総説

がんワクチン臨床開発の現状と今後の展望

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Current Status and Future Perspective of Cancer Vaccine Development: Tetsuro Sasada and Kyogo Itoh (*Dept. of Immunology and Immunotherapy, Kurume University School of Medicine*)

Summary

The field of cancer vaccines has moved forward dramatically, along with the progressive increase in basic knowledge of tumor immunology. During the last 20 years, a number of tumor-associated antigens have been identified, some of which have been clinically examined in patients, demonstrating encouraging results as immunotherapy against various types of cancers. However, most of the randomized clinical trials conducted to gain approval for official clinical use of the antigens have failed, due to an inability to demonstrate thair meaningful therapeutic benefit to patients over other existing treatments, with the exception of the dendritic cell (DC)-based vaccine (Provenge®), which has recently been approved as the first therapeutic cancer vaccine in the US. Such unexpected results have shed light on several important issues to solve in regard to further development of cancer vaccines. In particular, more attention should be paid to the fact that the characteristics of tumor cells and the immunological status against cancers differ widely among patients. Of note, the recent failure of cancer vaccines in clinical trials may be explained, at least in part, by the existence of a vaccine-specific adverse event; an induction of an "inconvenient immune response," that inhibits pre-existing host immunity. Development of a novel criteria and reliable biomarkers for selecting adequate patients and vaccine antigens would be a breakthrough for further cancer vaccine development. In this review, we will summarize the current status of cancer vaccine development and discuss how to overcome negative issues raised in recently conducted clinical trials of therapeutic cancer vaccines. **Key words**: Cancer vaccine, Clinical trial, Induction of inconvenient immune response, **Corresponding author**: Tetsuro Sasada, Department of Immunology and Immunotherapy, Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan

要旨 腫瘍免疫学の進歩とともに，がんワクチン療法は飛躍的な発展を遂げつつある。特にここ20年間にがん関連抗原が相次いで分子レベルで同定され，それらを標的としたがんワクチンの臨床応用が可能となった。初期・中期の臨床試験では従来では認められなかった優れた臨床効果が確認され，多くの後期ランダム化比較試験が実施されている。2010年4月には，自己樹状細胞を用いたがんワクチンProvengeが米国FDAより初めて承認され，この分野における画期的な第一歩といえる進展があった。一方，その他の後期ランダム化比較試験のほとんどで有意な臨床効果を立証できず，原点に立ち戻っての科学的・医学的検証が求められている。特にがん細胞や免疫系の多様性・多重性，ワクチンによる"不都合な免疫誘導"など，腫瘍免疫・がんワクチンの特性を再認識し，その知識を患者やワクチン抗原選択のための新しい基準の確立，バイオマーカー開発に反映させることが望まれる。この総説では，国内外のがんワクチン臨床開発の現状を総括した後，がんワクチン実用化のために今後克服すべき問題点について考察する。

はじめに

がん免疫療法は外科療法，化学療法，放射線療法に次ぐ，新世代がん治療法として注目を浴びてきた。特に1991年にベルギーのBoon博士らが細胞傷害性T細胞（cytotoxic T lymphocyte; CTL）の認識するがん関連抗原を報告して以来，各種がん関連抗原が相次いで分子レベルで同定され，それらを標的とした"がんワクチン"の臨床応用が可能となった。1990年代後半からの初期（第Ⅰ相）・中期（第Ⅱ相）臨床試験では従来では認められなかった優れた臨床効果が確認され，2000年代に入り多くの後期ランダム化比較試験が実施されている。基礎

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研究や初期・中期臨床試験の多くはアカデミア（大学・研究所）やベンチャー企業により開始されたが，製薬企業によるランダム化後期臨床試験へと展開され，現在承認をめざした開発競争も激化している。2010年4月には，自己樹状細胞を用いた“がんワクチン”が初めて米国のFDAにより承認されたが，その他の後期臨床試験のほとんどでワクチン治療の臨床効果を立証できていない。今後“がんワクチン”が第四のがん治療法として公認されるには，原点に立ち戻っての科学的・医学的検証が求められている。この総説では，国内外でのがんワクチン臨床開発の現状を総括した後，がんワクチン実用化のために今後克服すべき問題点について考察する。

Ⅰ．本邦におけるがんワクチン開発の現状

1．アカデミアにおけるがんワクチン開発

本邦のがんワクチン開発では，一貫してアカデミア（大学や研究所）が基礎研究・臨床研究（トランスレーショナルリサーチ）の両面において牽引役を果たしてきた。1980年代より腫瘍免疫に関する基礎研究が盛んとなり多くの優れた研究論文が発表されるとともに，研究会，学会にて活発な発表・討論がなされてきた。その結果，本邦のがんワクチン開発は基礎・臨床研究両面において世界のトップレベルにあるといえる。

これまでの研究開発の成果として，平成20年度から新設された先端医療開発特区（スーパー特区）を活用した研究事業に，以下の三つのがんワクチン関連の研究課題が採択された。

①「迅速な創薬化を目指したがんペプチドワクチン療法の開発」（研究代表者：東京大学 中村祐輔）は，80以上の大学や研究機関・国立がんセンターの関連部局が結集したプロジェクトである。具体的には，東京大学医科学研究所，久留米大学，札幌医科大学，国立がんセンター東病院が，ゲノム・遺伝子研究を介した扁平上皮がんへのペプチドワクチン，免疫学的研究を基盤としたテーラーメイドペプチドワクチン，熱ショック蛋白ワクチン，肝臓がんへのグリピカン3ペプチドワクチン，などの開発を実施している。

②「複合がんワクチンの戦略的開発研究」（研究代表者：三重大学 珠玖洋）は，三重大学，札幌医大，岡山大学，産業医大，東京大学，慶應義塾大学，北海道大学，金沢大学，理化学研究所を中心とした，免疫効果増強技術を加味したがんワクチン開発プロジェクトである。具体的には，ナノパーティクル包埋MAGE-A4蛋白ワクチン，サーバイビンペプチドワクチン，Th1細胞療法，などの実用化研究を実施している。

③「免疫先端医薬品開発プロジェクト」（研究代表者：大阪大学 岸本忠三）では，大阪大学が中心になり，新規アジュバントの開発，制御性T細胞除去療法の開発や，WT-1ペプチドワクチン開発を実施している。

本邦ではこれら先端医療開発特区（スーパー特区）に採択された研究チームが中心となり，ワクチン開発における様々な問題点の克服をめざして高水準の基礎・臨床研究を展開している。

