Systems biology as a compass to understand cancer-immune interactions in humans

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Chapter 8

Summary and discussion

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Major achievements in the field of immune oncology have demonstrated the ability of the immune system to induce a response against cancer. The prognostic impact of pre-existing immunity in several cancer types, including breast and colon cancer, demonstrates the influence of the immune system on disease progression[1–3]. At the same time, immunotherapeutic approaches that aim to enhance antitumor immune reactions have significantly improved the clinical outcome for a subset of patients. However, a large proportion of patients (60-80%) do not respond to immunotherapeutic treatments[4]. To extend the benefit of immunotherapeutic strategies to a larger number of patients, it is imperative to understand the mechanisms associated with immune responsiveness. Different variables have been described to influence the development of antitumor immunity in cancer patients, including the tumor's genetic program, the genetic makeup of the patients[5], and environmental factors such as the microbiome[6,7]. These factors likely act in concert to modulate antitumor immune responses[7]. This thesis aimed to dissect the molecular determinants of cancer immune responsiveness in human tumors. A systems biology approach was used to define underlying factors that shape the tumor microenvironment and reveal potential mechanisms of immune escape.

Molecular determinants of immune activation in colon cancer

The progression of colon cancer is influenced by a dynamic interplay between cancer cells and the tumor microenvironment (TME). The composition of the TME varies substantially between patients[8]. A subset of cancers has a high degree of immune cell infiltration and pre-existing antitumor immune reaction, whereas such an immune response is not apparent in others. In **Chapter 2**, we reviewed the literature for evidence of factors that determine the effect of intratumoral immune cells on clinical outcome in colon cancer. The type, location and density of tumor infiltrating lymphocytes (TILs) in combination with their functional molecular orientation, determines the effect on patient prognosis[1,9]. We argued that all cancers could be amenable to immunotherapy by shifting the tumor towards a reactive immune phenotype. In this chapter, we gave an overview of distinct modifiers of the immune phenotype that are distinct between the consensus molecular subtypes (CMS) of colon cancer. CMS1 tumors display an active, Th1-oriented immune activation status, that is commonly attributed to microsatellite instability (MSI) that is observed in CMS1 tumors. The increased mutational load and number of putative neoantigens in this context would lead to recognition of neoantigens by T cells. On the other hand, the tumor microenvironment of CMS4 tumors is characterized by transforming growth factor beta (TGF-ß) signaling and an increased abundance of fibroblasts. The immune suppressive role of TGF-ß would prevent an effective antitumoral immune activation[10–12]. Tumors of CMS2 and CMS3 subtypes display a "cold" immune phenotype, in which the degree of infiltration of immune cells is low. The mechanism of immune escape in these subtypes could be attributed to tumor-intrinsic oncogenic pathways are described to mediate immune avoidance[13]. As the factors that shape the TME are distinct between these CMSs, we proposed that distinct immunotherapeutic strategies are required for each CMS. For example, CMS1 tumors represent optimal candidates for immune checkpoint inhibition. A different strategy to re-engage the immune system can be envisioned for CMS4 tumors, for instance by combined TGF-ß pathway inhibition and immune checkpoint blockade.

While CMS classification helps to cluster cancer with comparable biological characteristics, it is important to note that molecular attributes that are described for each CMS only reflect molecular enrichments within these subtypes[14,15]. In other words, while TGF-ß activation and angiogenesis are enriched in CMS4 tumors, these processes are not restricted to this subtype.

Furthermore, not all CRCs within a specific CMS subgroup will have the exact same molecular attributes and respond similarly to treatment. Instead, the CMS classification provides a biologically relevant, conceptual framework that helps to define molecular processes in this heterogeneous disease. Therefore, the distinct therapeutic approaches provided for each CMS that are postulated in **Chapter 2** should be considered as such, and more specific biomarkers are required for appropriate allocation of (combination) therapies.

