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

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Chapter 1

General Introduction

General introduction

Tumor immunology

The immune system protects the body. The body's immune defense is directed against invading pathogens, including bacterial, viral, and parasitic infections, but also against cancer. Immune recognition and rejection of tumors has been demonstrated by the increased susceptibility to cancer in immunocompromised humans and by animal experiments[1]. The genetic and cellular alterations of cancer cells can result in the generation of cancer-associated antigens that can distinguish them from their normal counterparts[2]. Cancer immunity requires several important steps that are summarized by the cancer-immunity cycle[3] (**Figure 1**). After release of cancer cell antigens, they are captured by professional antigen presenting cells (APC) such as dendritic cells and presented on major histocompatibility class I and MHC-II molecules to T-cells. This results in the priming and activation of effector T cells responding against the cancer-specific antigens. Subsequently, T-cells traffic to the tumor and infiltrate in the tumor microenvironment. T-cells specifically recognize tumor associated antigens presented by the cancer cell surface bound to MHC-I on its T cell receptor (TCR), upon which effector T-cells kill the cancer cell.

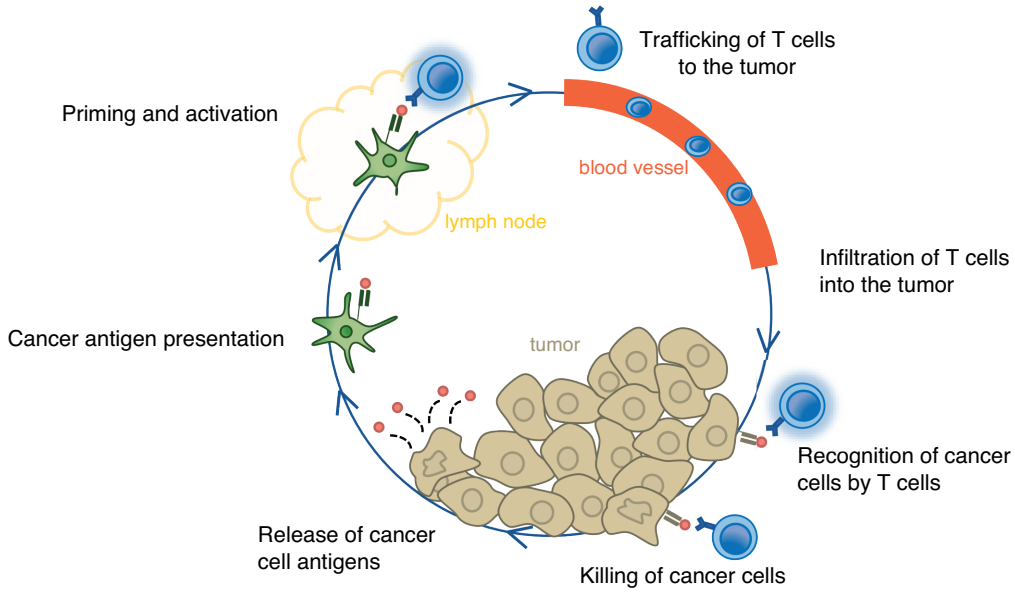

Figure 1. The cancer immunity cycle summarizes multiple steps that are required for an anticancer immune response to lead to effective killing of cancer cells.
Figure adapted from Chen and Mellman[3].

Major advances in the field of cancer immunotherapy have convincingly demonstrated that engaging the immune system to reject established tumors represents a highly effective therapeutic strategy. The enthusiasm for cancer immunotherapy has been reinvigorated by the advent of checkpoint inhibitors, such as CTLA-4 and PD-1 blockades. While this approach has led to dramatic therapeutic improvements in a subset of patients across several cancer types, the proportion of unresponsive patients (60-80%) still far exceeds those that do respond[4,5]. Therefore, the crucial challenge at this moment is to extend the benefit of immunotherapeutic treatments to a wider range of patients. This can be achieved from one side by identifying patients more responsive to immune interventions and from the other side by understanding the molecular bases of immune resistance, which in turn may lead to the identification of actionable targets.