2．製薬企業におけるがんワクチン開発

前述のごとく，本邦でのがんワクチン開発はアカデミアが主体となって推進されてきたが，製薬企業も国内外のバイオベンチャーとアライアンスを締結し実用化に向けて動きだしている。たとえば，キリンビールと米国Dendreon社とのアライアンスにより，国立がんセンターで再燃前立腺がん症例に対して前立腺関連抗原（PAP）とサイトカイン（GM-CSF）との融合蛋白をパルスした自己樹状細胞による免疫療法Provenge（Sipuleucel-T）の第Ⅰ相臨床試験が実施された。その後，日本での開発は中止になったが，米国での治験は継続され，2010年4月に米国FDAにより“がん治療薬”として初めて承認されるに至った。最近では，武田薬品工業と米国Cell Genesis社とのアライアンスによる，GM-CSF遺伝子を導入したがん細胞株ワクチン“GVAX”が話題になった。Cell Genesis社が実施した第Ⅱ相臨床試験までは良好な成績が報告されていたが，その後の二つのランダム化第Ⅲ相比較試験はいずれも成績不良で開発中止となった。

本邦の製薬企業によるペプチドワクチンの臨床開発は最近加速している。たとえば，大日本住友製薬は大阪大学で開発されたWT-1ペプチドワクチンの実用化を開始している。また，扶桑薬品，大塚製薬，塩野義製薬はオンコセラピー社とアライアンスを締結して，東京大学医科学研究所で開発されたペプチドワクチンの第Ⅱ相・第Ⅲ相臨床試験を膵臓がんなどに対して実施中である。臨床試験への患者登録も順調に進んでいるとのことで，ランダム化比較試験による生命予後延長が確認されれば，今後数年のうちに“がん治療薬”として実用化されるものと期待される。抗体医薬品開発では欧米に先んじられ，そのピークを過ぎた感もあることから，製薬企業によるがんワクチン開発が今後さらに進むものと推測される。

3．久留米大学における“テーラーメイド型”ペプチドワクチン

筆者の所属する久留米大学は，1999年よりペプチドワクチンの臨床試験を開始し，すでに1,000例を超える様々ながん種の患者を治療してきた。初期には，同一のワクチンを患者の免疫能に関係なく投与する“共通型”

れた（表1）。ワクチン投与（3回）の治療費が93,000米ドルと極めて高価な上に，製造施設が未整備なため年間にわずか約2,000名しか治療できない状態にあるが，この治療を求めて患者が殺到しているとのことである。ただし，標準治療法との比較試験はいまだ実施されておらず，Provengeの臨床上のインパクトが実際いかほどであるかについては評価が分かれると推定される。また，"がんワクチン"ではないが，T細胞表面に発現する抑制性分子CTLA-4を阻害する抗体（ipilimumab; Bristol-Myers Squibb社）が悪性黒色腫を対象とした第Ⅲ相ランダム化比較試験において，コントロール群と比較し有意に生存期間を延長することが示され，現在米国および欧州において医薬品承認を申請中とのことである（表1）。ただし，治療を受けた患者のうち臨床効果を示す者が20～25%と限定されているために，治療効果を予測するバイオマーカーの同定が急がれている。さらに，前立腺特異抗原（PSA）と三つの共刺激分子を搭載したウイルスによるワクチンProstvac（Therion Biologics社）が転移性ホルモン不応性前立腺がん症例での第Ⅱ相ランダム化比較試験で良好な成績を示し，国際共同第Ⅲ相試験が計画されている（表1）。

その他のがん治療ワクチンとしては，Oncophage（熱ショック蛋白—ペプチド複合体）がロシアで一部の腎臓がんに対して承認されたことも話題を集めたが，最近のランダム化比較試験では相反する結果も報告されている（表2）。また，大企業GlaxoSmithKline社により肺がんの再発予防ワクチンとしての承認をめざして，がん抗原MAGE-A3を用いた蛋白ワクチンの大規模ランダム化比較臨床試験が実施され，世界中から注目を集めている。さらには，ペプチドワクチン領域においても最近進展がみられている。たとえば，2009年にはヒトパピローマウイルス（HPV）陽性の外陰上皮内腫瘍に対して，HPV-16ウイルス腫瘍性蛋白E6・E7由来の合成長鎖ペプチドワクチンの接種により良好な臨床効果が報告された。この他にも，ペプチドワクチン療法では多数のペプチド投与，抗がん剤との併用など新しいアプローチにより生命予後が改善する可能性も示唆されているが，いまだ企業レベルでのランダム化比較試験の結果は報告されていない。

Ⅲ．がんワクチン開発における問題点

がんワクチンの実用化にはランダム化比較試験において優れた臨床効果を立証することが必要である。これまでに失敗に終わった臨床試験の結果からいくつかの解決すべき問題点が明らかとなっている。以下，その問題点と解決策について私見を踏まえて述べる。

1．新規ワクチン抗原の同定

がん抗原や免疫反応の特異性・多様性・多重性など，腫瘍免疫の特性を考慮すれば，同一のワクチンを患者の免疫能に関係なく投与する"共通型"ワクチンによって多数の患者に効果を期待するのは難しい。これまでに多くのがん関連抗原がcDNA発現クローニング法，SEREX法，リバースイムノロジー法，ゲノムレベルでの包括的遺伝子解析手法など，様々な手法で同定されてきたが，すでに十分なワクチン分子が同定されたとはいい難く，いっそうの研究が必要と思われる。また，既知のがん関連抗原に関しても，実際にがん細胞表面でHLA分子と結合して抗原提示され，免疫細胞の標的となり得るか否か，"ペプチドーム"レベルでの検証が必要であろう。

2．新規アジュバントの開発

これまでは，モンタナイドISA51などのFreund's incomplete adjuvantがペプチドワクチンと併用されることが多かったが，免疫賦活効果が弱いため，熱ショック蛋白（HSP）やtoll-like receptor（TLR）agonistなど，新しいアジュバントの開発が望まれる。また，すでに医薬品として承認されているという利便性からGM-CSFがワクチンと併用されることがこれまでには多かったが，その効果は"double-edged sword"ともいわれ，新規の免疫賦活，調節薬の開発が望まれる。

3．免疫モニタリング法の確立

がんワクチンの目的はがん細胞に発現する抗原に対する特異的な免疫反応を誘導することにある。したがって，ワクチン投与後にワクチン抗原に対する特異的な免疫反応が誘導されたか否かを検証することが重要である。特にワクチンによって誘導された免疫反応と臨床効果との相関を証明するためには，精確な免疫モニタリング法の確立が必須といえる。しかしながら，投与抗原に対する細胞性免疫反応を測定するために現在頻用されているサイトカイン測定法（ELISA，ELISPOT，細胞内サイトカイン染色法），HLA-Multimer法などの免疫モニタリング法は，いずれも再現性や感度上の課題を抱えている。血液中に抗原特異的CTLが存在するとしても，その頻度が低い（一般的には1万個～10万個に1個以下と推定）ために，患者から採取した血液細胞を直接に解析に用いるのは難しく，体外で一定期間抗原と培養する必要がある。研究者により血液細胞の培養条件や測定方法が異なるために再現性や感度上の問題が生じることがわかり，それを克服すべく検査法の標準化に向けて世界中で取り組みが開始されている。

一方，久留米大学では多数の検体をhigh throughputに解析できるLUMINEX法を用いて，血漿中の抗原特

異的な液性免疫反応，すなわち抗原特異的IgG抗体を測定し免疫反応の指標としている。T細胞から指令を受けた抗原特異的B細胞が形質細胞に分化して血液中に大量の抗体を産生するが，抗体測定技術としてすでに確立されているELISA法，LUMINEX法を，免疫モニタリングの一手法として活用すれば再現性や感度上の課題は解決されるものと推定される。

