Innovative therapy, monoclonal antibodies, and beyond: Highlights from the eighth annual meeting

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ABSTRACT

The eighth annual conference of "Innovative therapy, monoclonal antibodies, and beyond" was held in Milan on Jan. 26, 2018, and hosted by Fondazione IRCCS–Istituto Nazionale dei Tumori (Fondazione IRCCS INT). The conference was divided into two main scientific sessions, of *i*) pre-clinical assays and novel biotargets, and *ii*) clinical translation, as well as a third session of presentations from young investigators, which focused on recent achievements within Fondazione IRCCS INT on immunotherapy and targeted therapies. Presentations in the first session addressed the issue of cancer immunotherapy activity with respect to tumor heterogeneity, with key topics addressing: 1) tumor heterogeneity and targeted therapy, with the definition of the evolutionary Index as an indicator of tumor heterogeneity in both space and time; 2) the analysis of cancer evolution, with the introduction of the TRACERx Consortium—a multi-million pound UK research project focused on non-small cell lung cancer (NSCLC); 3) the use of anti-estrogen agents to boost immune recognition of breast cancer cells; and 4) the high degree of functional plasticity within the NK cell repertoire, including the expansion of adaptive NK cells following viral challenges. The second session addressed: 1) the effectiveness of radiotherapy to enhance the proportion of patients responsive to immune-checkpoint blockers (ICBs); 2) the use of MDSC scores in selecting melanoma patients with high probability to be responsive to ICBs; and 3) the relevance of the gut microbiome as a predictive factor, and the potential of its perturbation in increasing the immune response rate to ICBs. Overall, a picture emerged of tumor heterogeneity as the main limitation that impairs the effectiveness of anti-cancer therapies. Thus, the choice of a

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specific therapy based on reproducible and selective predictive biomarkers is an urgent unmet clinical need that should be addressed in order to increase the proportion of long-term responding patients and to improve the sustainability of novel drugs.

1. Introduction

National and international clinical and pre-clinical experts in immuno-oncology and cancer cell signaling gathered at the eighth annual edition of "Innovative therapy, monoclonal antibodies, and beyond," organized by Fondazione IRCCS Istituto Nazionale dei Tumori (Fondazione IRCCS INT) in Milan, Italy, on Jan. 26, 2018. State-of-the art approaches and the current and future challenges in the fields of immune-, targeted- and radio-therapies were presented. Specifically, Session I comprised the following topics: 1) tumor heterogeneity and targeted therapy; 2) repurposing anti-estrogens to boost drug immune-mediated activity in breast cancer; 3) functional diversification of human NK cells: implications for cell-based cancer immunotherapy; 4) neoantigen landscape and its impact on immune checkpoint inhibition; and 5) links between the gut microbiome and tumor immune surveillance. Session II dealt with: 1) MDSC scores; 2) radiotherapy in the era of ICI-based immunotherapy; and 3) Immune checkpoint inhibitors as new combinations in melanoma. Finally, the conference was closed with presentations in Session III from selected abstracts of junior researchers, on the topics of: 1) improvement of HER-2–positive gastric cancer patient selection for trastuzumab treatment; 2) the impact of cycling a fasting mimicking diet (FMD) on cancer patient metabolism; 3) upregulation of PD-L1 expression in triple-negative breast cancer stem cells as a potential shield against immune-mediated tumor rejection; and 4) the neutrophil extracellular traps and alteration of T-cell homeostasis in the bone marrow leukemic niche. Overall, clinicians and basic and translational scientists provided the audience with a broad overview of the most recent achievements that have been put forward as ways of best exploiting personalized anti-cancer treatment.

2. Session I: preclinical assays and novel biotargets

2.1. Tumor heterogeneity and targeted therapy

In Session I, Filippo De Braud (Fondazione IRCCS INT and Department of Oncology and Hemato-oncology, University of Milan) addressed the key topic of tumor heterogeneity as the crucial "cause" for anti-tumor therapies resistance and failure. In particular, and according to the extensive literature on this subject, the speaker distinguished two types of cancer-related heterogeneity as inter- and intra-tumor. As both of these types are associated with cancer stemness and clonal evolution theories, they are characterized by the possibility of dynamically changing over time, which delineates an evolutionary index (Evo Index). The Evo-index [1] is a combination of two fundamental tumor-specific components: the diversity (D) or intra-tumoral heterogeneity of the neoplasm, and the measure in which it changes over time. Evo-index quantifies heterogeneity in both space and time, and it is mostly responsible for the resistance to different agents that result in the failures of a therapeutic regimen and of valid identification by predictive biomarkers.

Solid tumors are characterized by the sequential accumulation of molecular alterations that are driven by a selective pressure and subsequent clonal evolution; these account for a high spatial and temporal heterogeneity [2] and are one of many contributing factors for resistance against targeted therapies [3]. A paradigm of genomic heterogeneity involvement in resistance to targeted therapies is represented by the acquired resistance to anti-EGFR monoclonal antibodies (mAbs) in patients with metastatic colorectal cancer (mCRC). Unlike the case of anti-EGFR–acquired resistance in NSCLCs [4], the high intralesional and interlesional tumor molecular heterogeneity that characterize secondary resistance to panitumumab or cetuximab in mCRC have prevented us until now from efficiently targeting all resistant clones with a single targeted agent [5]. PD-L1 expression is actually the most widely used biomarker-guided immune checkpoint–targeted therapy, but it is characterized by high levels of intralesional, interlesional, and temporal heterogeneity [6,7]. For example, in patients with muscle-invasive, platinum-resistant bladder cancer, platinum-based chemotherapy causes (as a resistance mechanism) molecular alterations in genes regulating cell cycle and up-regulation of PD-L1 [8]. Of note, this observation suggests that, if PD-L1 expression is used to select bladder cancer patients for treatment with PD-1/PD-L1 inhibitors after platinum failure, this temporal heterogeneity should be taken into account, and pre-chemotherapy tumor samples should not be tested for PD-L1. Moreover, immune checkpoint inhibitors efficacy can be impaired by intratumoral and spatial heterogeneity of inflammatory infiltrate [9,10] as well as of somatic mutational profiles [11] and by HLA loss of heterozygosity [12]. Tumor heterogeneity is also one of the main reasons why the novel breakthrough immunotherapy strategy, of the chimeric antigen receptor (CAR) T-cell adoptive therapy, currently lacks efficacy in solid tumors as compared to hematological malignancies [13]. In fact, tumor-associated antigens (TAAs) that can be targeted to solid tumors are subject to a more pronounced intra- and interlesional heterogeneity with respect to tumor-associated antigens, which are usually targeted to hematological malignancies [13]. A typical case is represented by the high intra-tumor heterogeneity of NY-ESO-1 expression, one of the most immunogenic TAAs in solid tumors [14]. Lastly, another context linked to intra-tumor heterogeneity is represented by metabolic reprogramming, an intriguing target for anti-cancer innovative therapies as its dysregulation sustains survival and rapid proliferation, immune-evasion, and resistance to targeted agents [15–17]. Taken together, these observations highlight the need of a deeper understanding of these phenomena, in order to improve clinical outcomes of cancer patients.