Gene signatures of immune-mediated tumor rejection

Early studies that identified an induction of inflammation at the tumor site upon immunotherapy were performed in the context of interleukin (IL)-2 treatment in metastatic melanoma. Tumor lesions obtained by fine needle biopsies before and early after systemic IL-2 administration showed increased markers of activation of antigen-presenting monocytes, production of chemoattractants (e.g. *CXCR3* and *CCR5* ligands such as *CXCL9*, *CCL3*, and *CCL4*), and upregulation of cytotoxic effector molecules characterizing T cells (e.g. calgranulin, grancalcin) and NK cells (e.g. *NKG5*, *NK4*)[6]. To define gene expression profiles that specifically associate with immune-mediated rejection, the changes induced by IL-2 and vaccination have been compared between complete responding and non-responding melanoma lesions. Whereas non-responding lesions did not show significant transcriptomic perturbations, responding tumors displayed various differentially expressed genes upon treatment, including upregulation of interferon regulatory factor 1 (*IRF1*), indicating an early switch from chronic to acute inflammation[7].

These initial studies focused on differential expression between groups on a gene by gene basis and employed prototype platforms investigating only a restricted number of transcripts. The implementation of platforms for genome wide transcriptional profiling paired to more sophisticated bioinformatic approaches enabled the analysis of molecular pathways[8]. Therefore, subsequent studies provided more insight in the underlying biology associated with immune related gene expression. In 2011, Weiss et al. demonstrated that high dose IL-2 treatment induced upregulation of various immunological processes, including upregulation of the CCR5/CCR5L pathway. Comparison of responding and non-responding lesions identified induction of IFNγ/IRF1 signaling in responding lesions[9]. Another study, comparing regressing and progressing melanoma metastases from patients with mixed responses to different forms of immunotherapy (autologous vaccination or IFNα), identified upregulation of antigen presentation pathway, interferon mediated response and cytotoxic T-lymphocyte-mediated apoptosis in regressing lesions[10]. For the highly clinically active agent imiquimod, a TLR-7 agonist, similar genes (i.e. IFN-stimulated genes, genes with cytotoxic effector function and *CCR5*- and *CXCR3* ligands) showed a marked upregulation. Notably, the level of upregulation was highest in lesions that were treated with the regimen associated with the highest effectiveness, implying a correlation between magnitude of local inflammation and clinical response[11].

The gene signatures found in these early studies on tumor rejection by immunotherapy strongly overlap with pathways that are upregulated during other instances of immune-mediated tissue rejection like graft versus host disease, allograft rejection or flares of autoimmunity[12]. This observation has led to the formulation of in the “immunologic constant of rejection” (ICR): immune-mediated tissue rejection, independent of its context, is associated with the coordinated activation

of IFN-stimulated genes driven by transcription factors *IRF1* and *STAT1*, including upregulation of *CCR5* and *CXCR3* ligands (i.e. *CCL3-5* and *CXCL9-11*), induced Th1 signaling (e.g. *IFNG*, *TXB21*, *CD8B*), and production of cytotoxic immune effector molecules (e.g. *GNLY*, *PRF*, and *GZMs*)[13–15]. In tumor samples, upregulation of these ICR pathways correlates with upregulation of immune-regulatory genes, suggesting a compensatory activation of suppressive mechanisms in these tumors (e.g. *IDO1*, *CTLA4*, *CD274*, *PDCD1* and *FOXP3*)[16–18] (**Figure 2**).

