naïve and memory T cells were needed, T cells were labeled with fluorescence-labeled antibodies specific for CD4 (BD Biosciences Cat# 555347, RRID: AB_395752), CD8 (Thermo Fisher Scientific Cat# 46–0087-42, RRID:AB_1834411), CD45RA (Thermo Fisher Scientific Cat# 11–0458-42, RRID:AB_11219672) and CCR7 (Thermo Fisher Scientific Cat# 25–1979-41, RRID:AB_2573421) and sorted using the cell sorter FACS ARIA II" (BD Biosciences). Events double-positive for CD45RA and CCR7 were sorted as naïve T cells, while the other events were sorted as memory T cells.

### Tumor sample processing and tumor-infiltrating lymphocytes isolation

Fresh tumor samples obtained from surgical resection of the primary breast cancer lesion were minced and digested with collagenase type VIII (0.56 mg/ml Sigma-Aldrich Cat# C2139-5G), under agitation, at 37°C for 2 h. Single-cell suspensions were separated from the non-digested fragments using sterile gauze and washed twice in RPMI-1640 (Thermo Fisher Scientific Cat# 31800–014). T-TIL were isolated from the cell suspension by magnetic positive selection with CD3 microbeads (Miltenyi Biotec Cat# 130–050-101), according to the manufacturer’s protocol. The negative fraction with the tumor cells was used, together with the fragments recovered after the tissue dissociation, for the generation of the tumor lysates. These materials were resuspended in PBS, submitted to five cycles of freeze/thaw in liquid nitrogen and centrifuged at 12,000 x g for 10 min. The supernatant was recovered in another tube and kept at –80°C to be used as tumor lysate. In the case of lysates from tumor cell lines, the same process was done using cell suspension from each cell line: MCF-7 (RRID:CVCL_0031), MDA-MB-231 (RRID:CVCL_0062) and SK-BR-3 (RRID: CVCL_0033). Mycoplasma-free cell lines were obtained from the "Rio de Janeiro Cell Bank" (BCRJ), and used within no more than 10 passages from thawing. Protein quantification was performed by the Bradford assay.

### Frequency of reactive T cells determination

T cells were labeled with 2.5 μM CFSE (Thermo Fisher Scientific Cat# C34554) and seeded in multiple independent wells of a 96-well U-bottom culture plate at a 10:1 lymphocyte:DC ratio. Dendritic cells were seeded after being labeled with CFSE and gamma-irradiated with 45 Gy. Tumor lysates were added to at least eight replicate wells, at a final concentration of 10 μg of protein/mL. Cells were cultured in a final volume of 200 μL RPMI-1640 supplemented with 5% human AB serum, 1% antibiotic-antimycotic (Thermo Fisher Scientific Cat# 15240–096), 1 mM sodium pyruvate (Lonza Cat# 13-115E), 1% Non-essential Amino Acid mixture (Lonza Cat# 13-114E), 2 mM L-glutamine (Lonza Cat# 17-605E) and 5 x 10^{-5} M β-Mercaptoethanol (Sigma-Aldrich Cat# M3148). After five days, each well was independently harvested, stained with a viability dye (Live/Dead®, Thermo Fisher Scientific Cat# L34955) and antibodies specific for CD25 (BD Biosciences Cat# 555432, RRID:AB_395826), CD3 (BD Biosciences Cat# 557852, RRID:AB_1834411), CD45RA (BD Biosciences Cat# 31800–014, RRID:AB_395752), CCR7 (BD Biosciences Cat# 25–1979-41, RRID:AB_2573421) and analyzed by flow cytometry. T cell proliferation was assessed by CFSE dilution and CD25 expression. Wells were considered as positive for T cell proliferation when the percentage of CFSE⁺CD25⁺ in that well was superior to a threshold value calculated as the mean plus 1.96 times.
Statistical analysis

All the results were analyzed with the Shapiro-Wilk normality test. Parametric statistical tests were used only when the samples passed the normality test (p > 0.05), whereas samples that did not pass the normality test were analyzed with non-parametric tests. For the statistical analysis between two groups, we used the paired and unpaired t-test (parametric) or Wilcoxon test (paired, non-parametric) and Mann–Whitney test (unpaired, non-parametric). The differences were considered statistically significant if the descriptive level was lower than 5%, that is, the p-value was less than or equal to 0.05. The statistical analysis and graphs presented were generated using GraphPad Prism® software version five for Windows. Graphs show means ± SD.

