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INVITED REVIEW

Towards the introduction of the 'Immunoscore' in the classification of malignant tumours

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Abstract

The American Joint Committee on Cancer/Union Internationale Contre le Cancer (AJCC/UICC) TNM staging system provides the most reliable guidelines for the routine prognostication and treatment of colorectal carcinoma. This traditional tumour staging summarizes data on tumour burden (T), the presence of cancer cells in draining and regional lymph nodes (N) and evidence for distant metastases (M). However, it is now recognized that the clinical outcome can vary significantly among patients within the same stage. The current classification provides limited prognostic information and does not predict response to therapy. Multiple ways to classify cancer and to distinguish different subtypes of colorectal cancer have been proposed, including morphology, cell origin, molecular pathways, mutation status and gene expression-based stratification. These parameters rely on tumour-cell characteristics. Extensive literature has investigated the host immune response against cancer and demonstrated the prognostic impact of the *in situ* immune cell infiltrate in tumours. A methodology named 'Immunoscore' has been defined to quantify the *in situ* immune infiltrate. In colorectal cancer, the Immunoscore may add to the significance of the current AJCC/UICC TNM classification, since it has been demonstrated to be a prognostic factor superior to the AJCC/UICC TNM classification. An international consortium has been initiated to validate and promote the Immunoscore in routine clinical settings. The results of this international consortium may result in the implementation of the Immunoscore as a new component for the classification of cancer, designated TNM-I (TNM-Immune).

Keywords: Immunoscore; colorectal cancer; colon carcinoma; tumour microenvironment; immune response; classification; prognostic markers; predictive markers; T cells

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Introduction

Cancer is a complex and dynamic disease characterized by major hallmarks. They include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism and evading immune destruction [1]. The most common system for classifying the extent of spread of cancer is the American Joint Committee on Cancer/Union Internationale Contre le Cancer (AJCC/UICC) TNM classification [2–4]. The tumour staging gives an estimation of the degree of tumour progression at the time of the surgical resection. The higher the degree of tumour progression, the greater the chance that the tumour will have undergone clonal evolution and acquired a set of unfavourable characteristics, such as the ability to invade lymphatic or blood vessels or to metastasize to distant sites. Furthermore, multiple tumour-cell parameters are an indication of the intrinsic biology of the tumour.

The TNM classification has been used for over 80 years and is valuable in estimating the outcome of patients for a variety of cancers [2–4]. There has been a continual refinement of the staging system. In 2009, the Union for International Cancer Control issued the seventh edition of the TNM classification guidelines. The pathologists generate data from a pathological snapshot. This static measurement integrating the tumour grade and stage is correlated with a dynamic process, such as the time to occurrence or death. The system is used in clinical trials to select patients who are eligible for inclusion, and in cancer registries to compare outcomes between different series, across different countries and over different time periods [5]. An accurate, stable internationally agreed staging system is essential to global progress in this disease. Its main aim should be to provide prognostic information and, based on this information, individual treatment decisions can then be made. In daily practice and in guidelines, the TNM category is directly linked to treatment strategies and, as such, changes in the TNM staging system have a considerable and direct impact on the cancer care that patients receive.

This TNM staging system has stood the test of time but provides incomplete prognostic information. Clinical outcome can significantly vary among patients within the same histological tumour stage [5]. In some patients, advanced-stage cancer can remain stable for years, and although rare, partial or full regression of metastatic tumours can occur spontaneously [6]. In contrast, relapse, rapid tumour progression and patient death is associated with approximately 25% of TNM I/II stage colorectal cancer (CRC) patients, despite complete surgical resection and no evidence of residual tumour burden or distant metastasis [6].

Unfortunately, the predictive accuracy of the traditional staging system relies on the assumption that disease progression is a tumour cell-autonomous process. The focus of this classification is solely on the

tumour cells and fails to incorporate the effects of the host immune response [7]. The phenotype of a tumour is not governed only by the epithelial component but also by the tumour environment, that is, other cells in contact with the tumour, the mesenchyme and the inflammatory infiltrate. These components determine the net inputs to the cell, which include ligands, cell–cell adhesion molecules, metabolites, oxygen and multiple soluble factors [1]. Relatively little information is available on the spatial organization of key proteins and cells, although new imaging techniques offer the potential for high-resolution measurements of the spatio-temporal dynamics of large numbers of proteins [8].

The TNM classification was never surpassed in multivariate analysis by alternative methods such as immunohistochemistry for tumour biomarkers, flow-cytometry for DNA content, molecular signatures or genetic features. However, it was shown that the analysis of a specific type of intratumoural immune response was indeed surpassing the TNM classification in multivariate analysis [9,10]. Thus, tumour progression has now to be considered as the result of a balance between an invasive tumour process and a defence system whose major component is constituted by the host immune response.

Molecular subtypes of colorectal cancer

Several ways to classify cancer have been proposed (Figure 1). These rely on tumour cell characteristics, including morphology, molecular pathways, mutation status, cell origin and gene expression-based methods, and allow the distinction of multiple, often overlapping, subtypes.

A morphology-based classification allows the distinction between five major types of CRC. More than 90% of CRCs are adenocarcinomas originating from epithelial cells. A number of histological variants are described: mucinous, signet ring cell, medullary, micropapillary, serrated, cribriform comedo-type, adenosquamous, spindle cell and undifferentiated. These histopathological criteria have modest prognostic value.

A second class is based on molecular pathways. CRC is molecularly defined into three groups: chromosomal unstable (CIN), microsatellite unstable (MSI) and CpG island methylator phenotype (CIMP). Most cases arise through the CIN pathway, with imbalances in chromosome number and loss of heterozygosity. These cancers have accumulation of mutations in specific tumour suppressor genes and oncogenes that activate pathways critical for CRC initiation and progression. A high degree of MSI (MSI-high) is present in 15% of CRCs and represents a specific type of genomic instability characterized by frequent microsatellite length mutations. Frameshift mutations in microsatellite instability high (MSI-High) colorectal cancers are a potential source of targetable neo-antigens [11]. Most MSI-high cancers are caused by epigenetic silencing of a mismatch-repair gene (*MLH1*). This silencing typically occurs in tumours of the CpG island methylator phenotype (CIMP-high) [12,13]. MSI-high cancers overlap with those of CIMP-high cancers [13,14]. CIMP-high represents a specific type of epigenomic instability that is characterized by widespread promoter CpG island methylation and epigenetic gene silencing [15]. Nonetheless, different from the MSI subgroup, CIMP-high patients have similar characteristics to MSI, and have been associated with old age, female gender, proximal tumour location, poor tumour differentiation, *BRAF* mutation, wild-type *TP53*, high-level of global DNA methylation and stable chromosomes (CIN) [12,13]. MSI tumours have a more favourable prognosis and are less prone to lymph node or distant metastasis [16]. CIMP-high might be a prognostic marker independent of the presence of MSI and *BRAF* mutation [17].