4．新規バイオマーカーの開発

ある特定のがんワクチンの治療効果は患者のがん種，病期や免疫状態によって異なり，すべての症例に治療効果を期待できない。たとえば，リンパ球増殖に関連する遺伝子群・抗原提示に関連する遺伝子群に突然変異や機能阻害がある症例や，免疫系からの逃避に関連する遺伝子群が作動している症例などにはワクチンの治療効果はあまり期待できない。また，がん細胞による宿主免疫系の抑制が強い場合には，免疫抑制の解除が必要になる。したがって，がんワクチンが医薬品として一般化するためには臨床効果と相関するバイオマーカーの開発が必須である。現在，臨床的有用性の確立したバイオマーカーは存在しないが，世界中でがんワクチンの効果予測に有用なバイオマーカーの同定が盛んに試みられている。たとえば，抗がん剤の分野で最近臨床応用されている遺伝子解析によるpharmacogenomicsのように，がんワクチン分野においても患者の治療適格性をゲノム・エピゲノムレベルで事前に診断する“vaccine genomics”の開発が望まれる。

5．患者の選択・除外基準の再検討

がんワクチン療法ではワクチン投与前の免疫状態，特にワクチン抗原に対する既存免疫の有無が重要であるが，一般的には適格・除外基準として評価されていない。たとえば，抗がん剤治療の分野では，患者個々に最適な薬剤の開発をめざしてpharmacogenomicsなど比較的新しい概念も採用され，抗がん剤感受性，過敏反応性などが選択・除外基準として考慮されつつある。一方，がんワクチン開発では従来からの薬剤開発に準じた基準が採用されることがほとんどである。臨床試験における症例選択・除外基準として，がん細胞の抗原発現量を解析することはあっても，宿主の免疫系については配慮されていない。投与予定のワクチンに対して免疫記憶がまったく存在しない場合には一次免疫反応から免疫誘導が開始されるため，特異免疫を付与するまでの期間は長期に及び，ワクチン開始初期においてがんが進行する可能性がある。また，投与するワクチン抗原に対する免疫寛容の有無などもワクチンの臨床効果に直接反映する。以上のような観点から，がんワクチン臨床試験にはがん特異免疫能へ配慮した適格・除外基準の設定が望まれる。

6．ワクチンに対する臨床効果の評価，判定基準の再検討

ワクチンに対する臨床効果の評価基準として，一般的にはRECIST（Response Evaluation Criteria in Solid Tumors）やWHO基準など従来の抗がん剤開発に準じた基準が採用され，既存の腫瘍サイズの変化，新病変出現の有無，などによって臨床効果が判定されることが多い。しかし，腫瘍サイズの変化と生命予後とが必ずしも相関しない，特に腫瘍サイズは変化しないで長期生存する患者（いわゆる“long SD”）が多く存在することや，生命予後にまったく関与しない新病変の出現を認めることが多い，など現基準の問題点も明らかとなり，がんワクチンの臨床的評価に特化した新しい基準の確立が望まれている。最近では，免疫療法患者の実際の臨床経過をもとにして考案された新しい基準“immune-related Response Criteria（irRC）”なども提唱されている。

Ⅳ．ワクチン特有の有害事象“不都合な免疫誘導”

がん患者個々の免疫状態は遺伝的に異なり，また病状とともに変化する。したがって，症例毎に異なるがん免疫の多様性，多重性を無視して“共通ワクチン”を開始すると一部の症例において予期せぬ結果も想定される。ここでは，“ワクチン投与により，患者に現存するがん特異免疫機能とまったく異なる特異免疫機能を生体内に誘導し，結果としてがん増殖を促進させる免疫反応”を“不都合な免疫誘導”と定義する。たとえば，がんワクチンにより強力な免疫賦活指令がだされると，既存の抗腫瘍免疫細胞との間でいわゆる“パイ”の奪い合いが起こり，既存の抗腫瘍免疫の抑制によりがん細胞の増殖が加速される可能性がある。すなわち，“不都合な免疫誘導”による既存の抗腫瘍免疫や感染防御免疫の抑制の結果として，ワクチン投与患者の早期死亡や早期のがん増悪が想定される。久留米大学においてもすべての患者に同じタイプのワクチンを投与する“共通ワクチン”の臨床試験において類似の経験があった。その結果，“共通ワクチン”の臨床試験を中断して，個々の患者に適したワクチン抗原を投与する“テーラーメイド型”のペプチドワクチンを開発し，実施している。

たとえば，Cell Genesis社，武田薬品工業の実施したGVAX前立腺がんワクチンの第Ⅲ相臨床試験の結果も“不都合な免疫誘導”が関与しているかもしれない。ホームページ上の情報だけではどのような原因，有害事象が関与したのか判断できないが，ワクチン投与群で対照群に比して多くの死亡者がでた。このワクチンで使用されたGM-CSFは，他の臨床試験においてもアジュバントとしてしばしば使用されてきたが，この試験と同様に患

者の予後が悪化したとの報告も散見され，その効果は"double-edged sword"ともいわれる。その原因は，現在汎用されている有害作用チェックリスト（CTCAE ver4.0）掲載の診断技術では特定できない"不都合な免疫誘導"による可能性も否定できない。

がんワクチン療法は一般的には比較的有害事象の少ない治療法といわれ，ワクチン特有の有害事象といえる"不都合な免疫誘導"は，その検出手段がないことから無視されてきた。しかし，がんワクチン療法の実用化・普及のためには，がんワクチンによりがん細胞に対する特異免疫が誘導されたか否かを検証すると同時に，"不都合な免疫誘導"により既存の抗腫瘍免疫や感染特異免疫が抑制されているか否かを検証する新しい診断技術の開発が不可欠であろう。

V．がんワクチン療法の限界と他治療との併用

がん細胞は様々な方法で免疫監視機構から逃れるため，がん免疫系が正常にもかかわらず，がんを完全に排除できず病態が進行することも多い。免疫系からの逃避のメカニズムとして，がん抗原やHLAの低下・消失，接着分子の脱落，免疫抑制因子の産生などが治療によりしばしば誘導される。したがって，がんワクチン療法単独では治療開始当初は有効な場合でも，逃避するがん細胞の増加とともに無効となることも多い。また，がん細胞は免疫系からの逃避に限らず，薬剤耐性遺伝子発現などによる抗がん剤治療からの逃避やホルモン受容体脱落によるホルモン療法からの逃避など，他の治療法に対しても抵抗を示すことがある。たとえば，前立腺がんでは免疫抵抗性（HLAクラスⅠ抗原消失もしくは発現低下）を示すがん細胞が根治手術時に，すでに腫瘍組織内の50%以上を占める。また，同じ腫瘍組織のなかには，ホルモンレセプターを消失したがん細胞，抗がん剤抵抗性遺伝子を獲得したがん細胞なども同時に存在するが，個々の抵抗性は重複しない場合も多い。したがって，いずれかの治療法に抵抗を示すがん細胞に対しても，ワクチン療法，ホルモン治療，抗がん剤治療を併用すれば治療効果を期待できる。特に最近では抗がん剤が，①"immunogenic cell death"の誘導，②抗原提示細胞の活性化，③免疫抑制性細胞群の阻害，などを介して，免疫療法の治療効果を相乗的に高める可能性が示唆されており，がんワクチンと抗がん剤との併用が盛んに臨床的に試みられている。