In **Chapter 3**, we used a multi-omics approach to precisely characterize the molecular landscape colon cancer and define molecular determinants of immune responsiveness. We generated a novel colon cancer dataset to dissect tumor, immunological, and microbial attributes in relation to patient outcome. RNA-seq, Whole Exome Sequencing (WES), 16S rDNA sequencing and TCR repertoire profiling were performed to capture both host and tumor-related processes. In this study, we validated the Immunologic Constant of Rejection (ICR) as signature with prognostic implications in colon cancer. Clustering based on expression of ICR genes defined ICR High, or immune "hot", ICR Medium, and ICR Low, or immune "cold" tumors. Interestingly, a large proportion of these "hot" tumors were CMS4 tumors. While CMS4 was associated with worse outcome, the ICR High CMS4 tumors had an improved survival, comparable to all other ICR High cases. This suggests that CMS4 tumors can develop a spontaneous, effective anti-tumor immune response.

Previously, it has been shown that only a small fraction of the T cell infiltrate is able to recognize tumor antigens (~10%)[16–18]. Therefore, the majority of TILs are considered as bystander T cells that infiltrate the site of the tumor but do not (directly) contribute to antitumor immunity[17]. This observation could dispute whether the evaluation of the total number of TILs or signatures that reflect immune activation in bulk tumor samples, are able to capture antitumor immune activation. Our work described in **Chapter 3**, demonstrated a near-perfect correlation between ICR score and the clonal expansion of T cells as reflected by TCR clonality. This suggests that clonal expansion of T cells is accompanied by increased expression of ICR genes. Furthermore, we proposed a novel marker summarizing the extent of the genetic immunoediting. Our multi-omics approach demonstrated that immune activation, expansion of TCR clones, and genetic immunoediting are parallel biological processes that are related to a favorable clinical outcome.

To evaluate the influence of the microbiota on antitumor immune responses, we explored associations between immune traits and tumor-associated microbiome. We found that a distinct microbiome composition associated with the functional orientation of the immune infiltrate. Specific microbial genera were associated with survival, independent of the immune phenotype of the tumor. Validation of these associations is required, and additional research will be necessary to underpin a mechanistic link.

Immune activation in breast cancer

Gene expression profiling studies in humans have enhanced our understanding of mechanisms associated with immune responsiveness. Researchers are highly encouraged and often required to share their transcriptomic datasets during publication of their research findings. The large amount of transcriptomic data provides a major resource for future investigations that were not anticipated at the time of publication or were not the main objective of the original studies. To facilitate and stimulate the re-use of previously published datasets, an interactive web application has previously been developed, the Gene Expression Browser (GXB)[19]. In **Chapter 4**, we describe a curated collection of 13 public datasets on human breast cancer, representing a total of 2142 transcriptome profiles[20]. These datasets were annotated with different immune based classification systems and uploaded to GXB. **Chapter 4** describes the composition, annotation, and harmonization of these datasets and demonstrates the utility of GXB by example

cases. To explore the expression of a gene of interest, we compared the expression of Human Leukocyte Antigen G (*HLA-G*) across ICR groups. In each of the 13 datasets, increased *HLA-G* expression was observed in breast tumors with a high expression of ICR genes. Next to exploration of a gene of interest, GXB also allows differential gene expression analysis between specific groups of interest. As example, the gene rank list of most differentially expressed genes between tumors of "Immune Benefit Status" were selected. Finally, the overlay feature of GXB was used to visualize the relationship between different categorical variables. Altogether, this work demonstrated the convenience of GXB to explore gene expression data in the context of breast cancer.