2.2. Repurposing anti-estrogens to boost drug immune-mediated activity in breast cancer

Elda Tagliabue (Fondazione IRCCS INT, Milan) provided the audience with the latest updates about the role of immune system in breast cancer. While breast carcinoma (BC) is not considered an immunogenic tumor, different clinical studies showed that the presence of tumor infiltrating lymphocytes play a prognostic and predictive role, and in particular in estrogen receptor (ER)-negative (ER–) tumors. Higher levels of TILs were found to be significantly associated with outcome disease in both TNBC and HER2-positive BC.

The presence of high levels of immune infiltrating cells may depends on cancer-specific mutations even though, as compared to other solid tumors, BCs have a low mutation rate with considerable variation between disease molecular subtypes. Aside from mutant peptides, the presentation of damaged intracellular proteins can generate tumor antigens. Tumor cell death, either spontaneous or induced by therapy, can lead to the release of immunogenic signals in the surrounding microenvironment. Chemotherapy and radiotherapy are not the only treatments that interact with tumor immune microenvironment. Indeed, part of the therapeutic activity of antibodies targeting tumor–cell surface antigens, such as the anti-HER2 mAb trastuzumab, is mediated through immunological mechanisms by sensing local immune cells that, in turn, activate an antigen-specific antitumor immune response [18]. Inflammatory mediators can be produced by activation of various types of oncogenes by mutation, chromosomal rearrangement, or

amplification, as well as the inactivation of tumor-suppressorgenes, thereby generating an inflammatory microenvironment. In this context, the team of Dr. Tagliabue recently determined that stimulation of HER2 + BC cells with anti-HER receptor ligands as EGF/HRG leads to the production of CCL2, which is a chemokine involved in monocyte recruitment *via* the PI3K/NF-kB signaling axis (Fig. 1). The concept is that the activated HER2 oncoprotein regulates recruitment and activation of pro-trastuzumabtumor-infiltrating immune cells through production of CCL2, which is supported by evidence that trastuzumab efficacy relies on CCL2 levels and monocytes present in the tumor microenvironment. A major role in the resistance to trastuzumab-based treatments is played by the expression and activity levels of ER in tumors. ER is a ligand-dependent transcription factor that regulates the expression of a variety of genes, both by binding to specific response elements located in their promoters and by modulating the function of other transcription factors, such as NF-kB.

In line with the ER repressive activity of NF-κB, Dr. Tagliabue´s group found that ER negatively controls the HER2-driven pro-trastuzumab tumor microenvironment, and that fulvestrant, a selective ER down-regulator, promotes increased NF-kB transcriptional activity and enhanced CCL2 expression. Therefore, ER inhibition may represent an avenue to increase the pro-trastuzumabtumor immune infiltration driven by HER2. Finally, ERs also regulate pathways in the innate and adaptive immune cells, mainly by enhancing their intrinsic immunosuppressive activity.

Overall, blocking estrogen signaling in patients with BC may represent a promising method of boosting the immune cell contribution to improve anticancer therapies, especially in triple-positive BCs. However, further studies considering the type of endocrine therapy and the host estrogen levels are needed to determine how to combine ER blocks with anti-tumor treatments (including emerging immunotherapies).

2.3. Functional diversification of human NK cells: implication for cell-based cancer immunotherapies

Representing key cellular components of the innate immune system, natural killer (NK) cells display an intrinsic capacity to kill tumor cells without prior sensitization [19]. Recent breakthroughs have revealed a high degree of functional plasticity within the NK cell repertoire, including the expansion of adaptive (memory) NK cells following viral challenges [20]. Karl-Johan Malmberg (University of Oslo, Norway, and Karolinska Institute, Stockholm Sweden) described their recent efforts to decipher fundamental mechanisms concerning the differentiation, plasticity, and functional regulation of diverse human NK cell subsets. Their ultimate goal is to harness these insights into the design of novel, targeted NK cell–based therapies against cancer.

Discrimination of self from non-self through the continuous selection of effector specificity is the backbone of effective immunity. For NK cells, this specificity is achieved by unique combination of variable germ-line receptors, able to recognize self-MHC antigens [21]. Inhibitory interaction between NK cell receptors and self-ligands is the key determinant in functional potentiation of pre-primed effector responses, a process termed NK cell education [22]. The calibration of effector potential to self-MHC allows for the rapid sensing of discontinuity in the level of MHC expression during infection, cellular stress or tumor transformation, whilst operating within a framework of overall tolerance to normal tissues. However, the cellular mechanism behind NK cell education remains poorly understood. By using high-resolution flow cytometry combined with confocal imaging and immuno-electron microscopy, Malmberg and his team discovered that educated NK cells display a unique accumulation of dense-core secretory lysosomes with high granzyme B content [23]. RNA-seq of sorted NK cell subsets showed that this discrete morphological phenotype persists in resting NK cells independently of transcriptional programs that regulate metabolism and lysosomal biogenesis. Further, pharmacological inhibition and gene-silencing experiments revealed a role for the lysosome-specific Ca^{2+}-channel TRPML1 and its upstream activator PIKfyve in the modulation of the secretory lysosomes and the global functional responsiveness of human NK cells. These results support a model in which unopposed chronic signaling through activating receptors disarms NK cells through TRPML1-mediated modulation of the acidic Ca^{2+} stores. This in turn is reflected in differences in the functional responsiveness between self and non-self KIR^{+} NK cells, providing important mechanistic insights into the connection between