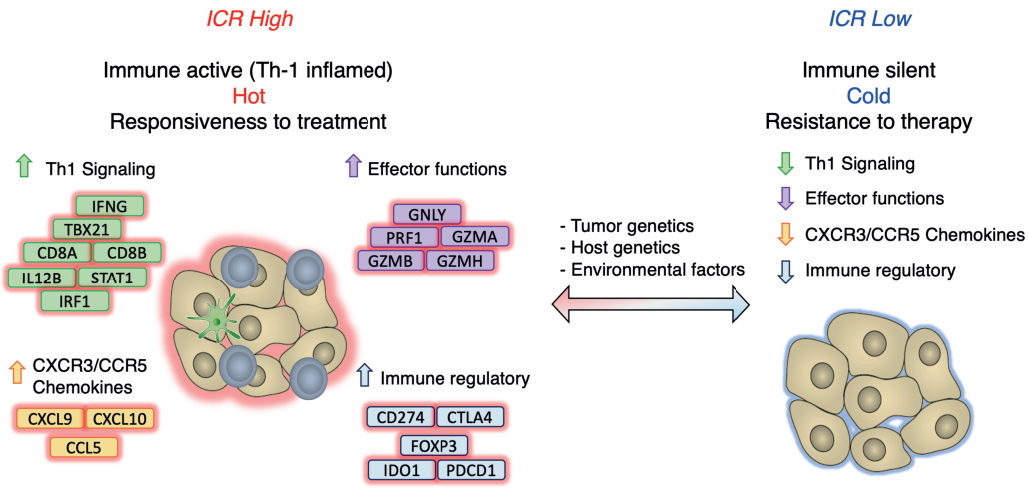

Figure 2. Schematic representation of two opposite cancer immune phenotypes based on the expression of genes that are typically associated with immune-mediated tissue rejection. The immune "hot" or active immune phenotype is characterized by upregulation of genes associated with Th1 signaling, cytotoxic effector molecules, CXCR3/CCR5 chemokines, and counter-activation of immune regulatory mechanisms. In the immune-silent or "cold" phenotype, on the other hand, the expression of these genes is low. ICR: Immunologic Constant of Rejection.

For immune checkpoint inhibition therapy (e.g. anti-PD-1/PD-L1 and anti-CTLA4), gene signatures similar to those observed in the context of IL-2, vaccination and adoptive cell transfer, have been described to reflect induction of immune-mediated tumor rejection. Anti-CTLA4 treatment in metastatic melanoma patients has been shown to increase expression of HLA class II genes, *IFNG*, CXCR3/CCR5 ligand genes and cytotoxic effector mechanisms genes. Importantly, this increase was higher in responders compared to non-responders[19]. Similarly, significant increases in immunoglobulins, *GZMB*, *PRF1*, *GNLY*, *CD8B* and *TCR-*α and *-*β genes were found in post-treatment biopsies of metastatic melanoma compared with baseline upon anti-CTLA4 treatment[20]. In a separate study, responding lesions from metastatic melanoma patients treated with anti-PD-L1 showed a gene expression pattern indicative of a generalized activation of CD8 and Th1 T cell response. In contrast, non-responding lesions displayed a lack of T cell infiltration and did not upregulate genes associated with enhanced T-effector cell activity[21]. Corresponding to these results, samples from melanoma metastasis collected early on anti-PD-

1 treatment, showed higher expression of HLA genes, Th1-IFNγ-related transcripts and chemokines in responding- compared to non-responding lesions[22]. Since the mechanism of action of immune checkpoint inhibitors is quite different, as they indirectly enhance inflammation by releasing immune inhibition, this convincingly supports the concept of the ICR, independent of the context, the pathways leading to eventual tumor destruction converge to a common mechanism[12].

Mechanisms of immune escape

Considering the large proportion of cancer patients that fail to establish an effective anti-tumor immune response when treated with immunotherapeutic approaches[23–26], it is highly relevant to define underlying mechanisms. Interference with these factors could potentially restore the anti-tumor immunity and hereby increase the number of patients that show clinical response to immunotherapeutic approaches.

Resistance to immunotherapy can clinically present as primary resistance, where a tumor fails to respond at all, or acquired resistance, where a tumor initially responds, but relapses or progresses after a period of time[27]. Focusing on immunotherapeutic failure, overlapping mechanisms have been observed in both scenario's. For example, an enrichment of mutations in the IFN-γ pathway genes has been observed in patients with primary resistance to anti-CTLA4 therapy[28]. Similarly, acquired resistance to anti-PD-1 was associated with loss-of-function mutations in interferon-receptor–associated Janus kinases (*JAK1* and *JAK2*) and a truncating mutation in antigen-presenting protein beta-2-microglobulin (B2M), resulting in defects in interferon-receptor signaling and antigen presentation, respectively[29].