Ancestry-associated disparity in breast cancer clinical outcome has been described and can only partly be attributed to socioeconomic factors[21,22]. Previous studies have reported an increased incidence of the more aggressive triple negative breast cancer (TNBC) subtype in patients of African ancestry (AA)[23–27]. In **Chapter 5**, we have explored molecular differences between tumors of patients of AA and those with a European ancestry (EA) by interrogation of The Cancer Genome Atlas (TCGA) breast cancer dataset. Interestingly, we observed that clinical outcome of breast cancer patients, irrespective of molecular subtype, was not different between patients of EA and AA. Instead, the difference in survival was specifically observed in tumors of a specific molecular subtype, the BasalMyo subtype. This molecular subtype is based on a novel refined classifier that uses Topological Data Analysis signatures of normal mammary cell types to subgroup breast tumors by seven clearly defined molecular subtypes[28]. The BasalMyo subtype is enriched for signatures of basal epithelial cells and myoepithelial cells. We focused our analysis to explore molecular alterations within this subtype that might contribute to ancestry-associated differences in survival outcome. We found that differences in survival rate of patients with BasalMyo tumors was even more apparent if the tumors exhibited an immune "cold" phenotype, defined by low ICR gene expression. A decreased estimated abundance of T-regulatory and T-helper type 2 cells was observed in patients of AA, both of which were related to worse outcome in AA patients. Analysis of 54 cancer-related pathways revealed differential enrichment of a select number of signaling pathways with prognostic connotation in either patients of African or European ancestry. Strikingly, we observed a differential enrichment of AMPK signaling with opposing prognostic significance in patients of African versus European ancestry. Due to the small size of the local Arab breast cancer dataset (n=16) we were not able to identify significant differences in molecular determinants, however, we did observe an enrichment of BasalMyo tumors with a trend for reduced T regulatory cell infiltration and differential enrichment of AMPK signaling.

Our findings indicated differences in cancer-related and microenvironmental features between AA and EA. These results support the notion to tailor cancer treatment based on population- or more specifically ancestry-specific molecular determinants.

Molecular determinants of immune disposition identified by pancancer analysis

Pre-existing intratumoral anti-tumor T helper 1 (Th-1) immune responses have been linked to favorable outcomes with immunotherapy, but not all immunologically "hot" cancers respond to treatment. In **Chapter 6**, we performed a pancancer analysis of data from the TCGA including 31 different histologies from 9282 patients to evaluate the prognostic connotation of Th-1 intratumoral immune responses using the ICR gene expression signature. Immune hot or an ICR High immune phenotype was associated with significantly improved survival for some cancer types including breast invasive carcinoma, skin cutaneous melanoma, uterine corpus endometrial carcinoma, and sarcoma while being linked to reduced survival in other cancer types such as uveal melanoma, low grade glioma, pancreatic adenocarcinoma and kidney renal clear cell carcinoma.

To define in which contexts the ICR has prognostic value, and in which ones it does not, we systematically evaluated molecular differences between these cancer types. Our results suggest that in tumors with high mutational burdens and/or high proliferation rates, ICR captures a true protective anti-tumor immune response, whereas in tumors that are dominated by oncogenic signaling ICR captures bystander or heavily suppressed immune infiltration with no protective effect. Analysis of samples from melanoma patients treated with checkpoint inhibitors in a publicly available dataset revealed the relevance of our findings in the context of immunotherapy. While ICR score was significantly increased in pretreatment samples of responding patients and was associated with improved survival, this survival benefit was precluded to samples with high proliferation scores. Conversely, ICR scores were only associated with survival in samples with low expression of TGF-ß pathway genes. These results could be used to refine patient stratification algorithms and to optimize strategies for immunogenic conversion of tumors to reach immune responsiveness in a wider range of patients.

Mechanisms of immune escape in the tonsillar crypts

Beyond in-depth analysis of cancerous tissues, insights on mechanisms of immune escape can also be obtained by investigation of normal healthy human tissues with immune regulatory functions, such as the human tonsils. The tonsils play an important role as first line of defense against foreign pathogens that enter the body through the oral cavity.