おわりに

1954年に長野泰一博士らによりインターフェロンがサイトカインとして初めて報告されたが，その後四半世紀を経て1980年代に各種サイトカインが日常臨床で使用できるようになった。また，1975年にはモノクローナル抗体の作製法が論文発表され，その四半世紀後（2000年）に各種がんに対するモノクローナル抗体薬が日常臨床で一般的に使用されるようになった。一方，"がん抗原ペプチド"の発見は1991年であるが，同様に推察すると，がんワクチンが日常臨床で活用できるのは四半世紀後（2016年）と予想される。したがって，期待と失望を繰り返してきたがんワクチン開発においても，今後5年間に飛躍的な進展を期待したい。そのためには，がん細胞・免疫系の多様性・多重性やがん細胞側の逃避現象など，腫瘍免疫の特性に関する理解を深めて，その知識を患者やワクチン抗原選択のための新しい基準の確立やバイオマーカー開発に反映させることが肝要であろう。また，各種ワクチン療法の特徴を理解してがん種や病期に適合したワクチン治療法を選択するとともに，がんワクチン特有の有害事象や限界を正しく理解し，各種標準治療と組み合わせた集学的治療法の開発が望まれる。

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Variation of tumor-infiltrating lymphocytes in human cancers: controversy on clinical significance

Tumors develop and progress under the influence of a microenvironment comprising a variety of immune cell subsets and their products. Recent studies have shown that tumor-infiltrating lymphocytes (TILs) are not randomly distributed, but organized to accumulate more or less densely in different regions within tumors, and interact with each other. Substantial evidence has suggested that not only $CD8^+$ and/or $CD4^+$ $\alpha\beta$ T cells but also other lymphocyte subsets, including $\gamma\delta$ T cells, B cells, NK cells, and NKT cells, infiltrate tumor tissues in variable quantities and play a key role in the regulation of antitumor immunity. In this article, we summarize available information regarding the diversity and composition of TILs, which may positively or negatively affect tumor growth and patient clinical outcomes. The clinical significance of TILs in human cancers remains unclear and is a subject of considerable controversy; largely due to the lack of functional data for TILs, as well as due to enormous variability of TILs in different tumors. A great deal more functional data about TILs needs to be obtained for individual tumors before TILs can be considered as a prognostic parameter in human cancers.

KEYWORDS: cytotoxic T lymphocytes prognosis regulatory T cells tumor microenvironment tumor-infiltrating lymphocytes

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After more than a century of debate, accumulating evidence now indicates that immune cells affect cancer development and/or progression [1]. For example, mice deficient in adaptive immunity or IFN-γ responsiveness have been reported to develop tumors with high frequency [1]. In addition, congenital or acquired immunodeficiencies have been known to be associated with increased prevalence of malignant diseases in humans [1]. These findings suggest that the immune system naturally possesses the ability to recognize tumor cells and has an active role in the surveillance against tumors. Nevertheless, in general, the immune system cannot inhibit malignant growth completely, since tumor cells do not elicit strong immune reactions owing to the lack of danger signals. In addition, although much has been learned about the potential of the immune system to control tumors in proof-of-principle animal model experiments, the promises that immunotherapies can boost the potential of the immune system for the benefit of cancer patients have not fully translated into clinical successes [2]. Therefore, more knowledge regarding cellular and molecular interplay between the immune system and tumors in humans remains to be discovered.

Tumors develop and progress under the influence of a microenvironment comprising various types of cells, including immune cells, fibroblasts, endothelial cells, blood vessels and their products, including cytokines, chemokines and metabolites. In particular, the biological and phenotypical characteristics of tumor cells have been reported to at least partly reflect the complex cellular and molecular interactions between the tumors and host immune systems. Tumor cells evoke recognition by both the innate and adaptive immune systems. The innate immune system, which is triggered and mediated by various types of cells, including granulocytes, macrophages, dendritic cells (DCs), NK cells, NKT cells, and $\gamma\delta$ T cells, quickly recognizes stress-induced molecules expressed on tumor cells via germline-encoded receptors. Following these initial innate immune responses, tumor cells stimulate the adaptive immune system by activating and expanding rare antigen-specific cells, which are mainly composed of T and B lymphocytes.

Various types of immune cell subsets, belonging to both the innate and adaptive immune systems, accumulate at tumor sites and play critical roles in promoting or inhibiting tumor development and/or progression [3–5]. The relationships between the immune system and tumors are diverse and dynamic. Immune surveillance can control or eliminate premalignant lesions and early cancers [1]. However, with time, tumor cells can undergo a process known as 'immunoediting' under selective pressure from the immune surveillance, and develop a resistant phenotype that is capable of manipulating immune cells,

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through secretion of various types of cytokines and chemokines, as a result of a failure in effective immune surveillance [1]. At this moment, tumor-promoting immune cells, such as tumor-associated macrophages and granulocytes, can accelerate tumor growth by generating inflammatory tumor microenvironments and producing proinflammatory factors that enhance tumor growth and angiogenesis [3–5]. In addition, immunoregulatory cells, such as regulatory T (Treg) cells and myeloid-derived suppressor cells, can also promote tumor growth through inhibition of other immune cells that are critical for effective antitumor immunity [3–5].

The diversity and composition of tumor-infiltrating immune cells have been reported to significantly affect clinical outcomes in cancer patients. Tumor-infiltrating lymphocytes (TILs) are composed of several different lymphocyte subsets, including $CD4^+$ and $CD8^+$ $\alpha\beta$ T cells, $\gamma\delta$ T cells, B cells, NK cells and NKT cells. Recent studies have indicated that not only $CD8^+$ and $CD4^+$ $\alpha\beta$ T cells, but also other lymphocyte subsets play a key role in the negative or positive regulation of antitumor immunity. In this article, we summarize and discuss current knowledge regarding the ways in which different subsets of TILs regulate tumor growth and affect patient clinical outcomes. Although other immune cells, such as macrophages, granulocytes, DCs and myeloid-derive suppressor cells, have also been demonstrated to infiltrate tumors and positively or negatively affect tumor growth via interaction with other immune cell subsets, this article focuses on the variation of TILs and controversy regarding their clinical significance in human cancers.