Tumor intrinsic pathways associated with decreased spontaneous anti-tumor immunity (MAPK, Wnt/β-catenin, PI3K signaling), have also been associated with primary resistance to immunotherapy. Both checkpoint blockade and adoptive transfer of T cells were ineffective in mice with upregulated Wnt/β-catenin signaling[30,31]. The inability of transferred effector T cells to restore anti-tumor immune responses was shown to result from failed recruitment of these cells to the tumor, caused by absence of *CXCL9-10*, which was found to be produced by CD103+ dendritic cells[31]. Similarly, PTEN-knockout tumors were less responsive to adoptive cell therapy compared with tumors expressing PTEN[32]. A link between MAPK signaling and responsiveness to immunotherapy is provided by preclinical studies demonstrating that MAPK inhibitors can enhance the efficacy of immunotherapeutic approaches[33–40].

Tumor immune microenvironment in specific cancer types

The effect of infiltrating immune cells on clinical outcome of cancer patients varies between cancer types[41]. For most cancer types, a pre-existing adaptive immune response within the tumor has been associated with improved clinical prognosis[42]. A positive association between T cell infiltration and clinical outcome has also been described for colorectal cancer[43] and breast cancer[44]. These two common malignancies are one of the major contributors to cancer-related deaths worldwide, accounting for an estimated 862,000 and 627,000 deaths in colorectal and breast cancer, respectively[45]. To increase the number of patients that could benefit from immunotherapeutic strategies, it is crucial to understand the interaction between the tumor and immune microenvironment.

Immune microenvironment in colon cancer

Colorectal cancer (CRC) is a heterogeneous disease. Traditionally, CRC has been classified based on cancers intrinsic pathological and molecular characteristics. In the clinical setting, CRC is stratified based on the Tumour, lymph Node, Metastasis (TNM) classification to guide treatment choice[46]. Histopathological assessment of tumor morphology is also applied[47], distinguishing for example adenocarcinoma from mucinous adenocarcinoma. Ultimately, cancer is a genetic disease caused by alteration of the genome, with alterations different molecular pathways leading chromosomal instability (CIN), microsatellite instability (MSI), or epigenetic deregulation in the CpG island methylator phenotype (CIMP)[48] (**Figure 3**).

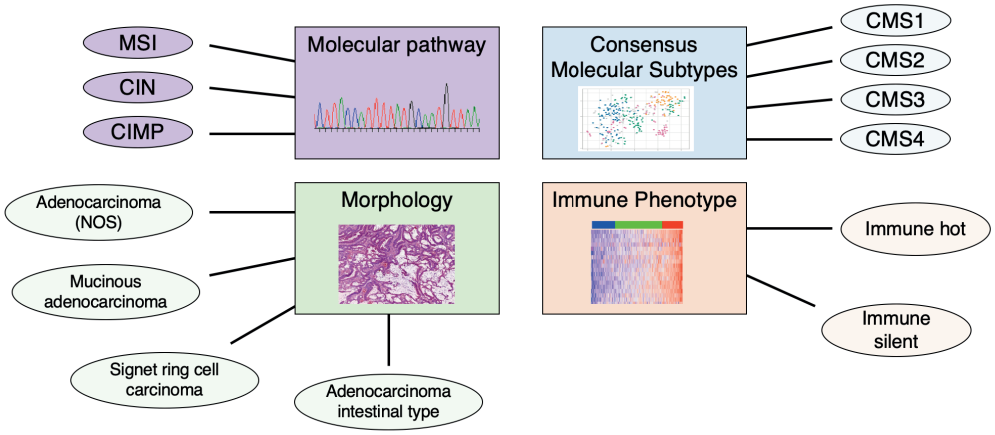

Figure 3. Classifications of colon cancer. MSI, Micro-satellite instability. CIN, Chromosomal instability. CIMP, CpG island methylator phenotype. NOS, not otherwise specified.