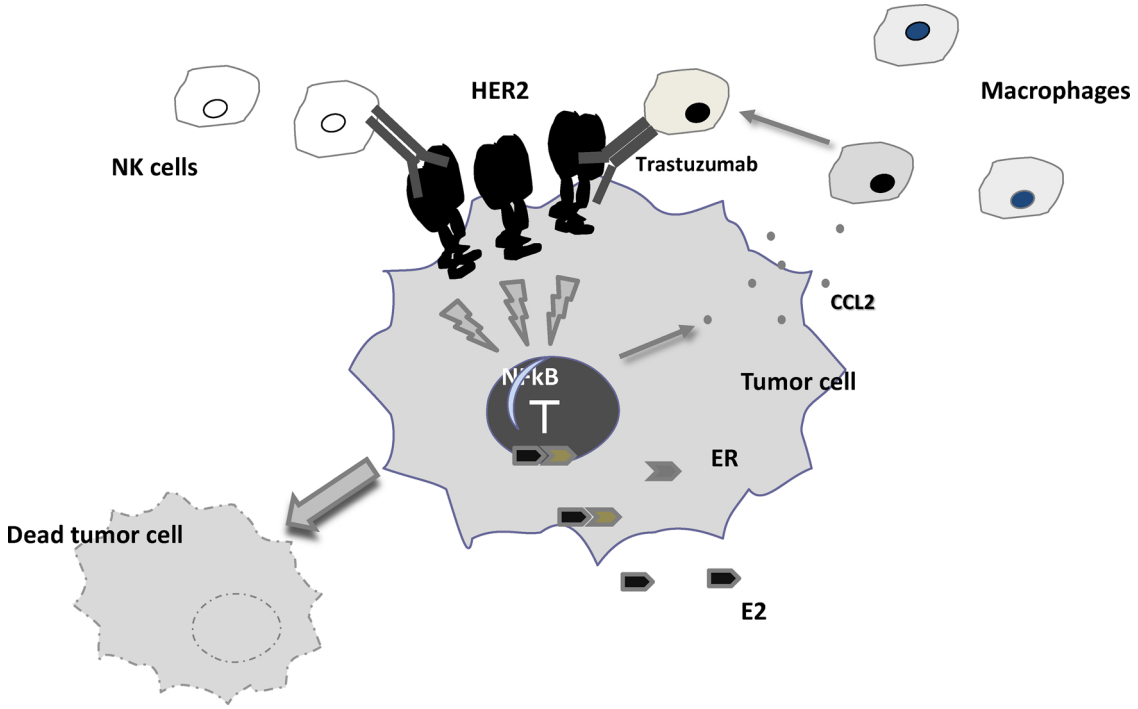

Fig. 1. HER2 oncogene induces the tumor production of CCL2 through PI3K-NF-kB signaling pathway. CCL2 recruits macrophages improving trastuzumab-dependent phagocytosis (ADCP). Estrogen (E2)-estrogen receptor (ER) complex inhibits NF-kB activity abrogating CCL2 production, macrophages recruitment and ADCP.

surface receptor input and the functional phenotype of educated NK cells. This new discovery suggests that NK cells process and integrate receptor input during education into a form of "molecular memory" stored in dense-core secretory lysosomes. Finally, Malmberg discussed the implications of these findings and the current efforts to selectively expand educated, highly functional NK cells for cell therapy against acute myeloid leukemia and myelodysplastic syndrome.

2.4. Neoantigen landscape and its impact on immune checkpoint inhibition

Nicholas McGranahan (Cancer Research UK Lung Cancer Centre of Excellence, London Cancer Institute) presented research into intra-tumor heterogeneity across cancer types, and introduced the audience to the TRACERx (TRAcking Cancer Evolution through therapy [Rx]) Consortium. The TRACERx study started in 2014 and will take place over nine years, with the goals of transforming our understanding of non-small cell lung cancer (NSCLC) and taking a practical step towards an era of precision medicine. The study aims to uncover mechanisms of cancer evolution by analyzing the intra-tumor heterogeneity in lung tumors from approximately 850 patients and then tracking their evolutionary trajectories from diagnosis through relapse. At £14 million, TRACERx represents the biggest single investment in lung cancer research by Cancer Research UK, and the start of a strategic UK-wide focus on the disease, aimed at making real progress for patients.

Led by Charles Swanton (Francis Crick Institute, UK) [24], the study will bring together a network of experts from different disciplines to facilitate integration of clinical and genomic data and to identify patients who could benefit from trials of novel targeted treatments. In addition, the study will use a whole suite of cutting-edge analytical techniques, giving unprecedented insight into the genomic landscape of primary and metastatic tumors.

In the future, TRACERx will enable us to define how intra-tumor heterogeneity impacts cancer immunity throughout tumor evolution and therapy. The primary objects of the consortium are:

- To define the relationship between intra-tumor heterogeneity and clinical outcome following surgery and adjuvant therapy (including relationships between intra-tumor heterogeneity, clinical disease stage and histological subtypes of NSCLC);
- To establish the impact of adjuvant platinum-containing regimens upon intra-tumor heterogeneity in relapsed disease compared to primary resected tumor.

Intra-tumor heterogeneity is increasingly recognized as a major hurdle to achieve improvements in therapeutic outcome and biomarker validation. Intra-tumor genetic diversity provides a substrate for tumor adaptation and evolution. However, the evolutionary genomic landscape of NSCLC and its changes throughout the disease course have not been studied in detail. The key secondary objects could be identified in the development and validation of intra-tumor heterogeneity (ITH) ratio index as a prognostic and predictive biomarker in relation to disease-free survival and overall survival. This would complete a picture of NSCLC evolutionary dynamics and define drivers of genomic instability, metastatic progression, and drug resistance by identifying and tracking the dynamics of somatic mutational heterogeneity, and chromosomal structural and numerical instability present in the primary tumor and at metastatic sites. The discovery of novel targets to improve outcome is a key element in any comprehensive program of lung cancer research. Whilst emerging evidence suggests that intra-tumor heterogeneity may significantly limit the anti-tumor activity of targeted therapeutics, its overall effect on the anti-cancer immune response may prove tractable, as high levels of intra-tumoral mutational diversity may generate neo-antigens perceived by the immune system as non-self, thus providing relevant targets for immune-based therapies. TRACERx will provide the future resource to define how intra-tumor heterogeneity impacts upon cancer immunity throughout tumor evolution and therapy. Such studies will help to define how the clinical evaluation of intra-tumor heterogeneity will guide patient stratification as well as the development of combinatorial therapies that incorporate conventional, targeted and immune-based therapeutics. This landmark study will bring together more than 80 lung cancer researchers in the UK, including oncologists, pathologists, laboratory researchers, and technicians, who are based in hospitals, universities, and research institutes.

2.5. Links between the gut microbiome and tumor immune surveillance

Laurence Zitvogel (University of Paris Medical School) presented a keynote lecture, in which presented her team's latest studies relating to the role of gut microbiome composition in cancer immunotherapy activities [25,26]. Immune checkpoint inhibitors (ICI) have now emerged as an innovative strategy for treating distinct cancers. However, most patients treated with ICIs do not benefit from immunotherapy. One of the putative mechanisms responsible for resistance to ICIs is the heterogeneity of gut microbiota. Indeed, the intestine represents the largest compartment of the immune system, and the recent advances in both sequencing technologies and culturomics have allowed microbiota to not only be characterized but also be defined with respect to their roles in human health and disease [27]. The existence of commensal bacteria represents a protection against pathogens and plays a pivotal role in some diseases, such as inflammatory bowel diseases, type 1 diabetes mellitus, metabolic and cardiovascular disorders, and cancer [28–31]. The influence on host immune systems may affect the efficacy of some agents; the mechanisms of action are currently under investigation. Notably, Prof. Zitvogel's team recently revealed that certain constituents of gut microbiota may influence the efficacy and toxicity of cancer immunotherapy, and specifically those directed to the CTLA-4 checkpoint blockade [25]. Further, Zitvogel and collaborators demonstrated that the therapeutic efficacy of anti–CTLA-4 antibodies is lost in mice treated with broad-spectrum antibiotics and, importantly, in mice kept under germ-free conditions that received fecal microbiome transplantation from non-responding human individuals [32–34). At the clinical level, it was observed that "generally" broad-spectrum antibiotic intake reduced survival of advanced cancer patients treated with PD-1/PD-L1 inhibitors. This effect was observed also in sarcomas, melanomas, and colon cancers. Of note, these findings will likely introduce a new direction in immuno-oncology research focused on testing adjunctive antibiotics that may facilitate the effectiveness of cancer immunotherapy by ensuring optimal microbiota composition. In each case, therapeutic efficacy was impaired when the gut microbiota was absent or manipulated and may differ for each treatment setting. Zitvogel detailed some of the major factors useful for deciphering the clinical importance of gut microbiome:

1 Fecal transplantation into mice and fecal composition, which influences the anti-tumor efficacy of anti-PD-1 Abs in mice;
2 Metagenomics analysis of the microbiota, in particular to determine the percentage of bacteria in the microbiota, which allows different levels of response to therapy;
3 The culturomics of fresh stool from patients, which could become a predictor of the treatment outcome.