A third method to classify CRCs is based on mutation analysis. Following the discoveries from Bert Vogelstein *et al* that tumours result from the sequential accumulation of alterations in oncogenes and tumour suppressor genes and the appearance of driver mutations, several mutations are now regularly tested in CRCs. These include the *APC*, *KRAS*, *TP53*, *BRAF*, *NRAS*, *PI3KCA* and *CTNNB1* genes. Somatic mutations in codons 12, 13 and 61 of *KRAS*, and more recently *NRAS*, predict innate resistance to mAbs targeting epidermal growth factor receptor [18–20]. The *BRAF* V600E mutation is an adverse prognostic factor in stage IV colorectal cancer but does not have clinical utility at present [21].

The fourth and fifth methods, assessing the cell of origin and gene expression, respectively, to classify CRCs are molecular based techniques. Using this approach, six groups of CRCs were recently defined, based on similarities with distinct cell types within the normal colon crypt and the response to classic chemotherapy and targeted therapies [22]. Other gene expression-based analysis described three groups of CRC patients [23], which correlated with two of the known molecularly defined groups, namely the MSI (renamed CCS2) and the CIN (CCS1) groups, whereas the third group corresponded partly with the CIMP group (CCS3). Further analysis of precursor lesions (serrated polyps) suggested that this latter group could be derived from the serrated pathway. Some partial overlap exists between both proposed gene expression-based classifications, especially with respect to the group that is defined by the MSI-high phenotype. Other gene signatures were also reported and, strikingly, most of the genes were unique to each signature. These signatures showed low prediction accuracy and moderate clinical usefulness [24].

Interestingly, both profiles emphasize the importance of *ZEB1*, a transcription factor regulating the epithelial–mesenchymal transition (EMT). However, EMT in CRC is generally not a feature of the whole

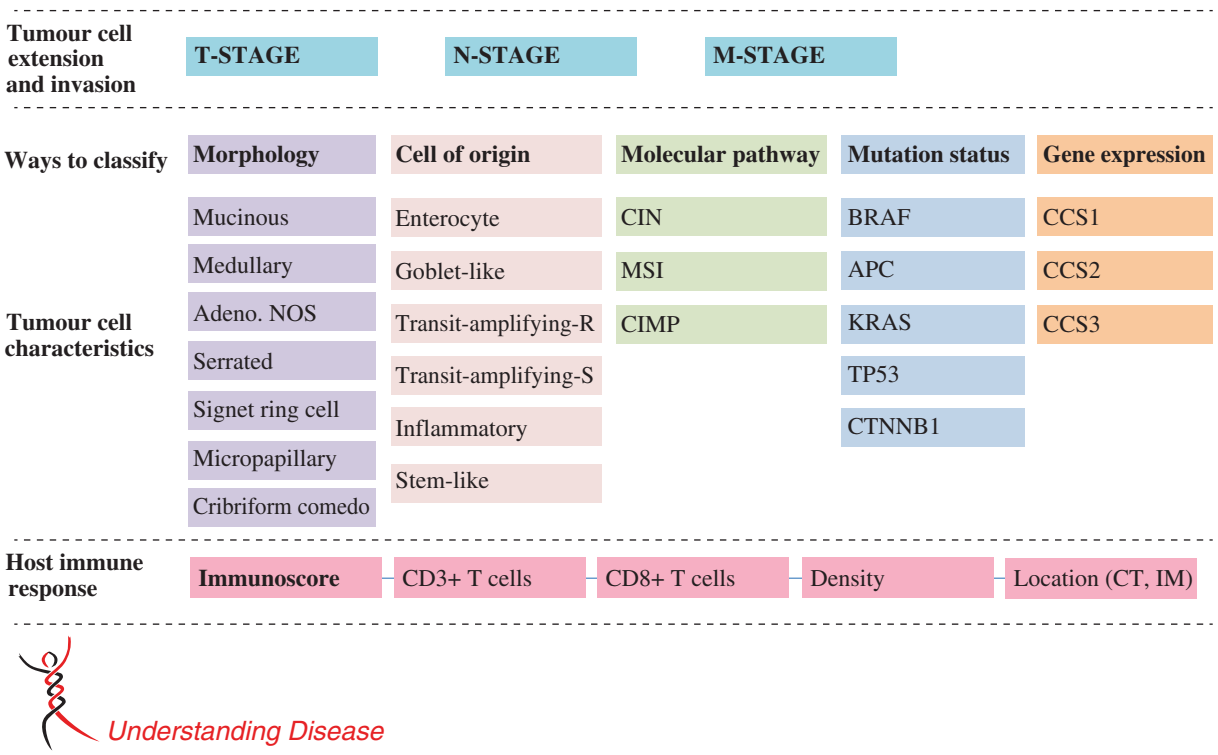

Figure 1. Classification of colorectal cancer. (Top) Classification based on tumour cell extension and invasion. (Middle) Classification based on tumour cell characteristics. Adeno NOS, adenocarcinoma not otherwise specified; Transit-amplifying-R, transit-amplifying-resistant; Transit-amplifying-S, transit-amplifying-Sensitive; CIN, chromosomal unstable; MSI, microsatellite unstable; CIMP, CpG island methylator phenotype. (Bottom) Classification based on the host immune response using the Immunoscore.

tumour but is seen at the invasive margin (IM). The stroma of human colorectal tumours was shown to contain TWIST1-positive cancer cells with mesenchymal phenotypes [25]. EMT is characterized by tumour cell budding, nuclear expression of β-catenin, loss of CDH1 (E-cadherin), gain of CDH2 (*N*-cadherin) and alteration of other epithelial cell adhesion molecules [26]. These features are often not molecularly defined but the result of interplay between the tumour and the microenvironment. Such local features lead to heterogeneity of the gene expression profile. Thus, gene expression classification may be prone to bias, due to different percentages of tumour cells in the sample, but also due to lack or uncontrolled presence of the IM in the sample used for RNA extraction. These issues were not addressed in either study [22,23]. Because of the limitation of gene expression signatures, and to facilitate implementation and testing in large patient series, both studies developed an immunohistochemistry approach based on gene expression data. Unfortunately, half of the patients turned out to be unclassifiable [22] and the absence of *CDX2* positivity in all CCS3 samples (25% of CRCs) [23] does not correspond with extensive literature data (98% positivity in CRCs) [27].

