Cancer Immunol Immunother (2014) 63:713–719
DOI 10.1007/s00262-014-1521-3

FOCUSSED RESEARCH REVIEW

Monitoring the immune competence of cancer patients to predict outcome

Serena Chang · Holbrook Kohrt · Holden T. Maecker

Received: 2 December 2013 / Accepted: 15 January 2014 / Published online: 1 February 2014

Abstract A new era of cancer immunotherapy has brought not only successful cancer vaccines but also immunomodulators, such as those that target checkpoint blockade in order to induce endogenous host immune responses. However, the immune system of cancer patients can be compromised through multiple means, including immune suppression by the tumor and by prior therapies such as chemotherapy and radiation. Therefore, a comprehensive means of assessing patient immunocompetence would seem helpful for determining whether patients are ready to benefit from immunotherapy, and perhaps even which immunotherapy might be most appropriate for them. Unfortunately, there are no standardized tests for immune competence, nor is there agreement on what to measure and what will be predictive of outcome. In this review, we will discuss the technologies and assays that might be most useful for this purpose. We argue for a comprehensive approach that should maximize the chances of developing predictive biomarkers for eventual clinical use.

This paper is a Focussed Research Review based on a presentation given at the Eleventh Annual Meeting of the Association for Cancer Immunotherapy (CIMT), held in Mainz, Germany, 14–16 May, 2013. It is part of a CII series of Focussed Research Reviews and meeting report.

S. Chang · H. T. Maecker (✉)
Institute for Immunity, Transplantation, and Infection, Stanford University School of Medicine, Fairchild Science Building, 299 Campus Drive, Stanford, CA 94305-5124, USA
e-mail: maecker@stanford.edu

H. Kohrt
Division of Oncology, Stanford University School of Medicine, Stanford, CA 94305, USA

Keywords CIMT 2013 · Immunocompetence · Immune monitoring · Mass cytometry

Introduction

In 1909, Paul Ehrlich hypothesized that the immune system played an important role in controlling the growth of cancer [1]. This hypothesis was supported by the classic experiments of Prehn and Main [2], which showed that mice could generate immune responses to methylcholanthrene-induced sarcomas. Yet for many years, surgery, chemotherapy, and radiation were used for the treatment of cancer, with little ability to successfully harness host anti-tumor immunity. Controversy about tumor immunogenicity certainly delayed progress in immunotherapy. Most cancers, unlike the chemically induced sarcomas and the relatively rare virus-associated tumors, were considered non-immunogenic or poorly immunogenic [3]. However, key discoveries in the 1980s showed that vaccination could change the immunogenic profile of naturally occurring tumors and that an effective immune response was required for this to occur [4, 5]. While tumors undoubtedly are selected to minimize their antigenic profile, it is now appreciated that most, if not all cancers induce some altered or overexpressed host proteins that can potentially function as antigens. In fact, the number of recognized tumor-associated antigens has grown tremendously [6], to include not only viral proteins (e.g., HPV) and chromosomal translocation products (e.g., bcr/abl), but also over-expressed normal proteins such as HER2/neu, telomerase, and mucin-1, aberrantly expressed germ line or differentiation antigens, and mutated normal proteins.

It is also now apparent that endogenous immune responses to these antigens can be detected, albeit at low

levels, in many (perhaps most) individuals with cancer. This was elegantly demonstrated in the work of Lee et al. [7] in melanoma patients, using MHC-peptide tetramers. Our group has also shown that breast cancer patients often have detectable responses to tumor antigens such as HER2/neu, CEA, and MAGE-A3 [8], as demonstrated by intracellular cytokine staining (ICS). However, many groups have also demonstrated ways in which endogenous immune responses to tumors are suboptimal. These can range from poor avidity of the T cells [9] to incomplete differentiation and restricted cytokine profiles [8]. Thus, the current generation of cancer immunotherapy seeks to overcome the inadequacies of endogenous tumor responses. This can be accomplished by supplying monoclonal antibodies to tumor antigens, bispecific antibodies that direct T cells to tumors, or engineered T cells that themselves target tumor antigens. In these cases, the host immune system is itself not directly altered, but immune molecules or cells are provided as an anti-tumor therapy. However, another approach is to target the immune system directly, rather than the tumor, by vaccination to induce stronger and more appropriate responses, by monoclonal antibodies inducing costimulatory pathways or inhibiting immune regulatory checkpoints, or by specific cytokine therapy to enhance immune responses, among other strategies.