Critically, the close interactions that exist between cancer, immunity, and microbiota could now be addressed using novel and established technologies such as metagenomics, metatranscriptomics, culturomics, and metabolomics. Finally, directed perturbation of the gut microbiome could provide new useful information for the quest of increasing the response rate to ICI therapy.

3. Session II: clinical translation

3.1. Radiotherapy in the era of ICI-based immunotherapy

Davide Franceschini (Humanitas Research Institute, Milan) reviewed the role of radiotherapy (RT) as a therapeutic tool for increasing the proportion of patients who will benefit from ICBs. RT presents an ideal tool, as it is easily accessible, deliverable to virtually any patient and any site, cost-effective, and already part of the standard of care for almost every cancer regime. More importantly, RT is immunogenic, as it provokes the release of new antigens in numerous ways, including: 1) *via* RT-killed tumor cells; 2) by the destruction of the tumor-supporting stroma; 3) by an increased expression of pro-inflammatory cytokines. Usually referred as "immunogenic modulation", all these radiation-induced molecular alterations in the biology of the cancer cell make the tumor more amenable to cytotoxic Tlymphocyte–mediated destruction [35]. This interaction has been proven in various pre-clinical experiences [36,37] and has its clinical counterpart in the so-called "abscopal effect". This term is used to identify a response of metastatic deposits that had not been directly irradiated, after the patient had received RT in another site. This effect is mediated by the immune system reactivated by the X-rays. The main limitation is that an abscopal effect is very rarely observed in the clinical practice when RT is used alone. Therefore, combining RT with immunotherapy (IT) could not only increase the efficacy of the drugs, but also increase the occurrence of an abscopal effect correlated with a better prognosis for patients [38]. Despite the great amount of preclinical studies exploring a combined RT-IT treatment, clinical data are rare and are mostly represented by retrospective experiences, mainly from melanoma patients. Altogether, these studies support a possible synergism between RT and IT, with survival improvements reported in most of these papers [39]. Information about safety of the combinations also emerges from these data; importantly, no significant increases in the expected toxicity have been observed.

Studies focusing on the possible combination of RT and IT have been conducted and are ongoing for patients with lung cancer as well. A subgroup analysis of the phase I study of pembrolizumab IT showed that patients with lung cancer who had also been treated with RT before the first administration of pembrolizumab had better progression-free survival (PFS) (of 4.4 compared to 2.1 months) and overall survival (OS) (of 10.7 compared to 5.3 months) than patients treated with pembrolizumab only. More recently, in the Pacific phase III trial, locally advanced NSCLC patients were treated with definitive chemo-radiotherapy for one year and thereafter with either durvalumab or placebo. Patients treated with durvalumab IT had a significantly better PFS than those treated with placebo (of 16.8 compared to 5.6 months) [40]. These promising results require further studies to better detail the mechanism(s) underlying their benefits and to address numerous unanswered questions. Aspects that remain unclear and should be addressed are: i) the ideal sequence of the combined RT-IT for maximizing the synergistic advantage; ii) the most suitable RT dosage and fractionation for stimulating the immune system; and iii) how to identify patients who can benefit from this combination treatment. Ongoing trials will hopefully answer these and other outstanding questions, so that the combination of RT-IT can be used to improve patient survival and prognosis.

3.2. MDSC score

It is well established that cancer immunity includes a complex and interconnected immunosuppressive processes that favor tumor growth by restraining adaptive immunity. Myeloid cells are one of the major mediators of this immunosuppression due to their high plasticity, their ability to undergo reprogramming in the periphery, and the profound conditioning effect that tumor exerts on myelopoieis [41,42]. In cancer patients, myeloid cell dysfunctions are detectable in lymph nodes and blood, implying that a systemic phenomenon plays a key role in disease progression [43]. Furthermore, their presence in peripheral circulation makes them an ideal source of prognostic and predictive biomarkers. In a population of 122 advanced melanoma patients undergoing therapy with immune checkpoint inhibitors (ICI) or a BRAF/MEK inhibitor (tyrosine kinase inhibitors, TKI), Licia Rivoltini (Fondazione IRCCS INT, Milan) reported that the frequency above cut-offs of defined immunosuppressive monocytic and granulocytic cell subsets (globally identified in a myeloid index score, MIS) in baseline blood predicts poor clinical outcome and rapid progression during therapy, independent of the type of treatment. Patients with MIS = 0 had a median OS of about 24 months, while patients with MIS > 0 had a severely reduced median OS, of only 8 to 2 months; this was confirmed in an independent validation case-set. Applying an *in vitro* model of myeloid cell conditioning by melanoma extracellular vesicles, a specific panel of miRNAs responsible for the generation of myeloid immunosuppressive cells (MDSC) could be identified. This MDSC-miR panel (including miRs known to regulate myeloid cell function, such as miR146a, miR125b, and miR155) was found to be amplified in circulating myeloid cells of melanoma patients with respect to healthy individuals, and in metastatic melanoma lesions in association with myeloid cell infiltrate. Most importantly, high levels of these miRs in baseline plasma of advanced melanoma patients predict resistance to therapy with ICI but not with TKI. This indicates that defined MDSC functional properties have an active role in restraining T-cell function upon ICI-mediated activation, making them good prognostic biomarkers and predictive factors of resistance to therapy. Altogether, the data indicate that systemic, cancer-related myeloid conditioning can profoundly affect responses to treatment in patients with melanoma; thus, they should be included in the evaluations required for screening patients and directing therapeutic choices.

3.3. Immune checkpoint inhibitors: new combinations in melanoma

Immune checkpoint inhibitors comprise the standard care in the treatment of several different tumors, but unfortunately, the response rates are not optimal for melanomas, with a maximum rate of 44% response indicating that most patients are unresponsive to these treatments (innate resistance). Further, as addressed by Michele Del Vecchio (Fondazione IRCCS INT, Milan), one-third of patients relapse after an initial response (due to adaptive resistance), which suggests the existence of multiple, non-redundant mechanisms of immunosuppression within the tumor microenvironment. Therefore, a combination strategy for treatment of these tumor would be desirable, in order to hit simultaneously more than one mechanism responsible for tumor immune escape. Del Vecchio's team has now determined that a combination of nivolumab plus ipilimumab obtained an overall response rate (ORR) of up to 58.3%, with 19.4% of patients obtaining a complete response (note that the median duration of response for these patients cannot be determined, as they have not yet relapsed). A major drawback of this treatment is the observation that it is associated grade 3/4 treatment-related adverse events (AE) in 58.8% of the cases; treatment was discontinued due to AEs (of any grade) in 39.3% of cases, even though 67% of these patients (for whom treatment was discontinued) are still alive at three years post treatment [44].