Human papillomavirus-associated head and neck squamous cell carcinomas (HPV-HNSCC) originate in the tonsils. Breaks in the basement membrane of in the tonsillar crypts are required for passage of lymphocytes, but at the same time expose the basal cell layer of the tonsil to HPV infection. The apparent paradox of HPV infection and malignant transformation of these epithelial cells in a lymphoid-rich microenvironment was previously explained by immune evasion by upregulation of PD-L1 by epithelial cells and macrophages in the tonsillar crypt[29]. In **Chapter 7**, we performed an in-depth analysis to further investigate the natural regulatory mechanisms that are activated in the tonsillar crypt. By comparing the transcriptional profile of distinct areas by laser capture microdissection within the human tonsil (i.e., surface epithelium, crypt epithelium, and germinal centers), we identified an enrichment of myeloid cells in the tonsillar crypt. We identified multiple additional immune checkpoints, beyond PD-L1, that were upregulated in the crypts and co-expressed by myeloid cells. Myeloid populations co-expressing PD-L1, CEACAM1, VISTA, and PVR are also found in HPV-HNSCC suggesting that these same myeloid populations might play a role in maintaining an immune suppressed microenvironment in HPV-associated tonsil cancer.

Future perspectives

This thesis provides novel insights into the interactions of human cancer with the tumor immune microenvironment. The results presented in this thesis suggest that an anti-tumor immune response is the result of a complex interplay between tumor infiltrating immune cells, tumor's genetic make-up, oncologic processes, and host-derived factors such as the microbiome.

To further increase our knowledge on the interactions between cancer cells and the surrounding microenvironment, it will be instrumental to integrate the generated genomic data with information on the spatial localization of cells in the tumor microenvironment. Novel technological approaches such as multispectral imaging, imaging mass cytometry, and spatial transcriptomics could be employed to localize cells in spatial context, providing valuable information that is not captured by bulk transcriptomics. Integration of these different levels of data obtained from the same tumors will help to better understand the relationship between the activation of immune-related pathways and localization of immune cells within the sample. These results can also shed light on the mechanisms of immune escape in tumors, for instance by defining whether immune evasion is caused by exclusion of immune cells from the tumor site or whether oncogenic signaling pathways or other factors prevent the activation of tumor infiltrating lymphocytes.

Diverse factors that may modulate antitumor immune responses were explored in this thesis, including oncogenic signaling, the tumor's genetic make-up, patient's ancestry, and tumor infiltrating microbiome. However, this is a non-exhaustive list, as additional factors contribute to antitumor immune activation. For instance, the host's genetic background has been demonstrated to contribute to the functional orientation of the tumor immune microenvironment[30]. Furthermore, the effect of epigenetic alterations in tumor and immune cells on anti-tumor immunity, and vice-versa, could be further investigated[31,32].

To gain mechanistic insights into the influence of distinct factors that shape anti-tumor immune responses, analyses of multiple samples/biopsies during (immunotherapeutic) treatment will be crucial. An improved understanding of the impact of therapeutic interventions on the tumor microenvironment over time, will be imperative to implement more effective immunotherapeutic approaches. This can be achieved on one hand by the identification of appropriate biomarkers that can predict immunotherapeutic response in pre-treatment or on-treatment biopsies, but also by understanding mechanisms of primary and acquired mechanisms of therapeutic resistance. From this thesis, it has become evident that oncogenic pathways are associated with immune evasion. Modulation of oncogenic pathways conductive to immunosuppression could dramatically increase the number of patients that will benefit from immunotherapeutic approaches. For instance, blockade of the MAPK-pathway on tumor cells promotes the development of an active tumor immune microenvironment, providing a rationale to combine MAPK-pathway inhibitors with immunotherapy[33].

To conclude, this thesis charted the landscape of anti-tumor immune activity. These novel biological insights as well as the data generated in this thesis, will aid future investigations to further disentangle the complex interactions in the tumor microenvironment. This will bring us one step closer to develop effective immunotherapeutic strategies for all cancer patients.

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