Within the past decade, many of these immunotherapy strategies have been proven effective, either alone or in combination with each other or with traditional therapeutic modalities. In terms of direct anti-tumor therapies, there are approved antibodies targeting receptors over-expressed in tumor cells (Cetuximab, or anti-EGFR, for colorectal and head and neck cancers [10], and Rituximab, or anti-CD20, for lymphoma [11]). Adoptive T-cell transfer has proven to have dramatic results in tumor eradication with metastatic melanoma patients [12]. Some of the most recent therapies to show promise in clinical trials are bispecific T-cell engagers (BiTEs) (e.g., blinatumomab, or anti-CD3/anti-CD19 antibody, for lymphoma and leukemia [13, 14]). In terms of "true" immunotherapies (that target the immune system and not the tumor directly), there are now approved cancer vaccines (Provenge for prostate cancer [15]) and monoclonal antibodies for checkpoint blockade (ipilimumab, or anti-CTLA-4, for melanoma [16]). Anti-PD-1 and anti-PD-L1, as well as other checkpoint antibodies, are in late-stage clinical testing. Several agonist antibodies to costimulatory receptors (CD40, CD137, and OX-40) are also in clinical trials. Yet the improvement in cancer patients treated with these immunotherapies, although dramatic, does not extend to the majority of patients. In general, many cancer clinical trials still fail to show clinical benefit, and this is in part related to heterogeneity in the host genetic background, the tumor, and the immune system.

Monitoring immune competence

Most of the current immunotherapies work at least in part by activating the immune system to target the tumor. But without knowing the immune competence of the patient, such strategies may fail, or work only in a fraction of patients. Tumors are known to induce immune suppression; over a decade ago, CD4+ T-cell anergy was reported as a common early event in tumor progression [17]. Immune suppression after radiation [18] and chemotherapy [19] has long been documented in cancer patients. These findings help explain tumor progression in spite of the presence of tumor-specific T-cell populations. They also argue for investigating immune competence in cancer patients as a prerequisite to initiating immunotherapy.

The argument has been made that pre-selection of patients with favorable immune profiles could be beneficial to trials of cancer vaccines [20]. In fact, the concept of the "immunoscore" as a fourth axis of tumor staging (after tumor size, nodal involvement, and metastases) has been widely publicized [21–23]. As put forth by Galon et al., the "immunoscore" is derived by immunohistochemical quantitation of infiltrating CD4+ and CD8+ T cells in tumor biopsies. This is undoubtedly an important development for the field, as the number and ratio of these cells appear to be prognostic of clinical outcomes.

However, despite the development and recognition received for the immunoscore, much more is possible with regard to immune competence measurement. For one thing, many specific phenotypes of CD4+ and CD8+ T cells can participate in either enhancing or blocking tumor immunity. These include cytotoxic T cells (CTL), helper T cells (Th1, Th2, or Th17), and regulatory T cells (Treg). For each of these classes, there are of course many phenotypic and functional variants [24]. Expression levels of specific activating or suppressing receptor/ligand combinations associated with T-cell activation, such as PD1/PDL-1 [25], CD137/CD137L [26], or CTLA-4/CD80 [27, 28] can be important for immune competence. There is also growing appreciation for the role of NK cells [29], as well as macrophages [30], in anti-tumor immunity induced by specific immunotherapies. Dendritic cells, B cells, and antibody production also have the potential to play an important role in anti-tumor immunity, and myeloid-derived suppressor cells (MDSC) have received recent attention as correlating with clinical outcome [31, 32].

Thus, a more comprehensive approach to measuring immune competence is needed. One way to assess the immune system on a relatively global level is to perform in vitro stimulation with a mitogen (such as PMA + ionomycin) and quantitatively analyze the cellular phenotypes that produce key cytokines in response to the stimulation.