A series of immune-modulating activities underlies the combination of targeted therapies, such as the TKIs BRAF and MEK inhibitors, with checkpoint blocker-based immunotherapy. In particular, BRAF inhibitors are able to oppose the constitutive internalization and endolysosomal sequestration of MHC class I molecules associated with activated MAPK signaling pathway, thereby: i) increasing the expression of melanocytic antigens and the tumor infiltration of CD8 + T cells; to reduce immunosuppressive cytokines (IL-6, IL-10, TGF-β); ii) enhancing the expression of immune inhibitory markers (*e.g.* PDL-1); and iii) decreasing the recruitment of immunosuppressive cells ($CCR2^+$Tregs, MDSCs) inside the tumor microenvironment [45,46].

Using the triple combination (anti-PD-1/PD-L1 + BRAF inhibitor + MEK inhibitor) has a main objective of putting together the positive features of the two single treatments: rapid activity for a large number of patients (from the targeted therapy), and prolonged response duration (from the immunotherapy). Clinical trials are ongoing to determine whether such approach is better than the double-combination, and whether it has an acceptable toxicity profile [47,48]. Finally, another method that is being tested is the combination of antagonist mAbs directed to different co-inhibitory receptors (*e.g.*, anti-PD-1 + anti-LAG-3).

The activation of the adenosinergic pathway inside the hypoxic tumor cells seems to contribute significantly to tumor growth and metastatization through effects on both tumor cells and stroma [49]. Early phase I clinical trials are ongoing for several different tumor types (such as the one in which the Del Vecchio team at the Fondazione IRCCS INT is involved with), even in combination with anti-PD-1 mAbs. Other clinical trials are ongoing to confirm the so called “abscopal effect”, that is, the activity of radiation therapy also in distant tumor sites different from the specific lesion irradiated, following the immunogenic cell death and the consequent cross-priming with the activation of the immune system [50].

Crucial issues that now should be addressed in the near future are finding an optimal combination of immune checkpoint inhibitors with vaccinations, and identifying potential biomarkers predictive of response/resistance to immunotherapy.

4. Session III: selected oral abstracts

4.1. Survival probabilities of gastric cancer patients

Gastric cancer (GC) is the second leading cause of cancer-related deaths worldwide, with unsatisfactory long-term survival following standard treatments [51]. HER2 overexpression/amplification is found in less than 20% of cases, but frequent heterogeneity of HER2 as a "leading" oncogenic driver poses fundamental clinical challenges. In metastatic GC (mGC), all major guidelines currently recommend HER2 testing to guide patient selection for trastuzumab treatment [52], because the addition of trastuzumab to double chemotherapy was shown to significantly improve all endpoints (overall response rate, PFS, and OS) in the pivotal ToGA trial [53]. However, the relatively unsatisfactory median PFS of 6.7 months in the trastuzumab arm, and the overlapping of PFS curve tails, clearly indicate the relevance on survival of primary and acquired resistance [54]. Additionally, subsequent trials with anti-HER treatments for mGC in different settings did not meet the expectations based on the positive data in HER2-positive breast cancer. In particular, the Jacob trial failed to demonstrate a statistically significant improvement in OS with the addition of pertuzumab to trastuzumab and chemotherapy in the first line setting, even if a 3.3-month increase in median OS was reported [55]. Therefore, there remains an unmet need for (a) an optimal clinical definition of primary and acquired resistance to anti-HER treatment; and (b) predictive biomarkers able to improve the design and the statistical power of future prospective studies.

Encouraging results for patients with HER2-positive gastric cancer (GC) whose long-term survival is still unsatisfactory following standard treatments were presented by Filippo Pietrantonio (Fondazione IRCCS INT and University of Milan). Pietrantonio and colleagues recently provided the first prospective demonstration of the clinical usefulness of candidate genomic alterations (the AMNESIA panel including EGFR/MET/KRAS/PI3K mutations and EGFR/MET/KRAS amplifications) [56]. The study design is summarized in Fig. 2.

Criteria for trastuzumab resistance were: progressive disease at the first CT scan; reassessment during trastuzumab plus chemotherapy; and stable disease lasting ≤ 4 months after treatment start. Criteria for trastuzumab sensitivity were initial partial/complete response and subsequently progressive disease at least 3 months after the last chemotherapy dose. The AMNESIA panel was able to predict primary resistance to trastuzumab in 55% of patients with HER2-positive mGC included in this case-control study (Fig. 3).

No patient included in the sensitive cohort (control) had the alterations present in the AMNESIA panel. Data demonstrated that patients with tumors bearing no candidate alterations had a significantly longer median PFS (5.2 compared to 2.6 months; HR, 0.34; 95% confidence interval, 0.07–0.48; p 0.001) and OS (16.1 compared to 7.6 months; HR, 0.38; 95% confidence interval, 0.09–0.75; p 0.015). The strength of the AMNESIA panel relies on: (a) the simultaneous assessment of multiple resistance mechanisms with an individual low frequency, but reaching 55% when combined together as "AMNESIA panel." This approach may provide a greater chance of validating genomic signatures as opposed to attempts of investigating just one biomarker at a time; (b) clinical selection of adequate patients, by adopting restrictive criteria for defining primary resistance *versus* clear sensitivity based on the combined assessment of RECIST response and time to progression, in order to overcome the challenge represented by the potential activity of chemotherapy; (c) the case-control study design based on a predefined statistical hypothesis. Thus, using the results of the AMNESIA panel provides a good strategy for improving patient selection for future clinical trials with anti-HER treatments; however, the lack of an untreated group in this study makes it necessary to further validate the panel before using it in prospective trials.

4.2. Impact of cyclic fasting mimicking diet (FMD) on cancer patient metabolism

Preclinical studies performed in recent years have suggested that reducing the concentration of glucose and growth factors, such as insulin and insulin-like growth factor 1 (IGF-1), in cancer cell growth media sensitizes tumor cells to the cytotoxic effects of several chemotherapeutic compounds, such as platinum salts, alkylating agents and doxorubicin, as well as to different molecular targeted therapies [57]. Many metabolic effects of *in vitro* starvation can be recapitulated *in vivo* by cyclic fasting, which reduces plasma glucose and amino acids, as well as serum growth factors [15]. In preclinical *in vivo* murine models, cyclic fasting concomitant with chemotherapy administration improves the anticancer effects of cytotoxic treatments, while protecting normal tissues from chemotherapy-induced toxicities [57]. So far, only few trials have assessed the safety of complete fasting for 1–3 consecutive days during chemotherapy administration. According to these studies, fasting was feasible and was associated with reduced chemotherapy-induced hematologic toxicities, including thrombocytopenia, erythrocytopenia, and DNA damage to circulating leukocytes [59,60]. However, the specific impact of fasting on human cancer growth and on mechanisms affecting the response to antitumor treatments is still unknown.