T cells

Among the various types of tumor-infiltrating immune cells, T lymphocytes have been most extensively studied. There has been considerable evidence that tumor-infiltrating T lymphocytes are clearly associated with antitumor immunity in cancer patients [6]. For example, Mihm and colleagues demonstrated a prognostic significance regarding levels of T-cell infiltrates in melanoma tissues in their pioneering and innovative studies. They showed that dense intratumoral, but not peritumoral, T-cell infiltrates in the vertical growth phase of primary melanoma were tightly correlated with prolonged survival and reduced incidence of metastatic diseases [7]. In ovarian cancers, the presence of $CD3^+$ T cells detected within tumor-cell islets, which was associated with increased expression of IFN-γ, IL-2 and lymphocyte-attracting chemokines within tumors, was reported to be independently correlated with delayed recurrence or delayed death in multivariate analyses [8]. Antitumor effects of tumor-infiltrating T cells have also been demonstrated directly by many clinical trials, in which infusion of TILs into cancer patients effectively controlled tumor growth [9]. For example, the transfer of *in vitro* expanded autologous T cells derived from TILs resulted in objective response rates as high as 72% in refractory metastatic melanoma [10].

$CD3^+$ T cells expressing T cell receptor (TCR) α and β chains ($\alpha\beta$ T cells) can be roughly classified as $CD8^+$ cytotoxic T lymphocytes (CTLs), which directly kill antigen-expressing target cells, and $CD4^+$ T cells, which secrete various types of cytokines. $CD4^+$ T cells are composed of different subsets, which elicit stimulatory or inhibitory/regulatory effects on antitumor immunity. $CD4^+$ helper T (Th) cells are crucial for efficient expansion and persistent accumulation of other antigen-specific immune cells. $CD4^+$ Th1 cells secrete type I cytokines, such as IFN-γ and TNF-α, which stimulate $CD8^+$ T cell responses via activation of antigen-presenting cells. $CD4^+$ Th2 cells secrete type II cytokines, such as IL-4, IL-5 and IL-13, which limit the activation of antigen-presenting cells and enhance humoral immunity. A newly identified $CD4^+$ T cell subset, Th17, elicits tissue inflammation related to autoimmune responses. $CD4^+CD25^+$ Treg cells inhibit T cell responses against self antigens via secretion of immunosuppressive cytokines, such as IL-10 and TGF-β, or direct interaction with other immune cells. Finally, T cells expressing TCR γ and δ chains ($\gamma\delta$ T cells) have been reported to play a unique and important role in stress-surveillance responses in numerous aspects of inflammation. Recent studies have shown the importance of the balance and interplay between these stimulatory and inhibitory/regulatory pathways in the tumor microenvironment, as a key determinant of biological behavior of tumors, as well as patient clinical outcomes [2–4].

Cytotoxic T lymphocytes

The existence of CTLs specific to tumors has been demonstrated by many previous studies, in which tumor-infiltrating T-cell lines, isolated from tumor tissues, were used to identify CTL epitopes from tumor-associated antigens in various types of cancers. Since the pioneering discovery of a CTL epitope from a melanoma-associated antigen by Boon and colleagues [11], numerous tumor-associated antigens have been identified. Kawakami and Rosenberg reported

several CTL epitopes from melanoma-associated antigens by using CTL lines infiltrating melanoma tissues [12]. Itoh and colleagues identified a number of CTL epitopes that were recognized by TIL clones established from resected tumor tissues in various kinds of cancers; including esophageal, colon, lung and gastric cancers [13,14]. Accumulating evidence has suggested that CTLs specific to tumor cells preferentially accumulate and/or expand within tumors. For example, in subcutaneous melanoma, the frequency of antitumor T cells within tumors was shown to be approximately tenfold higher than in the peripheral blood [15], and a significant difference in the frequencies of some *TCR-V* genes was observed in the metastatic tissues, compared with the blood [16], indicating that some specific T cells were enriched in the tumors. Similarly, in prostate cancers, $CD8^+$ TILs were found to exhibit a restricted *TCR Vβ* usage, and identical clones were identified in multiple sites within tumors [17]. These findings suggest that tumor-infiltrating T cells undergo clonal expansion and accumulation in response to specific tumor-associated antigens, although more direct evidence will be required through *in situ* analysis of antigen specificity.

There is limited information on detailed phenotypes of tumor-infiltrating CTLs. In murine tumor models and human clinical trials, TIL-derived $CD8^+$ T cells with the central memory phenotype have been reported to confer superior antitumor immunity, compared with those with the effector memory phenotype [9]. Although the naive/memory phenotypes of T cells in the peripheral blood have been well studied, those in TILs have not been precisely characterized. Li *et al.* showed that $CD8^+$ TILs freshly isolated from melanoma tissues have mostly a $CD27^+$ $CD28^+$ $CD57^-$ granzyme B^+ $perforin^{-/low}$ phenotype, indicating an early effector-memory stage of differentiation [18]. Anichini *et al.* also demonstrated that melanoma tissues contain an 'early-effector' phenotype of $CD8^+$ T cells expressing FoxP3, CD27, and CD28, but not CD45RA, CCR7, or CD57 [19]. These results suggest that some melanoma tissues contain TILs at earlier stages of activation/differentiation, which may possess more potential for clonal expansion. By contrast, Ahmadzadeh *et al.* recently reported that in melanoma tissues, the majority of TILs, including MART-1/Melan-A melanoma antigen-specific $CD8^+$ T cells, revealed a significantly higher frequency and level of programmed death (PD)-1, which correlated with an exhausted phenotype and impaired effector function, in contrast to T cells in normal tissues or in the peripheral blood [20].

Although tumor cells often express antigens that can be recognized by infiltrating T cells, immune-mediated complete rejection of tumors is rarely observed in humans. In fact, tumors have been reported to possess many different mechanisms to escape immune recognition [1,21]. For example, tumor cells can downregulate various target molecules, such as MHC molecules and tumor-associated antigens themselves, or generate a local suppressive milieu that affects the activity of infiltrating immune cells. They may produce inhibitory cytokines, such as IL-10 and TGF-β, and inhibitory metabolites, such as extracellular adenosine and prostaglandin E2, directly or indirectly via stimulation of other immune cells. They may also express ligands that directly interact with receptors on infiltrating immune cells, to provide negative signals associated with inhibition of their effector functions. For example, PD ligand-1 (PD-L1), which is abundantly expressed or induced in various types of tumor cells, has been shown to negatively regulate the TCR-mediated signaling after interaction with PD-1 expressed on antigen-specific T cells [17,20,22–24]. In addition, persistent expression of NKG2D ligands on tumors, or tumor-mediated production of soluble NKG2D ligands may cause downregulation of a key cytotoxic molecule, NKG2D, expressed in $CD8^+$ CTLs and NK cells, leading to tumor escape [25,26].