Table 1 Antibody panels for mass cytometry using ICS and phosphoepitope analysis (phospho-CyTOF)

CyTOF ICS				Phospho-CyTOF			
Metal label	Specificity	Clone	Source	Metal label	Specificity	Clone	Source
102–110Pd	barcoding (opt.)			102-110Pd	barcoding (opt.)		
113Cd	CD57	HCD57, BioLegend	In house	113Cd			
115In	Live/dead			115In	Live/dead		
139La	CD49d	9F10, BioLegend	In house	139La			
141Pr	CD45RA	HI100, BioLegend	In house	141Pr			
142Nd	CD19	SJ25C1, Southern Biotech	In house	142Nd	CD19	HIB19	DVS
143Nd	CD8	SK1, BioLegend	In house	143Nd			
144Nd	CD69	MCA 2806, AbD Serotec	In house	144Nd			
145Nd	CD4	RPA-T4	DVS	145Nd	CD4	RPA-T4	DVS
146Nd	Granzyme B	GB11, Abcam	In house	146Nd			
147Sm	CD20	2H7	DVS	147Sm	CD20	H1, BD	In house
148Nd	MIP1ß	D21-1351, BD	In house	148Nd			
149Sm	CD85j	292319, R&D Systems	In house	149Sm	CD7	CD7-687, BioLegend	In house
150Nd	CD45RO	UCHL1, BioLegend	In house	150Nd	CD3	UCHT1, BD	In house
151Eu	CD38	HB-7, BD	In house	151Eu	CD123	9F5	DVS
152Sm	TNF	Mab11	DVS	152Sm	CD27	0323, BioLegend	In house
153Eu	CD3	UCHT1, BD	In house	153Eu	CD45RA	H100	DVS
154Sm	CD107a	H4A3, BD	In house	154Sm	CD45 (opt.)	HI30	DVS
155Gd	GMCSF	BVD2-21C11, BD	In house	155Gd			
156Gd	CD94	HP-3D9, BD	In house	156Gd	pp38	D3F9	DVS
157Gd	IL-2	MQ1-17h12, eBioscience	In house	157Gd	CD24	ML5, BioLegend	In house
158Gd	IFNγ	4S.B3, BioLegend	In house	158Gd	pSTAT3	4/P-Stat3	DVS
159 Tb	HLA-DR	G46-6, BD	In house	159 Tb	CD11c	Bu15	DVS
160Gd	CD14	M5E2	DVS	160Gd	CD14	M5E2	DVS
161Dy	CD137	4B4-1, BD	In house	161Dy	IgD	IA6-2, BioLegend	In house
162Dy	IL-10 (indirect)	JES3-12G8, BioLegend	In house	162Dy	pErk1/2	20A, BD	In house
163Dy	CD137L	C65-485, BD	In house	163Dy	IkBtot	L35A5, CST	In house
164Dy	IL-17	N49-653	DVS	164Dy	CD25	M-A251, BD	In house
165Ho	CD127	A019D5, BioLegend	In house	165Ho	pS6	N7-548, BD	In house
166Er	CD33	P67.8, BD	In house	166Er	CD16	B73.1, BD	In house
167Er	CD27	L128, BD	In house	167Er	CD38	HIT-2	DVS
168Er	FcER1	9E1, Abcam	In house	168Er	CD8	SK1	DVS
169Tm	CCR7	150503, R&D Systems	In house	169Tm	pSTAT1	4a, BD	In house
170Er	PDL-1	10F.9G2, BioLegend	In house	170Er	CD3	UCHT1	DVS
171Yb	PDL-2	TY25, BioLegend	In house	171Yb	CD66 (opt.)	CD66a-B1.1	DVS
172Yb	PD1	EH12.1, BioLegend	In house	172Yb	pSTAT5	47/Stat5 (pY694), BD	In house
173Yb	Perforin	B-D48, Abcam	In house	173Yb	pPLCg2	K86-68937, BD	In house
174Yb	CD16	3G8, BioLegend	In house	174Yb	HLA-DR	L243	DVS
175Lu	CD56	NCAM16.2, BD	In house	175Lu	CD56	HCD56, BioLegend	In house
176Yb	CD25	M-A251, BD	In house	176Yb	CD127	A019D5	DVS

A variation of this approach was used in an early study from our group, in which the frequency of CD4+ T cells producing IFNγ in response to the superantigen SEB was assessed [33]. Despite the small sample size and heterogeneity of responsiveness to SEB in healthy controls, patients with multiple myeloma had generally much lower responses than controls, suggesting they were mostly immune suppressed.

Another functional readout that could be used to measure immune competence is intracellular phosphosignaling [34]. Recent work from our group (Shen-Orr, submitted for publication) has found variable levels of defective pSTAT

signaling in response to in vitro cytokine stimulation, in a subset of elderly individuals. These defects appear to be related to chronic inflammation.