The carcinogenic process that gives rise to an individual tumour is unique. It is postulated that tumours with similar characteristics share common pathogenic mechanisms and progression patterns. However, other major parameters have to be taken into consideration, in particular the tumour microenvironment. Importantly, neoplastic cells interact with host non-neoplastic cells (including immune cells) and extracellular matrix in the tumour microenvironment, and those components influence each other and modify an integral phenotype of any given tumour [28]. Many markers, signatures and methods have been described to evaluate the prognosis of cancer patients, yet very few such markers and laboratory assays translate into clinical practice or reach the statistical power of the TNM classification.

New ways to classify cancers using tumour microenvironment-related information

Modern classification of tumours is based on the recognition of disease entities that are characterized by morphological, phenotypical and genetic markers. Each classification system needs to be reliable, reproducible, clinically relevant and biologically meaningful. Many hurdles have to be taken into consideration. First, the inevitable presence of non-neoplastic cells, including immune cells, in 'tumour areas' means that DNA (or RNA) from the tumour area is not 'pure' DNA (or RNA) from neoplastic cells. These 'contaminants', often > 50% of non-tumour cells, may in fact have a profound biological meaning. Thus, the degree of immune cell infiltration may correlate with tumour molecular changes or may mask a correlation between

a tumour marker and the outcome, because 'contaminating' non-neoplastic cells can influence the results of a tumour molecular assay. For sensitive mutation detection, sequencing technologies allow the detection of approximately 5% of mutant alleles. For quantitative DNA methylation assays, a careful assessment of a potential influence of contaminating normal cells is necessary. This is even more problematic with gene expression signatures, where a different degree of non-neoplastic cells is observed among the assays.

Second, a precise characterization of specific tumour-infiltrating cells requires the use of an immunohistochemical technique to detect in the tissue the presence and localization of specific antigens expressed by subsets of immune cells. The evaluation of immune cells in haematoxylin and eosin (H&E)-stained sections can be done at a low cost compared to an immunohistochemical technique. However, an evaluation of a specific subset of immune cells is generally not possible with H&E-stained sections. For example, lymphocytes with opposite functions, such as $CD4^+$ T cells with Th1 orientation versus Th2 orientation versus immune cells with regulatory functions (Treg cells), or NK cells, NKT cells, B cells or cytotoxic CD8 T cells, are indistinguishable without proper marker evaluation and require antibody labelling by immunohistochemistry. Nowadays this is simple, as specific antibodies with high affinity and a high signal:noise ratio allow the detection of these immune cell subpopulations with a single staining.

Third, it is known that immune cells are scattered in the core of the tumour (CT) within the tumour stroma and the tumour glands, in the invasive margin (IM) and in organized lymphoid follicles distant from the tumour. A statistically significant correlation between immune cell density in each tumour region (CT and IM) and patient outcome has been shown in colorectal cancer [9]. Further, the combined analysis of tumour regions (CT plus IM) improved the accuracy of prediction of survival for the different patient groups compared with single-region analysis [9]. Given the major clinical importance of distinct tumour regions, it is appropriate to conduct immune cell infiltration evaluation systematically in the two separate areas, the core of the tumour and the invasive margin [9,10]. For routine practice, this requires immune cell evaluation on whole-tissue sections, taking into account their location.

Fourth, objective ways of counting immune cells need to be achieved in order to remove the subjectivity of field selection and imprecise semi-quantitative evaluation. Most of the tumour markers are generally more complicated to quantify than immune cells, since only a fraction of the tumour cells express the antigen and the staining intensity has often been taken into consideration. Standardized and reproducible measurement of the intensity of staining, and hence quantitation of protein expression, is intrinsically difficult using immunohistochemistry. In contrast, immune cell types are easier to quantify because well-characterized markers exist, giving complete membrane staining for immune cell subpopulations (eg $CD3^+$ for T cells), allowing counting cells as individual cells. Nowadays, digital pathology and image analysis software can detect stained immune cells and determine their densities (n cells/mm^2) in histological sections. Validation studies demonstrated the high concordance of these automated systems in comparison to optical counts [9,29]. This is particularly important to facilitate routine pathology and to speed up the process of quantification. In particular, given the huge number of infiltrating immune cells within a tumour (eg a mean of 75 000 $CD3^+$ cells present on a 4 μm-thick section of tumour slide from a CRC stage I/II patient), it would be unrealistic to ask pathologists to count them all. In addition, immune cells such as $CD3^+$ lymphocytes are often aggregated in complex cell clusters. Algorithms for segmenting clusters of densely packed cells permit a precise counting of cells. Given the major importance of the determination of the density of immune cells [9,30] to predict a patient's outcome, it is now required to take advantage of the digital pathology to determine the exact count of stained cells and the surface of the tissue analysed.

Fifth, the heterogeneity of a tumour applies to tumour cells but to the microenvironment as well. Upon evaluation of whole-tissue sections, a pathologist often needs to choose specific fields to perform detailed image analysis. The selection of the tissue areas to study may depend on subjective interpretation, and the evaluation needs to be validated by a second independent observer blinded for the other results. Inter-observer variability can reach very high levels [31]. The use of computer-assisted image analysis provides important advantages, as all the fields are analysed in the whole tumour section [32]. For example, the determination of the mean density of stained cells in a tumour region is supported by an objective computer-based cell-counting method, leading to a good level of reproducibility between users.