T-cell dysfunction, characterized by a decreased T-cell proliferation and diminished cytokine production, is frequently observed in tumor microenvironments [27,28]. Despite the recruitment of large numbers of tumor-specific $CD8^+$ CTLs into the tumors, T-cell hyporesponsiveness and/or other negative regulatory mechanisms may limit the effector phase of antitumor immune responses [29]. There are several different mechanisms that render T cells anergic or tolerant directly by tumors or indirectly via mediation of other immunosuppressive cells, such as myeloid-derived suppressor cells and macrophages. These include depletion of the amino acids arginine and tryptophan through overexpression of arginase I and indoleamine 2,3 dioxygenase (IDO), which may lead to downregulation or loss of the CD3ζ chain in T cells [30]. Production of reactive oxygen species and nitric oxide via nitric oxide synthase may also impair T-cell functions [30,31]. Recently, the binding of TCRs to galectin was reported to be linked to a dissociation between CD8 and TCRs, which leads to anergy of TILs [32]. In

addition, several different inhibitory receptors, such as PD-1, CTLA-4, B and T lymphocyte attenuator (BTLA), and LAG-3, have been shown to induce hyporesponsiveness of T-cells specific to tumor-associated antigens [17,20,22,24,33–35].

Dysregulation of lymphocyte trafficking may also be one of the mechanisms limiting immune responses against tumors. For example, significant defects in the expression of the vascular adhesion receptors, such as E-selectin, P-selectin, and ICAM-1, on the vessels within tumor boundaries, which may lead to a block in trafficking of effector T cells to tumors, have been observed in metastatic melanoma [36]. Similar findings were also described in other types of cancers, such as squamous cell carcinomas of the skin and lung carcinomas [37,38]. By contrast, highly migratory phenotypes of effector T cells were suggested to facilitate their ability to control tumors. In melanoma patients with stage III diseases, expression of CXCR3, a chemokine receptor related to lymphocyte migration, in $CD8^+CD45RO^+$ T cells, was significantly associated with enhanced survival [39]. Similarly, in metastatic melanomas, several lymphotactic chemokines, including CCL2, CCL3, CCL4, CCL5, CXCL9 and CXCL10, were reported in the tumor microenvironment to be related to the migration of activated $CD8^+$ effector T cells into tumor sites [40]. In colorectal cancers, expression of the adhesion molecule CD103 was reported to control the retention of memory $CD8^+$ cells within tumors [41].

Considerable evidence has accumulated, suggesting that the abundance of tumor-infiltrating $CD8^+$ CTLs is associated with improved clinical outcomes in various types of cancers [42–51]. In particular, recent studies have clearly demonstrated a clinical significance of $CD8^+$ CTLs in colorectal cancers [52–60]. A landmark study by Naito *et al.* demonstrated that the presence and location of one particular type of TILs, $CD8^+$ T cells, had a significant influence on clinical outcome in colorectal cancer [52]. Subsequently, Galon and colleagues disclosed that higher levels of tumor-infiltrating memory $CD45RO^+CD8^+$ cells were associated with the absence of early metastasis, a less advanced pathological stage and prolonged survival in colorectal cancers [53–55]. In addition, they reported that high expression of genes related to Th1 immune responses (*IRF-1*, *T-bet*, *IFN-γ*), cytotoxicity (*granulysin*, *granzyme B*), chemokines (*CX3CL1*, *CXCL10*, *CXCL9*) and adhesion molecules (*ICAM1*, *VCAM1*, *MADCAM1*) were associated with better clinical outcomes [53,54,57]. Following these observations, they have developed a novel immune scoring system, which reflects the densities of tumor-infiltrating $CD8^+$ and $CD45RO^+$ T cells, by quantifying them in the center and the invasive margin of tumor tissues in patients with the early stage [56] or any stages of colorectal cancers [58]. They reported two important findings: patients with higher immune scores (greater $CD8^+$ and $CD45RO^+$ T cell densities) showed increased disease-free and overall survival as compared with those with lower immune scores; and the immune score was superior to the standard prognostic clinical parameters, including the TNM staging system [58].

Recently, Nosho *et al.* also reported that tumor-infiltrating $CD45RO^+$ cell density was significantly associated with longer survival of colorectal cancer patients, independent of other clinical, pathological and molecular features, such as tumor microsatellite instability and LINE1 methylation [59]. Since accumulating evidence suggests that the density of infiltrating $CD45RO^+$ memory CTLs is an independent predictor of prognosis, the evaluation of these tumor-infiltrating cells could be part of the standard clinical practice for evaluating colorectal cancers at the time of diagnosis, although further studies will be required to validate its clinical utility. In addition, it would be of interest to examine whether a similar evaluation would also be useful for predicting the prognosis in other types of cancers. In fact, expression of CD45RO in TILs was reported to be also associated with improved survival in other cancers, including gastric [45] and esophageal cancers [61].

Since the quantities of TILs have been the only variables typically considered in most of the previous studies, there has been only limited information available regarding localization of $CD8^+$ T cells within tumors. Several studies characterized the relationship between localization of tumor-infiltrating T cells and patient clinical outcome. For example, Naito *et al.* clearly demonstrated that invasion of $CD8^+$ T cells inside cancer nests was significantly correlated with a favorable prognosis, whereas infiltrates at the tumor margins or in the stroma were not [52]. In stage IV non-small-cell lung cancer (NSCLC), patients with more tumor-infiltrating $CD8^+$ T cells in cancer nests than in cancer stroma (nests > stroma) showed significantly better survival, compared with those with more $CD8^+$ T cells in the stroma (nests < stroma) [44]. In esophageal squamous cell carcinoma, patient prognosis was correlated with the number of $CD4^+$ and $CD8^+$ cells in the stroma, and that of $CD8^+$ cells, but not of

$CD4^+$ cell, within the cancer nest [62]. In early-stage tongue cancers, the tumor nest-infiltrating $CD8^+$ T cells were shown to express PD-1 at a higher rate and NKG2D at a lower rate, compared with the $CD8^+$ T cells in the stroma, suggesting that exhausted and inactivated phenotypes were induced in TILs accumulating in the tumor nests [63]. Many of the previous studies suggested that $CD8^+$ T cell infiltration to cancer nests was prognostically most important, but more data need to be accumulated before definitive conclusions can be drawn. Since the impact of TILs on tumor progression and patient clinical outcomes appears to be strongly influenced by their localization within tumors, it is critical to precisely analyze both their localization and phenotypes in future studies.

Regulatory T cells

Substantial evidence has suggested that immunosuppressive cells infiltrating tumors provide one of the major barriers to effective antitumor immunity [3–5]. In particular, Treg cells have been reported to suppress effective antitumor responses of T cells and other immune cells and promote tumor progression [64]. In 1995, Sakaguchi *et al.* for the first time reported a T cell subset expressing both CD4 and IL-2 receptor α chain (CD25) on the cell surface, which they designated $CD4^+CD25^+$ Treg cells, as a distinct cell population that inhibits autoimmune diseases in murine models [65]. In humans, several groups identified this unique cell subset in the peripheral blood [66]. In addition to CD4 and CD25, Treg cells are phenotypically distinguishable by high levels of CTLA-4, GITR, and forkhead transcription factor FoxP3. Treg cells have been reported to arise in response to persistent antigen stimulation in the presence of TGF-β, particularly in the absence of inflammatory signals [66]. Antigen-specificity of Treg cells infiltrating tumors has not been extensively studied. Wang *et al.* reported that LAGE1 protein was recognized by tumor-specific $CD4^+$ Treg cell clones generated from the TILs of cancer patients [67]. They also identified ARTC1 as a target molecule of tumor-infiltrating Treg cells [68]. Nevertheless, more studies need to be done to clarify the origin and antigen specificity of tumor-specific $CD4^+$ Treg cells at tumor sites.