The main limitation related to complete fasting is its poor tolerability by most cancer patients, as demonstrated by poor accrual in

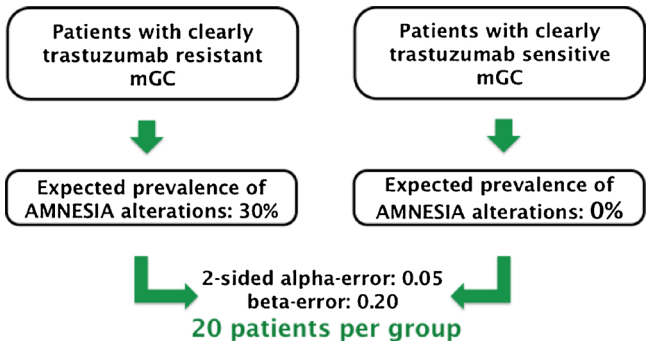

Fig. 2. Study flowchart.
Adapted from Pietrantonio F. et al., Clin Cancer Res. 2018 Mar

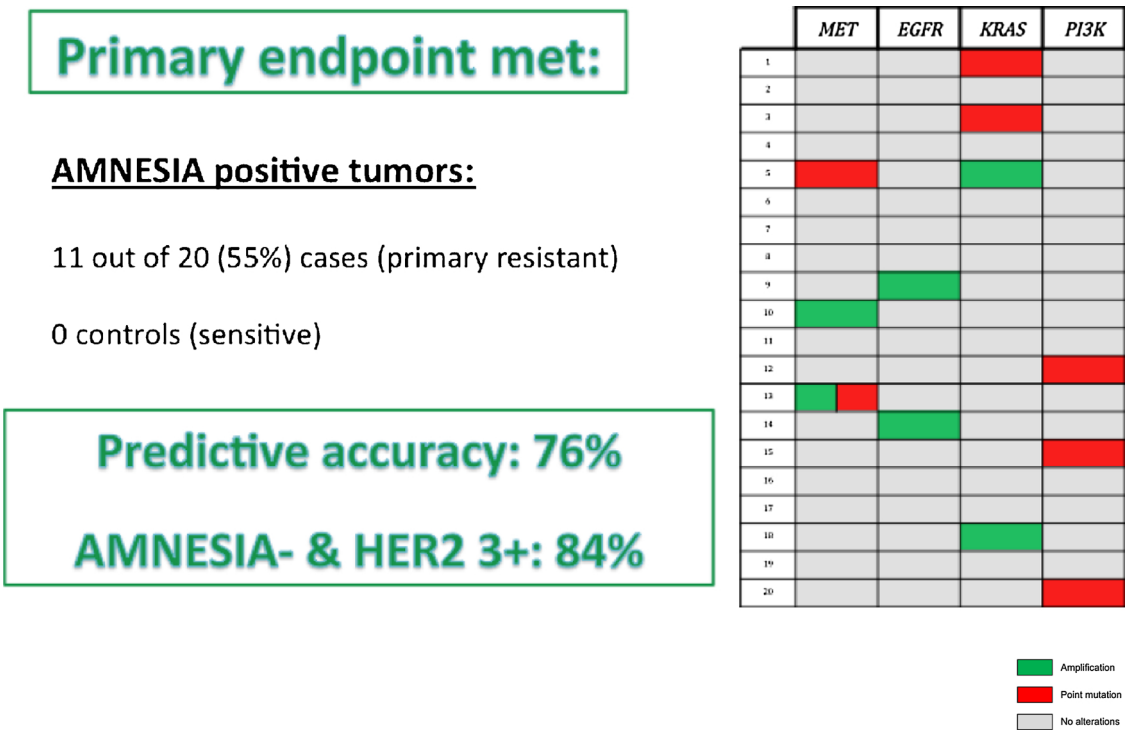

Fig. 3. Heatmap and predictive accuracy of AMNESIA panel.
Adapted from Pietrantonio F. et al., Clin Cancer Res. 2018 Mar