Sixth, specific markers need to be selected. They should be robust, well-established and have high predictive value. A growing body of literature [9,10,30,33–36] supports the hypothesis that cancer development is influenced by the host immune system. This may offer powerful prognostic information and facilitate clinical decision making regarding the need for systemic therapy [7]. Accumulat-ing evidence suggests that $CD3^+$ [9,10,30,34,37–43], $CD8^+$ [9,10,34,38,43–52] and $CD45RO^+$ [9,10,34,40,47,49,53,54] cells have roles in antitumour immune responses. Numerous data collected from large cohorts of human CRCs (with sample sizes $n = 415$, 599 and 602, respectively) demonstrated that the number, type and location of tumour immune infiltrates in primary tumours are prognostic for disease-free survival (DFS) and overall survival (OS) [9,10,30]. Altogether, these immune parameters are designated the 'immune contexture' [33,35,55]. Notably, several large studies of CRCs (with sample

sizes $n = 843$ and $n = 768$, respectively) have shown that tumour lymphocytic reaction and T cell subpopulations (CD8, CD45RO, FOXP3) are significant prognostic biomarkers, even after adjusting for stage, lymph node count and well-established prognostic tumour molecular biomarkers, including microsatellite instability (MSI), *BRAF* mutation and LINE-1 hypomethylation [54,56]. A possible association exists between MSI status and immune cell infiltrates [38]. MSI tumours often contain intra-epithelial T cells in response to the expression of neo-antigens on the cell surface [42], in particular those that do not undergo non-sense medicated decay [11]. This probably contributes to the better prognosis of patients with MSI tumours. Furthermore, CRCs from a large cohort ($n = 1197$) and external validation ($n = 209$) confirmed the prognostic value associated with $CD3^+$, $CD8^+$ and $CD45RO^+$ (PTPRC) T cell infiltration in CRCs [43].

A recent meta-analysis [55] summarized the impact of immune cells, including B cells, NK cells, MDSC, macrophages and all subsets of T cells on clinical outcome from more than 120 published articles. Importantly, the beneficial impact of the immune infiltrate with cytotoxic and memory T cell phenotype has been demonstrated in cancers from diverse anatomical sites; including colorectal cancer but also malignant melanoma, lung, gastric, oesophageal, head and neck, breast, bladder, urothelial, ovarian, cervical, prostatic and pancreatic cancer, hepatocellular carcinoma, medulloblastoma and Merkel cell carcinoma [55]. It is interesting to note that the implications of this immune phenotype apply not only to various organs of cancer origin, but also to various cancer cell types, ie adenocarcinoma, squamous cell carcinoma, large cell cancer and melanoma. Thus, general characteristics emerge in which cytotoxic T cells, memory T cells and T_H1 cells are associated with prolonged survival [35,57,58]. In contrast, the prognostic impact of other immune cells, such as B cells, NK cells, MDSCs, macrophages and a subset of T-helper populations (T_H2, T_H17, T_{REG} cells), differ depending on the type of cancer and on the cancer stage [55]. Altogether, the publications indicate that a precise analysis of the immune component of the tumour microenvironment by computer-based analysis may be essential to managing patients better. Thus, precise analysis of the tumour microenvironment by pathologists may be essential for future clinical implementation and better patient management. An expertise in this new emerging field is now warranted to translate it into the clinical practice.

'Immunoscore' as a new approach for the classification of cancer

A potential clinical translation of these observations is the establishment of a scoring system designated 'Immunoscore' (Figure 2), derived from the

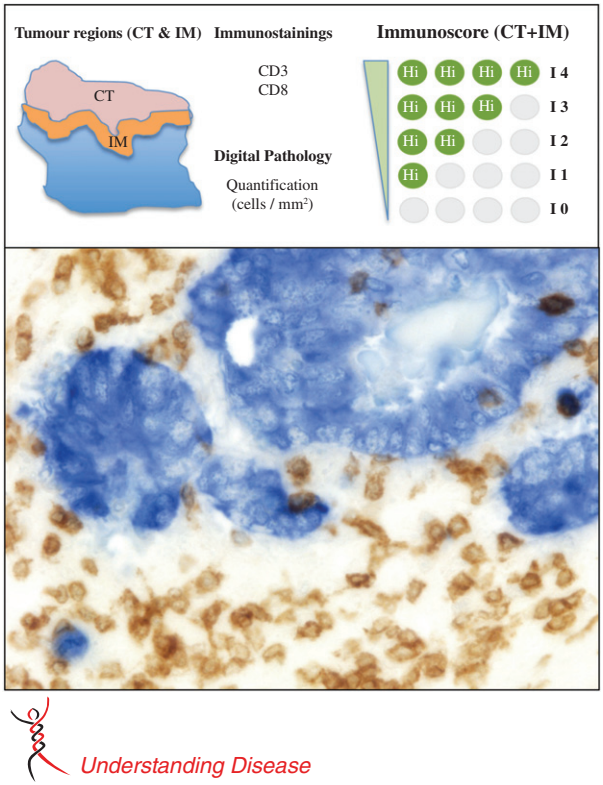

Figure 2. (Top) Immunoscore definition and methodology. (Bottom) Immunohistochemistry of a colorectal tumour stained for $CD3^+$ T cells (brown).

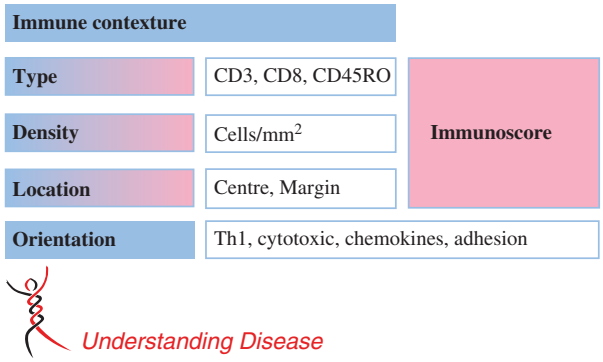

Figure 3. Correspondence between the immune contexture and the Immunoscore.

immune contexture (Figure 3) [33,35,55], and based on the numeration of two lymphocyte populations (CD3/CD45RO, CD3/CD8 or CD8/CD45RO), both in the core of the tumour (CT) and in the invasive margin (IM) of tumours, as a clinically useful prognostic marker in colorectal cancer [34]. Detailed description of the immune contexture in comparison to the Immunoscore has been described previously [35,36]. The Immunoscore provides a score ranging from Immunoscore 0 (I0) when low densities of both cell types are found in both regions, to Immunoscore 4 (I4) when high densities are found in both regions. This test has a dual advantage: first, it appears to be the strongest prognostic factor for DFS, disease-specific (DSS) and

OS, including at early-stage colorectal cancers; and second, it has biological meaning (adaptive immune response to tumours) and provides a tool or a target for novel therapeutic approaches, including immunotherapy (as recently illustrated in clinical trials boosting T cell responses with anti-CTLA4, anti-PDCD1 (PD-1) and anti-CD274 (PD-L1) [59–62]. Current immunohistochemical technologies allow the application of such analyses in routine diagnostic pathology. Thus, considering the probable universal character of the immune control of tumours, it is essential for patients to take into account the immune parameter as a prognostic factor and to introduce the Immunoscore as a component of cancer classification [6,7,35,63,64].