The technology of choice: mass cytometry

Today, the readout of either cytokines or phosphoprotein signaling, in combination with cell phenotyping, is routinely done by multiparameter flow cytometry [34, 35], among other methods. However, the number of markers required to comprehensively subset immune cells, even at a fairly granular level, is on the order of 20 or so. Adding in a variety of either cytokine or phosphoepitope antibodies would bring the panel upwards of 30 markers or more. This is clearly beyond the ability of traditional multicolor flow cytometry.

Fortunately, a new generation of flow cytometry instrumentation, based on mass spectrometry readout of heavy metal ion-labeled probes, has been developed [36–38]. Termed "mass cytometry," and supported by a commercial instrument (CyTOF, DVS Sciences, Toronto, CA), this technology has a twofold benefit over traditional fluorescence-based flow cytometry. First, the number of labels that can be measured per sample is greatly enhanced (presently about 40, but increasing regularly). Second, the "spillover" of signal from one detector channel to another is dramatically reduced, eliminating the need for calculating compensation matrices and reducing the noise associated with such spillover.

The first application of mass cytometry to large-scale immune profiling was carried out by Bendall et al. [39] using a panel of 34 cell surface and phospho-specific antibodies. Additional studies have used ICS, along with cell surface phenotyping, on this platform as well [40]. Extensive discussion of the platform's benefits and caveats have been published elsewhere [38, 41] and will not be repeated here. However, it suffices to state that this platform is ideally suited, and in fact already proven, for readout of highly multiparameter phosphoepitope and intracellular cytokine analysis.

Our laboratory has recently built 31–39 parameter CyTOF panels for each of these types of applications (phosphoepitope and intracellular analysis). The antibodies in these panels are shown in Table 1. The intracellular cytokine panel is currently being used with PBMCs from cancer patients prior to and after immunotherapy. The cells are stimulated for 4 h with PMA + ionomycin, and cell phenotypes and functions are analyzed.

Analyzing the data for biomarkers

A complexity of the mass cytometry approach is how to analyze 40-parameter single-cell data. While one can certainly perform directed gating using bivariate dot plots, this soon becomes overwhelming and is likely to miss potentially key populations or responses. More global visualization approaches have been published, including SPADE [42], viSNE [43], and heat maps [44]. While SPADE performs clustering of related cells, and viSNE does not, both SPADE and viSNE display the data of a single file in a two-dimensional representation of relatedness. Cells (in viSNE) or clusters (in SPADE) are displayed as circles in a nearest neighbor analysis, where those most related to each other are grouped closest together.

Another analytical approach that has been applied to CyTOF data is principal components analysis (PCA) [40]. This is useful for showing differences between particular cell populations, e.g., influenza-specific versus CMV-specific T cells.

We have begun to use SPADE to compare the mass cytometry profiles of PMA + ionomycin-stimulated PBMCs of cancer patients and healthy controls (Fig. 1). A number of cytokines in stimulated baseline cancer patient samples (IL-17, IL-10, granzyme B, GM-CSF, CD107a) showed diverse maps compared to controls that had very homogeneous maps. This type of analysis suggests that the cancer patients are more heterogeneous in their immune profiles than controls, sometimes showing increases and sometimes decreases in specific cytokine responses. We therefore have some hope that there will be immune phenotypes that correlate with therapy outcome.

A variety of informatics approaches will likely be needed to mine data such as the CyTOF profiles now being generated. Eventually, these should lead us to refine our assays, perhaps focusing in more detail on subsets and functions that are most promising. Finally, simpler assays than the CyTOF will undoubtedly be possible to design, and these simpler assays will likely be the ones that make it into clinical diagnostics, if there are to be any diagnostics of this kind. But at present we are still casting a wide net: we do not yet know which, if any, immune competence markers will correlate with clinical outcomes in particular cancer types; and we do not know how immune competence might best be modulated to achieve more successful cancer therapies.