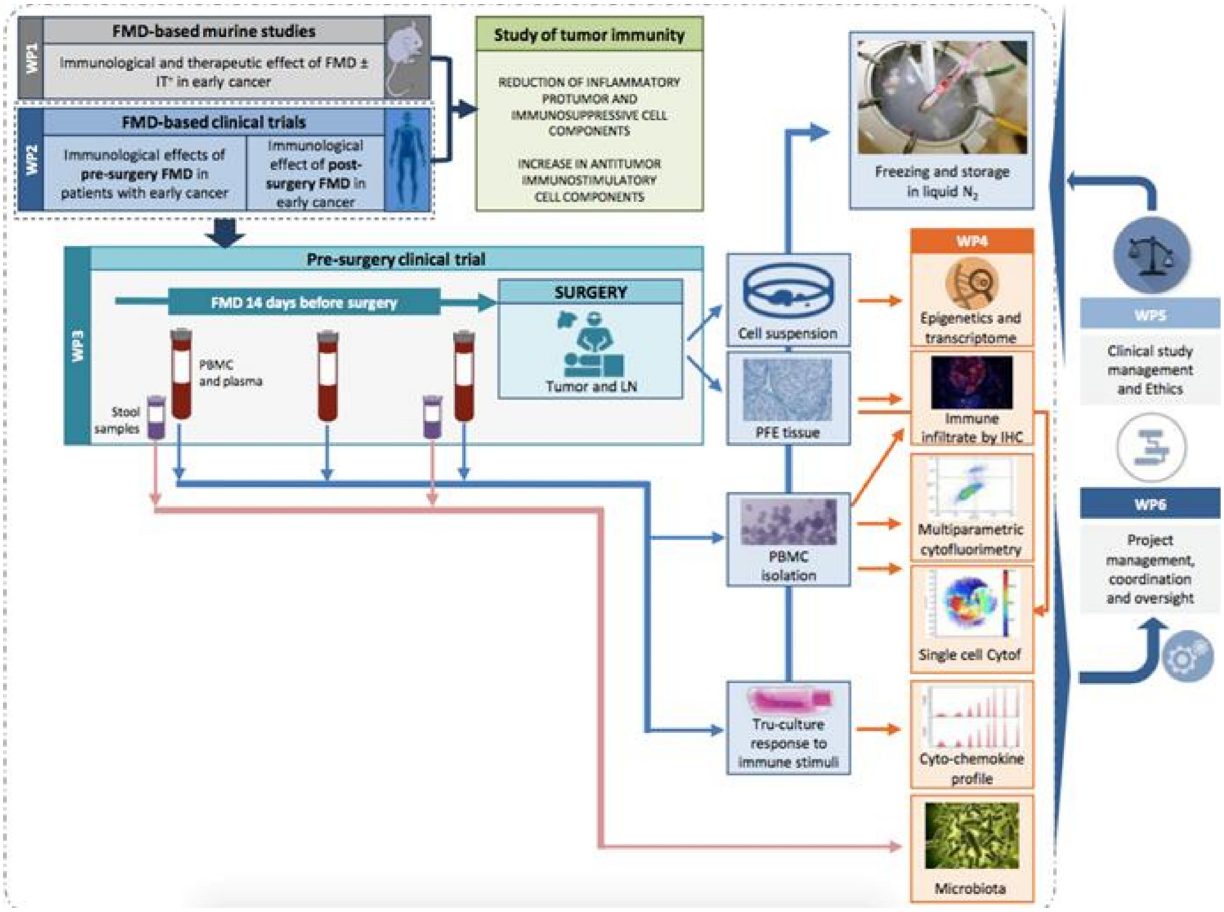

Fig. 4. DigesT trial (NCT NCT03454282) flowchart: patients with breast cancer or melanoma who are candidate to be treated with curative surgery will undergo one 5-day FMD cycle about 1 week before undergoing surgery.

clinical trials that were started in the past decade. In recent years, the group of Valter Longo proposed a dietary regimen able to produce similar biological effects as complete fasting, while being potentially more tolerated by cancer patients [61]. This approach is known as "fasting-mimicking diet" (FMD) and consists in a cyclic, calorie-restricted (400–800 Kcal/day), low-carbohydrate, low-protein diet to be administered cyclically [61]. In preclinical experiments, FMD inhibited *in vivo* tumor growth similarly to complete fasting, while synergizing with cytotoxic chemotherapy to activate antitumor immunity and to inhibit tumor growth [62]. In healthy volunteers, every-four week FMD was quite well-tolerated, and associated with reduced total and trunk fat, diastolic/systolic blood pressure, and reduced plasma IGF-1 levels [61].

Claudio Vernieri (Fondazione IRCCS INT, IFOM, and FIRC Institute of Molecular Oncology, Milan) and colleagues are now conducting the clinical trial NCT03340935, aimed at assessing the safety, feasibility and metabolic effects of 5-day FMD in patients with different tumor types and stages, or 5-day FMD concomitant with different treatments. Patients who are shown to be highly motivated to fast during their standard anti-tumor treatments are considered for enrollment in the study. The same FMD scheme is prescribed to all patients, and the diet is repeated every 3 to 4 weeks, with an allowed maximum of 8 consecutive FMD cycles. To date, 63 patients have been enrolled from February to November 2017. All patients undergo blood and urine sampling before the initiation and at the end of each FMD cycle to measure changes in the concentration of plasma glucose, triglyceride and cholesterol, serum insulin, and IGF-1 and urine ketone bodies is measured. Based on preliclinical evidence showing an impact of the FMD in activating antitumor immunity, a new study, namely the DigesT trial (NCT NCT03454282), will be started soon to assess the effects of one FMD cycle on patient peripheral blood immune profile. In particular, patients with breast cancer or melanoma who are candidate to be treated with curative surgery will undergo one 5-day FMD cycle about 1 week before undergoing surgery. As shown in the Fig. 4, blood, urine and stool samples will be collected before and at the end of the FMD, as well as on the day of surgery and at one month after surgery, to evaluate the effect of the FMD on metabolic and immunological parameters (composition of PBMC populations, transcriptome and epigenetic modifications) as well as on the bacterial composition of the gut microbiota. Immunohistochemical analyses will be also performed in the tumor tissue and lymph nodes to study the modifications induced by the diet on lymph node populations and, in case, on tumor biology.

This "window of opportunity" study, which will be conducted in patients not receiving other medical treatments, will give the opportunity to test the effect of the FMD where used alone, *i.e.* without the confounding effect of other treatments, such as chemotherapy, immunotherapy or other biological treatments. In parallel with the clinical trial, preclinical experiments in mouse models of breast cancer and melanoma will evaluate the anticancer activity of the FMD, alone or in combination with anti-PD1 monoclonal antibodies.

4.3. Triple-negative breast cancer stem cells up-regulate PD-L1 expression as a potential shield against immune-mediated tumor rejection

TNBC is a molecular BC subgroup characterized by worse prognosis and currently still orphan of specific therapeutic targets whose identification is an urgent medical need [63]. Recently, immune checkpoint inhibitors, which block the PD1/PD-L1 axis, have been identified as promising therapeutics capable of restoring the T-cell immune response and achieving an effective tumor inhibition [64]. Promisingly, the administration (in the NCT01375842 trial) of atezolizumab, a monoclonal anti-PD-L1 antibody, induced 19% of ORR in metastatic TNBC [65]; nonetheless, the clinically objective responses fall short of making a significant improvement. In this context, evidence underlined the implication of cancer stem cells (CSC), a small but biologically relevant tumor cell subset involved in tumor-initiation, progression, and metastatization [66], as well as in evasion from anti-tumor immune attack in the early phases of carcinogenesis [67]. In addition, the expression of PD-L1 has recently been reported to be in the stem cell compartments of some solid tumors; however, while no findings are yet available for TNBC stem cells (TNBC-SCs).

Research presented by Lorenzo Castagnoli (Fondazione IRCCS INT, Milan) is now focused on the analysis of PD-L1 expression in TNBC-SCs to assess its role as potential immune-modulator of the anti-tumor immune response. In-house profiled human TNBCs ($n = 158$) that were molecularly stratified for high levels of PD-L1 (PD-L1^{High}) showed significantly enriched expression of immune and cancer stemness pathways as compared with those with low PD-L1 expression (PD-L1^{Low}). In addition, the PD-L1^{High} cases were significantly associated with a signature of a high stemness score (SS^{High}). Functional bioassays strongly suggested that PD-L1 up-regulation was orchestrated by activation of the WNT signaling genes pathway. In keeping with these results, Castagnoli and colleagues also detected, in five distinct TNBC cell lines, a specific higher expression of PD-L1 in the ALDH-positive and CD44^{High} TNBCSC subsets as compared to their negative counterparts (Fig. 5). Further, they observed that PD-L1-positive tumor cells had a significantly higher mammosphere-forming efficiency as compared to PD-L1-negative cells. Remarkably, human TNBC samples contained tumor elements co-expressing PD-L1 with ALDH1A1 and/or CD44v6 cancer stemness biomarkers.