The Immunoscore was shown to be very powerful, for instance, in CRC patients with clinically localized colorectal cancer, with no detectable tumour spreading to lymph nodes or distant organs. These patients are usually treated only by a surgical removal of the tumour; however, approximately 25% of these patients will have recurrence of their disease, indicating that occult metastases were already present at the time of curative surgery. No tumour-associated marker predicts the recurrence of this subgroup of patients that could have a benefit for an adjuvant therapy. The Immunoscore (I) approach (with an enumeration of $CD8^+$ and $CD45RO^+$ cells in the CT and the IM) was applied to two large independent cohorts ($n = 602$). Only 4.8% of patients with a high I4 relapsed after 5 years and 86.2% were still alive. In contrast, 72% of patients with a low score (I0 and I1) experience tumour recurrence and only 27.5% were alive at 5 years. These I0 and I1 patients could potentially have benefited from adjuvant therapy if the Immunoscore had been incorporated into the tumour staging [34].

The Immunoscore classification, demonstrating the prevalence of immune infiltrates, was shown to have a prognostic significance superior to that of the AJCC/UICC TNM classification system. For all patients with CRC stages I/II/III, multivariate Cox analysis revealed that the immune criteria remained highly significantly associated with prognosis (DFS, DSS, OS). In contrast, the histopathological staging system (T stage, N stage and tumour differentiation) was no longer significant [10]. Tumour invasion was shown to be statistically dependent on the host immune reaction. Indeed, the immune pattern remained the only significant criterion over the classical AJCC/UICC TNM classification for DFS and OS, and led to an editorial entitled 'TNM staging in colorectal cancer: T is for T cell and M is for memory' accompanying the publication by Mlecnik *et al* [10,65]. It has thus been suggested that the prevalence of tumour immune infiltrates, more than the tumour status, could be a key indicator for recurrence, metastasis and therefore clinical outcome (Figure 4). These results suggest that once human cancer becomes clinically detectable, the adaptive immune response plays a critical role in preventing tumour recurrence. The ability of effector-memory T cells to recall previously encountered antigens leads to a protective response. Following primary exposure to antigen, memory T cells disseminate and are maintained for long periods of time [66]. The trafficking properties and the long-lasting antitumour capacity of memory T cells could result in long-term immunity in human cancer. Over the past few years, the area of immune regulation at the level of the tumour microenvironment has gained a forefront position in cancer research, in CRC [9,10,30,34,55], melanoma [67] and all other cancer types [7]. The Immunoscore, initially described several years ago [9] as a prognostic factor [10,34], could also play a role as a marker to predict the response to biotherapies targeting the immune checkpoints [63,64],

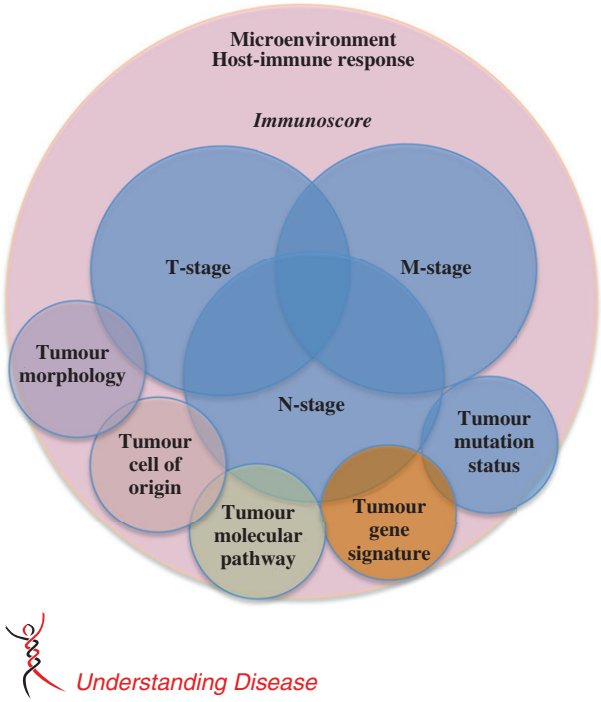

Figure 4. Schematic representation of cancer classification based on tumour-microenvironment-related parameters (Immunoscore), based on tumour extension and invasion (T, N, M stages), and based on five alternative tumour-related methods.

The inherent complexity of immunohistochemistry, in conjunction with protocol variability, contributes to the variability of the results obtained. A standardized consensus method is required. Large-scale assay harmonization efforts have already been witnessed, conducted for commonly used immunological assays of peripheral blood immune cell populations [68,69]. It is therefore essential to pursue assay uniformity to reduce these limitations. A clinical validation of the Immunoscore with standardized procedures is necessary to reach clinical applicability for individual patients.