Conclusions

We are clearly only at the beginning of developing the knowledge required to do effective patient prognosis on the basis of immune competence measurements. However, we have argued here that the new wave of cancer immunotherapies should greatly benefit from comprehensive immune competence measurement and that the platform of mass cytometry provides an ideal discovery tool for doing

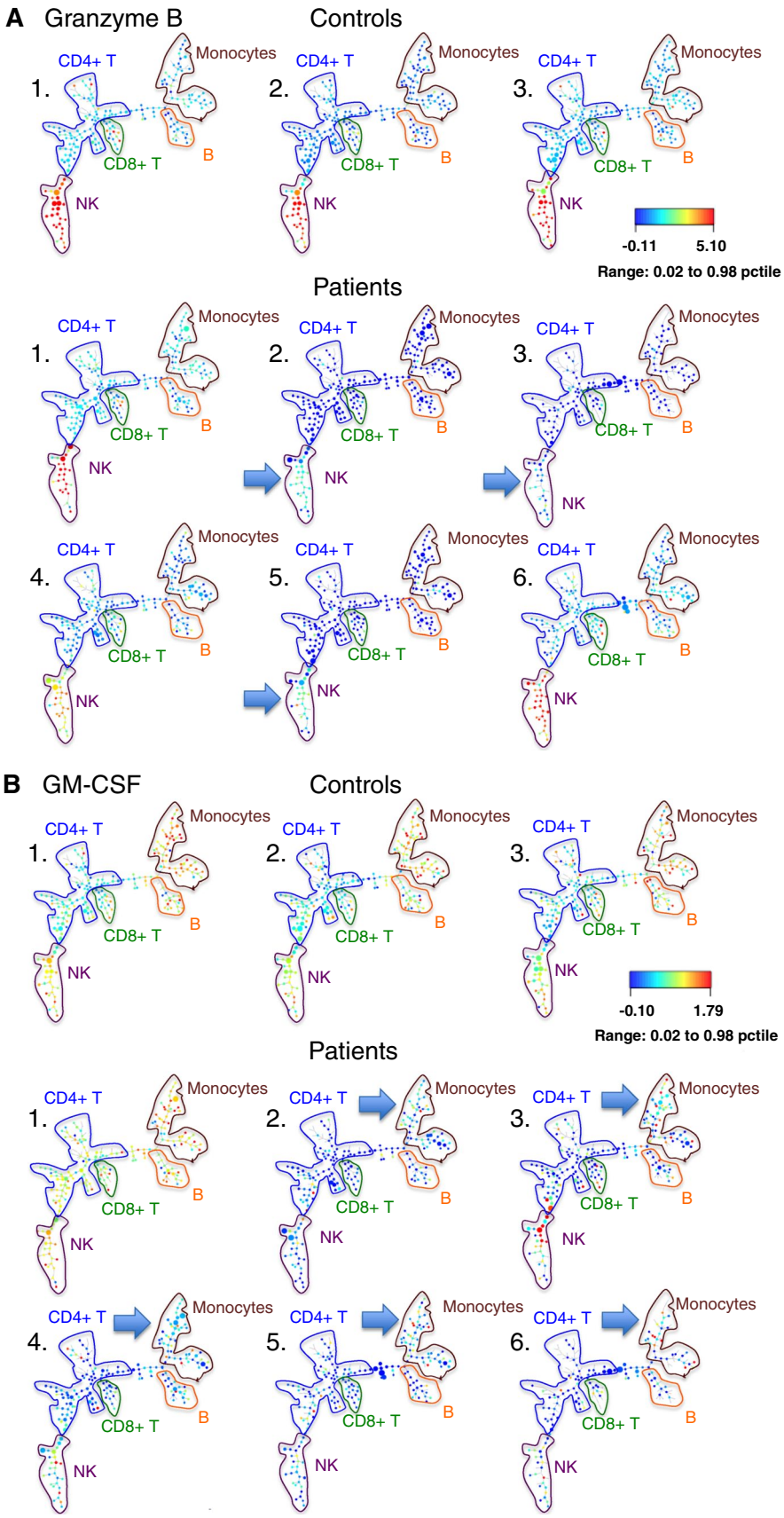

Fig. 1 SPADE analysis of PMA + ionomycin-stimulated PBMCs from three healthy controls (*top row*) and six head and neck cancer patients (*bottom row*) prior to immunotherapy. Major cell lineages are annotated based on lineage marker expression. Cell clusters are colored for granzyme B (**a**), or for GM-CSF (**b**) median intensity (arbitrary units), and cluster size is proportional to cell number. *Note* the lack of granzyme B expression in NK cells and lack of GM-CSF expression in monocytes, in certain patients compared to controls (*blue arrows*)

such measurements. With the first steps already being taken by the development of the immunoscore, we look forward to seeing more sophisticated tests of immune competence for cancer patients being applied in the near future. It then remains to be seen what biomarkers will emerge for prognosis of outcome or choice of immunotherapy modality.

Conflict of interest The authors declare that they have no conflict of interest.

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