June and colleagues first reported an increased proportion of $CD4^+CD25^+$ Treg cells, which secrete an immunosuppressive cytokine, TGF-β, in tumor specimens obtained from patients with early stage NSCLC and late-stage ovarian cancer [69]. Subsequent studies have shown increased frequencies of $CD4^+CD25^+$ Treg cells in the peripheral blood as well as within tumors in various types of cancers [70–72]. Curiel *et al.* first demonstrated a significant association between an increased density of $CD4^+CD25^+$ Treg cells within tumors and poor prognosis in patients with ovarian cancers [73]. They also found that Treg cells expressed a chemokine receptor, CCR4, on their cell surface, which recruited them to tumors via its ligand chemokine, CCL22, produced by tumor cells. Subsequently, considerable evidence has accumulated suggesting that an increased frequency of Treg cells within tumors or tumor-draining lymph nodes is associated with negative clinical outcomes in patients with various types of cancers [43,74–78]. Sato *et al.* demonstrated that an increase in the ratio of intraepithelial $CD8^+$ cells to Treg cells, which reflects the balance between effector and regulatory T cells, was associated with a favorable prognosis in ovarian cancer [79]. Similar findings were also reported in cervical and colorectal cancers [80,81]. In addition, Hodi *et al.* performed a detailed pathological analysis in tumor biopsy specimens from melanoma patients, treated by vaccination with irradiated, autologous tumor cells engineered to secrete GM-CSF, followed by antibody blockade of CTLA-4, and demonstrated that the extent of tumor necrosis was related to the ratio of $CD8^+$ T cells to $FoxP3^+$ Treg cells [82]. Considering the prognostic significance of tumor-infiltrating Treg cells, depleting this cell subset *in vivo* might enhance effective antitumor T cell responses. Although there are still no definitive data showing an improved clinical outcome after Treg cell depletion in human cancer patients, several studies showed a greater induction of antitumor effector T cells when Treg cell numbers were reduced by the administration of a CD25-targeted toxin [83–85].

Interestingly, an accumulation of $CD4^+FoxP3^+$ Treg cells within tumors has recently been reported to be associated with a better prognosis in some types of cancers. Loddenkemper *et al.* showed that intratumoral infiltration of $FoxP3^+$ cells was significantly higher in localized diseases than in metastatic diseases in 40 colorectal cancers [86]. Salama *et al.* demonstrated that a high density of intratumoral $FoxP3^+$ cells was a favorable factor for improved prognosis in multivariate analysis with 967 tumor samples from patients with stage II and III colorectal cancers [87]. Similarly, Correale also reported that the infiltration of $FoxP3^+$ Treg cells was a favorable factor for progression-free and overall survival in 57 relapsed colorectal cancer patients undergoing

chemotherapy or chemoimmunotherapy [88]. In addition, in patients with follicular lymphoma and Hodgkin's lymphoma, high numbers of intratumoral Treg cells were associated with longer disease free and overall survival [89,90]. Moreover, in patients with head and neck cancer, tumor infiltration by FOXP3$^+$ Treg cells was positively associated with better locoregional control of the tumors [91]. Although the exact biological mechanisms remained to be clarified, similar findings have been demonstrated in several different types of cancers, suggesting that such positive prognostic effects of intratumoral Treg cells might not be a disease-specific observation. The favorable prognosis in patients with a high density of tumor-infiltrating FOXP3$^+$ Treg cells may be explained by their capacity to downregulate proinflammatory, tumor-promoting immune responses that are generated by infectious stimuli from bacteria, translocated through the mucosal barrier and promoted by proinflammatory immune cell subsets, such as Th17 cells [92]. This may be a typical example that a unique tumor microenvironment can shape the composition and function of TILs in a unique way.

It should also be noted that activated human CD4$^+$CD25$^-$ effector T cells transiently express FoxP3 without acquisition of suppressive functions [93–95], although FoxP3 expression is often used to assess the frequency/density of intratumoral Treg cells. In addition, subsets of CD8$^+$ T cells [95–97], as well as tumor cells [98] may also express FoxP3. Therefore, although FoxP3 is currently the best marker available for immunohistochemistry (IHC) staining to identify Treg cells, the simple use of this marker may overestimate the frequency/density of intratumoral Treg cells. In the cases with high numbers of FoxP3$^+$ activated T cells in tissue specimens, the simple enumeration of FOXP3-positive cells without other markers [99] or functional analyses [100,101] may lead to the misinterpretation of data.

In addition to CD4$^+$CD25$^+$FoxP3$^+$ Treg cells, other immunosuppressive subsets, such as T regulatory cells type 1 (Tr1), Th3, and CD8$^+$FoxP3$^+$ T cells, have also been reported to infiltrate tumor tissues [96,97,102,103]. However, little information is available regarding these immunosuppressive cell types, and their biological and clinical significance within tumor microenvironments remains to be determined.

Th1 & Th2 cells

Most studies assessing tumor-infiltrating immune cells have focused on the analysis of CD8$^+$ T cells and/or CD4$^+$CD25$^+$ regulatory T cells, which are thought to be the main mediators/regulators of antitumor immunity. Therefore, helper CD4$^+$ cells within tumors have not been extensively studied, although the available evidence suggests that they play significant roles in the immune responses to cancers. In a melanoma patient with a dramatic clinical response to adoptive transfer of *ex vivo* expanded TILs, CD4$^+$ T cells that react with multiple tumor antigens in an HLA Class II-restricted manner were identified in TILs, suggesting that tumor-specific CD4$^+$ T cells might be associated with *in vivo* tumor regression [104]. In NSCLC, CD4$^+$ T cells in cancer stroma, but not CD8$^+$ T cells in cancer nests, were reported to be associated with favorable prognosis [105]. In head and neck squamous cell carcinoma, activated CD4$^+$CD69$^+$ T cells within tumors but not CD8$^+$ T cells, were positively correlated with a good prognosis [91]. Recently, a marked increase in IFN-γ-secreting CD4$^+$ T cells expressing ICOS (CD4$^+$ICOS^{hi}) was reported to be accompanied by an increase in the ratio of the effector to regulatory CD4$^+$ T cells (Teff/Treg) in the peripheral blood as well as within tumor tissues in bladder cancer patients receiving anti-CTLA-4 blockade therapy [106].