We performed multiple Immunoscore quality controls to test the accuracy and repeatability of the method. We first observed that the automated cell-counting method achieved a very good level of correlation with the optical counting for CD3 and CD8 immunostainings and an excellent reproducibility of

Good biomarker	Immunoscore characteristics
Routine	☑ Technique to be performed by pathologist using bright field and precise cell evaluation
Feasible	☑ Established pathology techniques, using 2 regular whole slide FFPE sections
Simple	☑ Automatized immunochemistry
Inexpensive	☑ Two simple stainings less costly than complicated molecular techniques
Rapid	☑ Autostainers, scanner, digital pathology reduce the time to perform an Immunoscore
Robust	☑ Two strong membrane stainings, with no background, allowing the enumeration of individual cells
Reproducible	☑ Inter-observers variability is removed by the use of digital pathology, taking into account cell location and counts
Quantitative	☑ Precise cell density (cell number / surface area)
Standardized	☑ Standardized operating procedure should be performed to ensure reproducibility and worldwide comparisons
Pathology-based	☑ Necessity of pathologist expertise to validate cell type, cell location, and cell counts perform by digital pathology
Powerful	☑ The Immunoscore has a prognostic value highly significant even in Cox multivariate including TNM classification [9, 10]

Understanding Disease

Figure 5. Characteristics of a good biomarker and of the Immunoscore.

the count of stained cells. In addition, the variablity between users for the determination with the software of the immune cell densities in tumour regions was 5.2% and 2.5% for CD3 and CD8, respectively (unpublished data). All the tests indicated a reliable assessment of the Immunoscore. To be used globally in a routine manner, evaluation of a novel marker should have the following characteristics: do-able in routine, feasible, simple, inexpensive, rapid, robust, reproducible, quantitative, standardized, pathology-based and powerful. The Immunoscore has a potential to fulfil these key aspects (Figure 5). Importantly, for patients with rectal cancer treated by chemoradiotherapy (pCRT) before surgery, preoperative chemoradiation therapy induces histological reactions precluding the realization of an Immunoscore, since the delineation of the analysed tumour regions (CT and IM) is often no longer practicable. Also, assessment of anti-tumour immunity by the Immunoscore is inappropriate in biopsies, since the limitation of the material precludes a precise delimitation of the tumour and the invasive margin. However, immune cell quantification remains an interesting possibility in tumour biopsies.

To assess the Immunoscore in clinical practice and measure its prognostic value, we are conducting a prospective multicentre study (French PHRC programme) for 600 patients from seven hospitals bearing a colorectal cancer treated by primary surgery. In an effort to promote the utilization of the Immunoscore in routine clinical settings, we initiated a worldwide Immunoscore consortium, with the support of the Society for Immunotherapy of Cancer (SITC) [64]. Several thousands of tumours from patients will be Immunoscore-tested by the 23 centres from the worldwide consortium. The worldwide Immunoscore consortium, composed of international expert pathologists and immunologists, identified a strategy for the organization of worldwide retrospective study for the validation of the Immunoscore in colon cancer. Evidence-based selection of specific markers for the Immunoscore was discussed. Because of background staining and loss of antigenicity in stored sections (CD45RO) and granular staining (GZMB), it was decided to employ the two easiest membrane stains, CD3 and CD8. Thus, the combination of two markers ($CD3^+$ and $CD8^+$) in two regions (CT and IM) was agreed for validation in standard clinical practice. Precise quantification is currently performed on whole slide sections (Figure 2), following the recommended initial guidelines.

The purpose of the ongoing Immunoscore worldwide consortium is to validate the following points: first, to demonstrate the feasibility and reproducibility of the Immunoscore; second, to validate the major prognostic power of the Immunoscore in routine for patients with colon cancer stages I/II/III; and third, to demonstrate the utility of the Immunoscore to predict stage II colon cancer patients with high risk of recurrence. Twenty-three international pathology expert centres are now participating in the Immunoscore enterprise. These participants represent 23 centres from 17 countries, including Asia, Europe, North America, Australia and the Middle East (Australia, Austria, Canada, China, Czech Republic, France, Germany, India, Italy, Japan, The Netherlands, Qatar, Spain, Sweden, Switzerland, the UK and the USA). It is hoped that this initiative will result in the implementation of the Immunoscore as a new component for the classification of cancer, TNM-I (Immune). The Immunoscore should better define the prognosis of cancer patients, better identify patients at high risk of tumour recurrence [70], help to predict and stratify patients who will benefit from therapies [35] and, ultimately, help save the lives of patients with cancer.

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Author contributions

JG is coordinating the Worldwide Immunoscore initiative, conceived the study and wrote the manuscript; JG and FP initiated the Immunoscore project; BM, GB, FP, CL, AB and JG performed the initial experiments related to the Immunoscore; FP and HKA participated in the drafting of the manuscript. AL, CB, GB, FT, PD, AH, MA, LL, MM, FG, FP, FMM, BAF and JG were experts involved in the design of the Immunoscore study and expert pathologists participating to the inaugural Immunoscore workshop. All authors, JG, BM, GB, HKA, AB, CL, AL, IZ, AH, CB, IDN, RP, GVM, GB, FT, PD, MM, LL, FG, MA, CDA, FVV, EZ, LC, PSO, SHB, BGW, MR, LN, YK, SH, KO, SO, PG, PW, NS, TT, KY, PSP, SNS, YW, SK, FAS, PAA, FMM, BAF, VS, FP, are participants of the initial worldwide Immunoscore task force study and have read and approved the manuscript.