Acknowledgments

The authors thank Dr. Fabio Laginha and Henrique Gomes Guedes from the Pêrola Byington Hospital for providing the patient samples, the blood bank from Oswaldo Cruz Hospital for providing the leukoreduction chamber and the Irradiation Centre of the IPEN for performing the irradiations.

Disclosure of potential conflicts of interest

The authors report no conflict of interest.

Funding

This work was supported by the Sao Paulo Research Foundation - FAPESP under Grant (2014/25988-1 and 2016/10137-8); the Coordination for the Improvement of Higher Education Personnel – CAPES; and the National Council for Scientific and Technological Development - CNPq under Grant (409825/2016-6) and (308053/2017-6).

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References

1. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100(1):57–70. doi:10.1016/S0092-8674(00)00204-9.
2. Coulier PG, Van Den Eynde BJ, Van Der Bruggen P, Boon T. Tumour antigens recognized by T lymphocytes at the core of cancer immunotherapy. Nat Rev Cancer. 2014;14(2):135–146. doi:10.1038/nrc3670.
3. Vigneron N. Human tumor antigens and cancer immunotherapy. Biomed Res Int. 2015;2015:948501. doi:10.1155/2015/948501.
4. Bioley G, Jandus C, Tuyaets S, Rimoldi D, Kwok WW, Speiser DE, Tiercy JM, Thielemans K, Cerottini JC, Romero P. Melan-A/MART-1-specific CD4 T cells in melanoma patients: identification of new epitopes and ex vivo visualization of specific T cells by MHC class II tetramers. J Immunol. 2006;177(10):6769–6779. doi:10.4049/jimmunol.177.10.6769.
5. Gros A, Parkhurst MR, Tran E, Pasetto A, Robbins PF, Ilyas S, Prickett TD, Gartner JJ, Crystal JS, Roberts JM, et al. Prospective identification of neoantigen-specific lymphocytes in the peripheral blood of melanoma patients. Nat Med. 2016;22(4):433–438. doi:10.1038/nm.4051.
6. Tonegawa S, Brack C, Hozumi N, Matthysssens G, Schuller R. Dynamics of immunoglobulin genes. Immunol Rev. 1977;36:73–94. doi:10.1111/imr.1977.36.001.
7. Pittet MJ, Valmori D, Dunbar PR, Speiser DE, Lienard D, Lejeune F, Fleischhauer K, Cerundolo V, Jeremic J, Romero P. High frequencies of naive Melan-A/MART-1-specific CD8(+) T cells in a large proportion of human histocompatibility leukocyte antigen (HLA)-A2 individuals. J Exp Med. 1999;190 (5):705–715. doi:10.1084/jem.190.5.705.
8. Barnes TA, Amir E. HYPE or HOPE: the prognostic value of infiltrating immune cells in cancer. Br J Cancer. 2017;117 (4):451–460. doi:10.1038/bjc.2017.220.
9. Corti D, Sallusto F, Lanzavecchia A. High throughput cellular screens to interrogate the human T and B cell repertoires. Curr Opin Immunol. 2011;23(3):430–435. doi:10.1016/j.coi.2011.04.006.
10. Geiger R, Duhun T, Lanzavecchia A, Sallusto F. Human naive and memory CD4+ T cell repertoires specific for naturally processed antigens analyzed using libraries of amplified T cells. J Exp Med. 2009;206(7):1525–1534. doi:10.1084/jem.20090504.
11. Trager U, Sierra S, Djordjevic G, Bouzo B, Khandwala S, Meloni A, Mortensen M, Simon AK. The immune response to melanoma is limited by thymic selection of self-antigens. PLoS One. 2012;7(4):e35005. doi:10.1371/journal.pone.0035005.