It is now well recognized that beyond the cancer cells themselves, the tumor microenvironment (including tumor stroma and infiltrating immune cells) also impacts tumor progression. This is reflected in the recently introduced classifications of CRC, including Consensus Molecular Subtypes (CMS) of CRC and the tumor immune phenotype (**Figure 3**). CMS classification is based on gene expression profiles from bulk tumor samples (including both tumor and stromal compartments) and was delineated by forming a consensus between previously proposed gene expression–based CRC subtyping algorithms[49]. A detailed description of the four CMS subtypes and clinical implications is provided in **Chapter 2**. The contribution of the immune phenotype of colon cancer on disease progression has conclusively been demonstrated by histological quantification and localization of cytotoxic and memory T cells in the center of the tumor and invasive margin[43,50]. A pre-existing active tumor microenvironment is associated with improved survival in colon cancer[50].

Immune checkpoint blockade is now approved by the FDA for the specific subgroup of CRC with MSI, or defective DNA mismatch repair[51]. Mismatch repair deficiency (MMRd) occurs in approximately 15% of colorectal carcinomas, either caused by a germline mutation in genes

responsible for DNA MMR (3%) or sporadically by somatic inactivation of the same pathway, most commonly through hypermethylation of the *MLH1* gene (12%)[52]. The increased number of mutations that arise as a consequence of MMRd lead to an increased number of neoantigens that are presented to the immune system, which makes these immunogenic tumors ideal candidates for immune-based approaches.

To expand the reach of immunotherapy beyond MSI-H tumors, different strategies can be envisioned that aim to covert immune "cold" tumors, to "hot" tumors (described in detail in **Chapter 2**). With this objective in mind, it will be very important to better define underlying factors that shape the tumor immune microenvironment, including influence from the tumor (e.g. specific mutations, molecular pathway), host-derived factors (e.g. genetics of the host, epigenetic changes), and environmental factors (e.g. life style, microbiome).

Immune microenvironment in breast cancer

Like CRC, prognostication of breast cancer is traditionally based on tumor intrinsic factors, including TNM classification and histopathological characteristics like tumor size, grade, number of affected lymph nodes, hormone receptor- (i.e. estrogen, progesterone and androgen receptors) and HER2 status. Gene expression profiling has added an important classification based on expression of 50 genes (PAM50) that defined different intrinsic molecular subtypes (IMS) with differential prognosis[53–56]. Four major IMS of breast cancer have been identified: Luminal A, Luminal B, Her2-enriched and Basal-like. More recently, a refined classifier of PAM50 has been proposed that utilizes a combination of Topological Data Analysis signatures of normal mammary cell types (basal epithelial cells, luminal epithelial cells, myoepithelial cells, and Her2-related expression) to classify breast cancer in seven distinct molecular subtypes with prognostic value[57].

Although the immune landscape has shown to relate to the above factors to various extents, it represents an additional factor with independent prognostic value[58–61], indicating its significant influence on cancer progression. Tumor infiltrating lymphocytes are associated with a better prognosis in Basal-like or triple negative breast cancer (TNBC) and Her2-positive tumors[44,62]. TNBC are more likely to respond to immunotherapy compared to other breast cancer subtypes, caused by i) pre-existing infiltration of lymphocytes to the tumor[63], ii) a higher number of mutations[64], and iii) increased *PD-L1* expression[65].

As only a small proportion of luminal cancers (15%) display an active Th-1/ICR High immune phenotype[18], this subtype is not directly considered as a candidate for immunotherapy. However, combination of immune checkpoints with MAPK-inhibition treatment could represent an effective strategy for immunogenic conversion of immune-silent breast cancer to immune-active tumors[33]. In a systematic analysis, Hendrickx et al.[18] previously investigated the relationship between tumor genetic programs and immune responsiveness in breast cancer. This analysis included copy number variation, somatic mutations, and transcriptomics from >1000 samples from The Cancer Genome Atlas (TCGA). Mutations in *MAP2K4* or *MAP3K1* genes were 10-fold enriched in immune-silent (ICR Low) tumors compared to immune active (ICR High) tumors. MAPK pathways deregulation was associated with ICR Low tumors in all IMSs, suggesting that dysregulation of the MAPK pathways, either sustained by *MAP3K1* or *MAP2K4* mutations or alternative mechanisms, could be implicated in the development of the unfavorable cancer immune phenotype[18]. Indeed, MAPK pathway inhibition has been shown to increase breast cancer immunogenicity[34,66]. These findings highlight the relevance of elucidation of the relationship between tumor genetic programs and immune responsiveness.