References

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144:** 646–674.
2. Locker GY, Hamilton S, Harris J, *et al.* ASCO 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer. *J Clin Oncol* 2006; **24:** 5313–5327.
3. Sobin L, Wittekind C. *TNM Classification of Malignant Tumors*. Wiley-Liss: New York, 2002.
4. Weitz J, Koch M, Debus J, *et al.* Colorectal cancer. *Lancet* 2005; **365:** 153–165.
5. Nagtegaal ID, Quirke P, Schmoll HJ. Has the new TNM classification for colorectal cancer improved care? *Nat Rev Clin Oncol* 2011; **9:** 119–123.
6. Mlecnik B, Bindea G, Pages F, *et al.* Tumor immunosurveillance in human cancers. *Cancer Metast Rev* 2011; **30:** 5–12.
7. Bindea G, Mlecnik B, Fridman WH, *et al.* Natural immunity to cancer in humans. *Curr Opin Immunol* 2010; **22:** 215–222.
8. Faratian D, Clyde RG, Crawford JW, *et al.* Systems pathology – taking molecular pathology into a new dimension. *Nat Rev Clin Oncol* 2009; **6:** 455–464.
9. Galon J, Costes A, Sanchez-Cabo F, *et al.* Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006; **313:** 1960–1964.
10. Mlecnik B, Tosolini M, Kirilovsky A, *et al.* Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction. *J Clin Oncol* 2011; **29:** 610–618.
11. Williams DS, Bird MJ, Jorissen RN, *et al.* Nonsense mediated decay resistant mutations are a source of expressed mutant proteins in colon cancer cell lines with microsatellite instability. *PLoS One* 2010; **5:** e16012.
12. Ogino S, Cantor M, Kawasaki T, *et al.* CpG island methylator phenotype (CIMP) of colorectal cancer is best characterised by quantitative DNA methylation analysis and prospective cohort studies. *Gut* 2006; **55:** 1000–1006.
13. Samowitz WS, Albertsen H, Herrick J, *et al.* Evaluation of a large, population-based sample supports a CpG island methylator phenotype in colon cancer. *Gastroenterology* 2005; **129:** 837–845.
14. Nosho K, Irahara N, Shima K, *et al.* Comprehensive biostatistical analysis of CpG island methylator phenotype in colorectal cancer using a large population-based sample. *PLoS One* 2008; **3:** e3698.
15. Zlobec I, Bihl M, Foerster A, *et al.* Comprehensive analysis of CpG island methylator phenotype (CIMP)-high, -low and -negative colorectal cancers based on protein marker expression and molecular features. *J Pathol* 2011; **225:** 336–343.
16. Laghi L, Malesci A. Microsatellite instability and therapeutic consequences in colorectal cancer. *Dig Dis* 2012; **30:** 304–309.
17. Ogino S, Nosho K, Kirkner GJ, *et al.* CpG island methylator phenotype, microsatellite instability, *BRAF* mutation and clinical outcome in colon cancer. *Gut* 2009; **58:** 90–96.
18. Laurent-Puig P, Cayre A, Manceau G, *et al.* Analysis of *PTEN*, *BRAF*, and *EGFR* status in determining benefit from cetuximab therapy in wild-type *KRAS* metastatic colon cancer. *J Clin Oncol* 2009; **27:** 5924–5930.
19. Karapetis CS, Khambata-Ford S, Jonker DJ, *et al.* *K-ras* mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med* 2008; **359:** 1757–1765.
20. Peeters M, Oliner KS, Parker A, *et al.* Massively parallel tumor multigene sequencing to evaluate response to panitumumab in a randomized phase III study of metastatic colorectal cancer. *Clin Cancer Res* 2013; **19:** 1902–1912.
21. Tie J, Gibbs P, Lipton L, *et al.* Optimizing targeted therapeutic development: analysis of a colorectal cancer patient population with the *BRAF* (V600E) mutation. *Int J Cancer* 2011; **128:** 2075–2084.
22. Sadanandam A, Lyssiotis CA, Homicsko K, *et al.* A colorectal cancer classification system that associates cellular phenotype and responses to therapy. *Nat Med* 2013; **19:** 619–625.
23. De Sousa EMF, Wang X, Jansen M, *et al.* Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions. *Nat Med* 2013; **19:** 614–618.