In addition, *in vivo* analysis of the tumor-forming ability of a high-grade murine mammary tumor cell line, sorted according to PD-L1

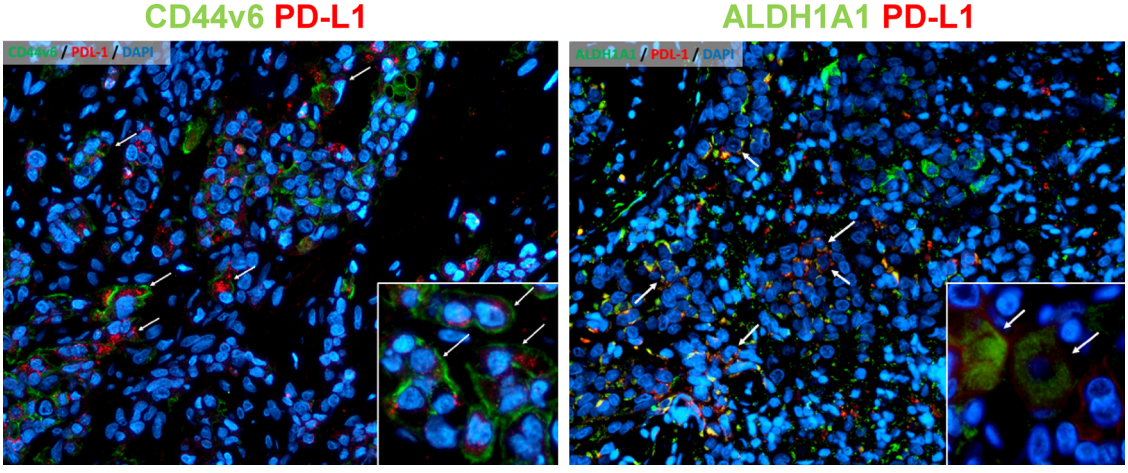

Fig. 5. Representative IF analysis of co-localization of human canonical cancer stemness biomarkers (CD44v6 and ALDH1A1) and PD-L1 in TNBC specimens.

expression levels, showed a significantly increased tumor uptake of PD-L1^{High} *versus* PD-L1^{Low} cells. Further, treatment of TNBC cells with two distinct selective WNT inhibitor or activators down- or up-regulated PD-L1 expression, respectively, implying a functional cross-talk between WNT activity and PD-L1 expression. Overall, the results from Castagnoli and colleagues suggest that PD-L1–positive tumor elements with a stemness phenotype may participate in the complex dynamics of TNBC-related immune evasion, which might be targeted and restrained through WNT signaling inhibition for future treatment strategies.

4.4. Neutrophil extracellular traps and alteration of T cell homeostasis in the bone marrow leukemic niche

Bone marrow (BM) malignancies are clonal disorders believed to result from the neoplastic transformation of hematopoietic stem cells (HSC) or progenitors cells. However, increasing evidence suggests that such a transformation could also occur in a context of deregulated or dysfunctional BM stroma.

Sabina Sangaletti (Fondazione IRCCS INT, Milan) and colleagues now hypothesize that a dysfunctional BM stroma could be induced in AML also as a consequence of a peripheral aberrant immune stimulation (*i.e.*, autoimmunity) that alters the BM immune regulatory state. Indeed epidemiological studies support a link between autoimmunity and myeloid malignancies. Neutrophil extracellular traps (NETs), which are networks of extracellular fibers primarily composed of DNA from neutrophils and that bind pathogens, can be seen more specifically as DNA-threads decorated with anti-microbial proteins that are normally released by neutrophils to control infections. NETs are pathogenically and chronically released during autoimmunity. Sangaletti and colleagues have now shown that NETs stimulate the activation and loading of DCs with neutrophils-Ag to promote the development of autoantibodies and SVV, and that NETosis can be inhibited by ECM proteins. The absence of SPARC, a master collagen regulator, promotes NET formation in peripheral organs of autoimmune Fas$^{lpr/lpr}$ knockout mice, leading to a malignant transformation of CD5+ B-cells. They thus evaluated the hypothesis that persistent immune stimulation (such as infection or autoimmune conditions) alters the immune cell status in the BM, thereby promoting a set of BM stromal changes that enable NET formation; in turn, this affects normal or mutated HSCs. To induce autoimmunity, naïve mice were immunized with a dendritic cell–based immunization protocol [68] mice that developed auto-antibodies were sacrificed, and their BM were analyzed for stromal modification and compositions. Sangaletti and colleagues found that autoimmunity caused down-regulation of the matricellular protein SPARC and collagen type-I in the BM stroma and altered T-cell homeostasis by i) reducing the frequency of regulatory T-cells (Treg); and ii) increasing the activation of T-effector cells (Teff, CD4+Foxp3-) that produced the pro-inflammatory cytokines TNF and IFN. These conditions unleashed NET formations in the BM that, in turn, increased the take of the murine AML cell line C1498.

Next, they evaluated the significance of NET in the context of human AML a disease characterized by the abnormal proliferation of immature cells and blasts. By applying a previously described NET-related signature to a panel of AML, they found that AMLs could be separated according to the NET-related gene signatures, from healthy BM, MDS and CML. Analyzing in more detail AMLs, they found that 20 genes from the NET-related gene signature were differently expressed in AML BM as compared to normal BM. Thus, AML cases can be subdivided into two clusters, characterized by a different enrichment in inflammatory genes programs and NPM1-signalling. Extending their supervised analysis to a larger cohort of AML cases for which the NPM status and clinical data were available, they further found that the NET signature defined two clusters in which mutated and wild-type NPM1 cases were significantly segregated. The presence of NET in human NPM-1 mutant AML was confirmed by *in situ* analysis, showing the preferential enrichment in NET in NPM1-mutant AML.

Finally, to assess the clinical impact of NET-related inflammatory features, Sangaletti and colleagues applied their signature to a cohort of high-risk AML patients who had been treated with a combination of chemotherapy (with citarabine) and immunomodulation (with lenalidomide). According to the NET-related inflammatory signature, AML cases could be subdivided into two groups, which differed significantly from each other in OS. A similar result was obtained using a minimal signature consisting of three genes belonging to the NET-related inflammatory signature. The relevance of NET, in NPM-1–driven myeloproliferation, was further demonstrated by immunizing NPM1-mutant mice, which showed a worsened myeloproliferation and the presence of myeloid cell blasts. In sum, their data indicate that stromal/ECM changes, NETs, and BM inflammatory conditions can support genetic events that move towards the development and progression of a myeloid malignancy.

5. Conclusions

Overall, the eighth annual conference of “Innovative therapies, monoclonal antibodies, and beyond” provided a wide picture of recent advances in the understanding of the role of the immune system in cancer, the molecular mechanisms responsible for neoplastic transformation, the biology of cancer cells, and the immunological mechanisms regulating tumor-host interaction. Key features emerged from the final discussion mainly related to the mechanisms of tumor clonal evolution as well as tumor escape and resistance from immuno-surveillance or checkpoint inhibitors. The role of tumor heterogeneity, tumor microenvironment, the gut microbiome, and the practice of using a fasting mimicking diet (FMD) for tumor growth control were presented and discussed. The possibility that tumor refractoriness in TNBC to immunotherapy regimens is potentially due to PD-L1–expressing CSC compartment represents a yet-unexplored mechanism of therapy resistance that opens the way to future intra-Institutional investigations. Gaining an in-depth comprehension of biological, molecular, and immunological mechanisms currently represents a major challenge for all clinicians and scientists involved in the debate, and represents the necessary future direction for modern cancer immunotherapy.

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