Outline of this thesis

The work presented in this thesis aims to identify biomarkers of immune-responsiveness and their prognostic implications in human carcinomas. Underlying factors that shape the tumor microenvironment and potential mechanisms of immune evasion are investigated using immunogenomic profiling of tumor samples.

Part 1 of this thesis specifically focusses on the immunogenomic profiling of colon cancer. In **Chapter 2**, evidence for the impact of the tumor immune microenvironment in colorectal cancer is described in a literature review. Immunogenomic classifications of colorectal cancer and their prognostic and predictive implications are presented. The importance of the tumor immune phenotype and associations with molecular attributes in colon cancer is thoroughly investigated in **Chapter 3**. This research article presents a new colon cancer cohort that was extensively profiled on a molecular level, including RNA sequencing, Exome Sequencing, T-cell receptor sequencing, and microbiome 16S profiling. Integrative analysis of data from these different platforms provided novel insights in the molecular correlates of the tumor immune microenvironment and a better understanding of immune mediated tumor rejection in colon cancer.

In the second part of the thesis (**Part 2**), immunogenomic profiling is applied to breast cancer. In **Chapter 4**, we classified samples of 13 public datasets of human breast cancer by transcriptomic profile, focusing on immune-based classifications. We contributed to the implementation of the interactive data browsing and visualization web application, “Gene Expression Browser (GXB)”, to facilitate utilization of these datasets. Examples of interactive data exploration are provided to demonstrate the use of GXB to evaluate cancer gene expression across immunologic classifications of breast cancer. In **Chapter 5**, we have focused our analysis to explore molecular alterations that might contribute to ancestry-associated disparity in breast cancer clinical outcome, with a focus on patients of African ancestry. Our study is the first study that utilized a unique approach combining the use of curated survival data from The Cancer Genome Atlas Pan-Cancer clinical data resource, SNP-based inference of ancestry[67], and a novel Topological data based- breast cancer subtype classification system[57]. Through a comprehensive transcriptomic analysis of breast tumors from the The Cancer Genome Atlas breast cancer cohort and a small local cohort from Qatar, we identified differences in cancer-cell intrinsic and microenvironmental features by ancestry.

The final part of this thesis (**Part 3**) aims to identify potential mechanisms of tumor immune evasion using our immunogenomic approach. The chapters of this section describe studies on distinct datasets across different settings, with the common objective to better understand the ways tumors can evade the immune system. In **Chapter 6**, a pan-cancer analysis of data from The Cancer Genome Atlas encompassing 31 different histologies from 9,282 patients, demonstrates that cancer-specific pathways modulate the prognostic power of favorable intratumoral immune responses. A high expression of the ICR signature was associated with significant survival benefit for some cancer types including breast invasive carcinoma, skin cutaneous melanoma, uterine corpus endometrial carcinoma, and sarcoma while being linked to significantly reduced survival in other cancer types such as uveal melanoma, low grade glioma, pancreatic adenocarcinoma and kidney renal clear cell carcinoma. Systematic analysis encompassing transcriptomic and genomic attributes suggest that in tumors with high mutation

burdens and/or high proliferation, ICR captures a true protective anti-tumor immune response, whereas in tumors dominated by cancer signaling ICR captures bystander or heavily suppressed immune infiltration with no protective effect. In **Chapter 7**, a study is presented that aimed to characterize the natural regulatory mechanisms that support immune privilege within the tonsillar crypt. Transcriptional profiling of different regions of the normal human tonsil confirmed a suppressed immune microenvironment specifically in the crypts compared to lymphoid rich germinal centers, and surface epithelium. We identified differentially upregulated immune checkpoints within the crypts. Understanding of the natural immunosuppressive microenvironments could provide insights to the immune resistance in cancer setting.

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