24. Sanz-Pamplona R, Berenguer A, Cordero D, *et al.* Clinical value of prognosis gene expression signatures in colorectal cancer: a systematic review. *PLoS One* 2012; **7:** e48877.
25. Celesti G, Di Caro G, Bianchi P, *et al.* Presence of Twist1-positive neoplastic cells in the stroma of chromosome-unstable colorectal tumors. *Gastroenterology* 2013; **145:** 647–657.
26. Gosens MJ, van Kempen LC, van de Velde CJ, *et al.* Loss of membranous Ep-CAM in budding colorectal carcinoma cells. *Mod Pathol* 2007; **20:** 221–232.
27. Werling RW, Yaziji H, Bacchi CE, *et al.* CDX2, a highly sensitive and specific marker of adenocarcinomas of intestinal origin: an immunohistochemical survey of 476 primary and metastatic carcinomas. *Am J Surg Pathol* 2003; **27:** 303–310.
28. Ogino S, Galon J, Fuchs CS, *et al.* Cancer immunology – analysis of host and tumor factors for personalized medicine. *Nat Rev Clin Oncol* 2011; **8:** 711–719.
29. Vayrynen JP, Vornanen JO, Sajanti S, *et al.* An improved image analysis method for cell counting lends credibility to the prognostic significance of T cells in colorectal cancer. *Virchows Arch* 2012; **460:** 455–465.
30. Pages F, Berger A, Camus M, *et al.* Effector memory T cells, early metastasis, and survival in colorectal cancer. *N Engl J Med* 2005; **353:** 2654–2666.
31. Rubin MA, Zerkowski MP, Camp RL, *et al.* Quantitative determination of expression of the prostate cancer protein α-methylacyl-CoA racemase using automated quantitative analysis (AQUA): a novel paradigm for automated and continuous biomarker measurements. *Am J Pathol* 2004; **164:** 831–840.
32. Angell HK, Gray N, Womack C, *et al.* Digital pattern recognition-based image analysis quantifies immune infiltrates in distinct tissue regions of colorectal cancer and identifies a metastatic phenotype. *Br J Cancer* 2013; **109:** 1618–1624.
33. Galon J, Fridman WH, Pages F. The adaptive immunologic microenvironment in colorectal cancer: a novel perspective. *Cancer Res* 2007; **67:** 1883–1886.
34. Pages F, Kirilovsky A, Mlecnik B, *et al. In situ* cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer. *J Clin Oncol* 2009; **27:** 5944–5951.
35. Angell HK, Galon J. From the immune contexture to the Immunoscore: the role of prognostic and predictive immune markers in cancer. *Curr Opin Immunol* 2013; **25:** 261–267.
36. Galon J, Angell HK, Bedognetti D, *et al.* The continuum of cancer immunosurveillance: prognostic, predictive, and mechanistic signatures. *Immunity* 2013; **39:** 11–26.
37. Dahlin AM, Henriksson ML, Van Guelpen B, *et al.* Colorectal cancer prognosis depends on T-cell infiltration and molecular characteristics of the tumor. *Mod Pathol* 2011; **24:** 671–682.
38. Guidoboni M, Gafa R, Viel A, *et al.* Microsatellite instability and high content of activated cytotoxic lymphocytes identify colon cancer patients with a favorable prognosis. *Am J Pathol* 2001; **159:** 297–304.
39. Laghi L, Bianchi P, Miranda E, *et al.* $CD3^+$ cells at the invasive margin of deeply invading (pT3–T4) colorectal cancer and risk of post-surgical metastasis: a longitudinal study. *Lancet Oncol* 2009; **10:** 877–884.
40. Peng RQ, Wu XJ, Ding Y, *et al.* Co-expression of nuclear and cytoplasmic HMGB1 is inversely associated with infiltration of $CD45RO^+$ T cells and prognosis in patients with stage IIIB colon cancer. *BMC Cancer* 2010; **10:** 496.
41. Sinicrope FA, Rego RL, Ansell SM, *et al.* Intraepithelial effector ($CD3^+$)/regulatory ($FoxP3^+$) T cell ratio predicts a clinical outcome of human colon carcinoma. *Gastroenterology* 2009; **137:** 1270–1279.
42. Tougeron D, Fauquembergue E, Rouquette A, *et al.* Tumor-infiltrating lymphocytes in colorectal cancers with microsatellite instability are correlated with the number and spectrum of frameshift mutations. *Mod Pathol* 2009; **22:** 1186–1195.
43. Zlobec I, Karamitopoulou E, Terracciano L, *et al.* TIA-1 cytotoxic granule-associated RNA binding protein improves the prognostic performance of CD8 in mismatch repair-proficient colorectal cancer. *PLoS One* 2010; **5:** e14282.
44. Deschoolmeester V, Baay M, Van Marck E, *et al.* Tumor infiltrating lymphocytes: an intriguing player in the survival of colorectal cancer patients. *BMC Immunol* 2010; **11:** 19.
45. Menon AG, Fleuren GJ, Alphenaar EA, *et al.* A basal membrane-like structure surrounding tumour nodules may prevent intraepithelial leucocyte infiltration in colorectal cancer. *Cancer Immunol Immunother* 2003; **52:** 121–126.
46. Naito Y, Saito K, Shiiba K, *et al.* CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. *Cancer Res* 1998; **58:** 3491–3494.
47. Oberg A, Samii S, Stenling R, *et al.* Different occurrence of $CD8^+$, $CD45R0^+$, and $CD68^+$ immune cells in regional lymph node metastases from colorectal cancer as potential prognostic predictors. *Int J Colorectal Dis* 2002; **17:** 25–29.
48. Prall F, Duhrkop T, Weirich V, *et al.* Prognostic role of $CD8^+$ tumor-infiltrating lymphocytes in stage III colorectal cancer with and without microsatellite instability. *Hum Pathol* 2004; **35:** 808–816.
49. Salama P, Phillips M, Grieu F, *et al.* Tumor-infiltrating $FOXP3^+$ T regulatory cells show strong prognostic significance in colorectal cancer. *J Clin Oncol* 2009; **27:** 186–192.
50. Suzuki H, Chikazawa N, Tasaka T, *et al.* Intratumoral $CD8^+$ $T/FOXP3^+$ cell ratio is a predictive marker for survival in patients with colorectal cancer. *Cancer Immunol Immunother* 2010; **59:** 653–661.
51. Tosolini M, Kirilovsky A, Mlecnik B, *et al.* Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, th2, treg, th17) in patients with colorectal cancer. *Cancer Res* 2011; **71:** 1263–1271.
52. Yoon HH, Orrock JM, Foster NR, *et al.* Prognostic impact of $FoxP3^+$ regulatory T cells in relation to $CD8^+$ T lymphocyte density in human colon carcinomas. *PLoS One* 2012; **7:** e42274.
53. Lee WS, Park S, Lee WY, *et al.* Clinical impact of tumor-infiltrating lymphocytes for survival in stage II colon cancer. *Cancer* 2010; **116:** 5188–5199.
54. Nosho K, Baba Y, Tanaka N, *et al.* Tumour-infiltrating T-cell subsets, molecular changes in colorectal cancer and prognosis: cohort study and literature review. *J Pathol* 2010; **222:** 350–366.
55. Fridman WH, Pages F, Sautes-Fridman C, *et al.* The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* 2012; **12:** 298–306.
56. Ogino S, Nosho K, Irahara N, *et al.* Lymphocytic reaction to colorectal cancer is associated with longer survival, independent of lymph node count, microsatellite instability, and CpG island methylator phenotype. *Clin Cancer Res* 2009; **15:** 6412–6420.
57. Ascierto ML, De Giorgi V, Liu Q, *et al.* An immunologic portrait of cancer. *J Transl Med* 2011; **9:** 146.
58. Pages F, Galon J, Dieu-Nosjean MC, *et al.* Immune infiltration in human tumors: a prognostic factor that should not be ignored. *Oncogene* 2010; **29:** 1093–1102.
59. Brahmer JR, Tykodi SS, Chow LQ, *et al.* Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med* 2012; **366:** 2455–2465.
60. Hodi FS, O'Day SJ, McDermott DF, *et al.* Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 2010; **363:** 711–723.

61. Robert C, Thomas L, Bondarenko I, *et al.* Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med* 2011; **364:** 2517–2526.
62. Topalian SL, Hodi FS, Brahmer JR, *et al.* Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* 2012; **366:** 2443–2454.
63. Galon J, Franck P, Marincola FM, *et al.* Cancer classification using the Immunoscore: a worldwide task force. *J Transl Med* 2012; **10:** 205.
64. Galon J, Pages F, Marincola FM, *et al.* The immune score as a new possible approach for the classification of cancer. *J Transl Med* 2012; **10:** 1.
65. Broussard EK, Disis ML. TNM staging in colorectal cancer: T is for T cell and M is for memory. *J Clin Oncol* 2011; **29:** 601–603.
66. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol* 2004; **22:** 745–763.
67. Ascierto PA, De Maio E, Bertuzzi S, *et al.* Future perspectives in melanoma research. Meeting report from Melanoma Research: A Bridge Naples–USA. Naples, 6–7 December 2010. *J Transl Med* 2011; **9:** 32.
68. Fox BA, Schendel DJ, Butterfield LH, *et al.* Defining the critical hurdles in cancer immunotherapy. *J Transl Med* 2011; **9:** 214.
69. van der Burg SH, Kalos M, Gouttefangeas C, *et al.* Harmonization of immune biomarker assays for clinical studies. *Sci Transl Med* 2011; **3:** 108ps144.
70. Bindea G, Mlecnik B, Tosolini M, *et al.* Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity* 2013; **39:** 782–795.