12. Thommen DS, Schumacher TN. T cell dysfunction in cancer. Cancer Cell. 2018;33(4):547–562. doi:10.1016/j.ccell.2018.03.012.
13. Boog CJ, Kast WM, Timmers HT, Boes J, de Waal LP, Melief CJ. Abolition of specific immune response defect by immunization with dendritic cells. Nature. 1985;318(6041):59–62. doi:10.1038/318059a.
14. Steinman RM. Dendritic cells: understanding immunogenicity. Eur J Immunol. 2007;37(S1):553–560. doi:10.1002/eji.200737400.
15. Chen AM, Khanna N, Stohlman SA, Bergmann CC. Virus-specific and bystander CD8 T cells recruited during virus-induced encephalomyelitis. J Virol. 2005;79(8):4700–4708. doi:10.1128/JVI.79.8.4700-4708.2005.
16. Lurquin C, Lethé B, De Plaen E, Théate I, Van Baren N, Coulier PG, Boon T. Contrasting frequencies of antitumor and anti-vaccine T cells in metastases of a melanoma patient vaccinated with a MAGE tumor antigen. J Exp Med. 2005;201 (2):249–257. doi:10.1084/jem.20041378.
17. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SAJR, Behjati S, Biankin AV, Bignell GR, Bolli N, Borg A, Børresen-Dale AL, et al. Signatures of mutational processes in human cancer. Nature. 2013;500(7463):415–421. doi:10.1038/nature12477.
18. Mazzocchi A, Belli F, Mascheroni L, Veggetti C, Parmiani G, Anichini A. Frequency of cytopotoxic T lymphocyte precursors (CTLp) interacting with autologous tumor via the T-cell receptor: limiting dilution analysis of specific CTLp in peripheral blood and tumor-invaded lymph nodes of melanoma patients. Int J Cancer. 1994;58:330–339.
19. Germeau C, Ma W, Schiavetti F, Lurquin C, Henry E, Vigneron N, Brassier F, Lethé B, De Plaen E, Velu T, et al. High frequency of antitumor T cells in the blood of melanoma patients before and after vaccination with tumor antigens. J Exp Med. 2005;201(2):241–248. doi:10.1084/jem.20041379.
20. Ertel A, Verghese A, Byers SW, Ochs M, Tozener A. Pathway-specific differences between tumor cell lines and normal and tumor tissue cells. Mol Cancer. 2006;5(1):55. doi:10.1186/1476-4588-5-55.
21. Ehrlich P. Über den jetzigen Stand der Karzinomforschung. Ned Tidschr Geneesk. 1909;5:273–290.
22. Petrova G, Ferrante A, Gorski J. Cross-reactivity of T cells and its role in the immune system. Crit Rev Immunol. 2012;32(4):349–372. doi:10.1615/CritRevImmunol.v32.i4.
23. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity’s roles in cancer suppression and promotion. Science. 2011;331(6024):1565–1570. doi:10.1126/science.1203486. (80)
24. Liu Z, Li M, Jiang Z, Wang X. A comprehensive immunologic portrait of triple-negative breast cancer. Transl Oncol. 2018;11(2):311–329. doi:10.1016/j.tranon.2018.01.011.
25. Spitzer MH, Carmi Y, Reticker-Flynn NE, Kwek SS, Madhireddy D, Martins MM, Gherardini PF, Prestwood TR, Chabon J, Bendall SC, et al. Systemic immunity is required for effective cancer immunotherapy. Cell. 2017;168(3):487–502e15. doi:10.1016/j.cell.2016.12.022.
26. Henry C, Marbrook J, Vann DC, Kodlin D, Wofsy C. Limiting dilution analysis. Sel Methods Cell Immunol. In: Mishell BB, Shiggi, SM, editors. Selected methods in cellular immunology. San Fracisco: W. H. Freeman & Co;1980;138.