It should be noted that the roles of tumor-infiltrating CD4$^+$ cells are considered to be a double-edged sword, since immune responses to tumors are controlled by the balance of cytokines produced by two distinct helper T cell subsets, Th1 and Th2 cells [107]. Several studies evaluating gene and/or protein expression profiles in a variety of primary human tumors have demonstrated that a Th1-type signature is associated with improved clinical outcomes [108–111]. In renal cell carcinoma, the upregulation of the Th1-type immune response, particularly an increase in Th1-associated chemokines, was reported to be associated with a favorable prognosis [108]. In human papillomavirus (HPV)-18-positive high-grade cervical lesions, higher numbers of T cells positive for CD4 and T-bet, a transcription factor inducing Th1-type gene expression, were correlated with a favorable clinical outcome [109]. In colorectal cancers, patients with high expression of the Th1-related gene cluster (*T-bet*, *IRF-1*, *IL-12Rβ2*, *STAT4*), but not those of the Th2-related gene cluster (*IL-4*, *IL-5*, *IL-13*), showed improved prognosis [111]. Collectively, these findings suggest that Th1 cells play critical roles in antitumor immunity.

Conversely, De Monte *et al.* recently found that the ratio of Th2 (GATA-3$^+$)/Th1 (T-bet$^+$) tumor-infiltrating lymphocytes was a negative predictive marker of survival in pancreatic cancer

patients [112]. They demonstrated that Th2-type inflammation induced by thymic stromal lymphopoietin (TSLP), which was produced as a result of the cross-talk between tumor cells and cancer-associated fibroblasts, was associated with reduced patient survival. Aspord and colleagues demonstrated that a tumor-infiltrating $CD4^+$ T cell subset secreting type 1 (IFN-γ) as well as high levels of type 2 (IL-4 and IL-13) cytokines, facilitated tumor development in breast cancer [113]. They also recently reported that breast cancer cell-derived TSLP contributes to the inflammatory Th2 microenvironment, conducive to breast tumor development by inducing OX40L expression on DCs [114]. Since only limited information is currently available, more data need to be accumulated in order to clarify the precise roles of tumor-infiltrating $CD4^+$ cells within tumor microenvironments.

Th17 cells

A novel type of $CD4^+$ helper T cells, Th17, which secretes the inflammatory cytokine, IL-17, has recently been identified as a distinct T cell subset [115,116]. Some combinations of TGF-β, IL-6, IL-1β, IL-21 and/or IL-23, which are secreted by malignant cells and associated stromal cells, are reported to be essential for Th17 differentiation and expansion. However, the precise mechanisms for Th17 development are still not completely understood and remain controversial. Early differentiation of naive $CD4^+$ T cells to Treg and Th17 cells have both been reported to require the immunosuppressive cytokine, TGF-β, suggesting that there may be a substantial plasticity between Th17 and Treg cell development. Indeed, *in vitro* stimulation of naive $CD4^+$ T cells with TGF-β alone induces Treg cell differentiation, whereas TGF-β in combination with IL-6, IL-21 or IL-23 promotes Th17 cell differentiation.

Accumulating evidence suggests that Th17 cells play important roles in inflammation and autoimmune diseases [115,116], but their specific roles in the tumor microenvironment have not been clearly determined. Th17 cells have been reported to possess both protumorigenic and antitumorigenic effects [117,118]. The protumorigenic effects of Th17 cells rely on their capacity to enhance angiogenesis via induction of a wide range of angiogenic factors, such as VEGF, and to activate tumor-promoting transcription factors, such as Stat3. Th17 cells also can promote tumorigenesis by recruiting inflammatory cells, such as neutrophils, via the production and release of proinflammatory cytokines and chemokines. By contrast, Th17 cells may be converted into IFN-γ-producing Th1 cells that mediate tumor rejection. In addition, Th17 cells can elicit a protective inflammation that promotes the activation of tumor-specific $CD8^+$ T cells. Although Th17 cells constitute a minor population in human peripheral blood or lymph nodes in cancer patients, they are reported to be detected within tumor tissues at higher frequency in various types of cancers, including ovarian [119], hepatocellular [120], prostate [121], small cell lung [122], colorectal [111,123] and gastric cancers [124]. Tumor-infiltrating Th17 cells were found to express several types of receptors for trafficking in the peripheral tissues, such as CXCR4, CCR6 and CD161, which may be associated with a selective migration and retention within tumor tissues [119]. In addition, tumor cells and tumor derived fibroblasts were reported to produce cytokines RANTES and MCP-1 that recruit Th17 cells and provide the cell–cell contact engagement that facilitates generation and/or expansion of Th17 cells in the tumor microenvironment [125].

There has been relatively little information regarding the clinical significance of tumor-infiltrating Th17 cells. In ovarian cancer, the levels of tumor-infiltrating Th17 cells positively predicted patient outcome [119]. By contrast, the accumulation of intratumoral Th17 cells was associated with a poor prognosis in hepatocellular carcinoma and colorectal cancer patients [111,120,123]. Further studies will be required to define the roles of this cell subset in the tumor microenvironment and clarify their clinical significance.

γδ T cells

γδ T cells represent only a small subset of the total T cell population in the peripheral blood. This 'unconventional' T cell subset has been reported to play an important role in stress-surveillance responses in numerous aspects of inflammation, including infectious diseases, autoimmunity and tumor immunity [126]. γδ T cells can directly recognize specific antigens without antigen-processing or -presentation by MHC molecules. Following the engagement of specific antigens, γδ T cells can provide cytokines and/or chemokines that affect downstream, adaptive immunity. In addition, γδ T cells can directly influence the adaptive immunity by functioning as antigen-presenting cells [127].

In multiple experimental models, γδ T cells were reported to play important roles in antitumor immune responses, predominantly as a positive regulator. γδ T cells produce inflammatory cytokines such as IFN-γ and show high cytolytic

activity. Transformation-induced changes in tumors are suggested to cause stress-surveillance responses mediated by γδ T cells and enhance antitumor immunity [126]. In murine studies *in vivo*, intraepithelial γδ T cells were shown to be rapidly recruited to tumor sites, in order to produce high levels of IFN-γ and control cutaneous tumor development. Mice lacking γδ T cells revealed an increased susceptibility to chemically induced cutaneous carcinogenesis [128]. In addition, it has recently been demonstrated that IL-17-producing γδ T lymphocytes play a decisive role in chemotherapy-induced anticancer immune responses in mice [129]. However, these positive roles have not been supported in other settings. In another murine model, transplanted tumors were found to be better controlled in mice lacking γδ T cells, suggesting that γδ T cells could also play negative roles in antitumor immunity [130].Although the adoptive transfer of *ex vivo* expanded γδ T cells has been performed in some early-phase clinical trials [131,132], there has been little information regarding the clinical significance of tumor-infiltrating γδ T cells in human cancers. Tumor-infiltrating γδ T cells were reported to be significantly correlated with a brief disease-free interval in advanced serous ovarian carcinoma [133]. However, in renal cell carcinoma, the percentages of intratumoral γδ T cells were consistently low (~1%) in nearly all of 248 tumor specimens examined, and failed to correlate with patient prognosis [134]. Peng *et al.* recently found high percentages of γδ1 T cells, which strongly inhibited the activation of conventional T cells and the maturation of DCs via a unique toll-like receptor signaling pathway, among TILs in human breast and prostate cancers [135].

B cells

Humoral responses to tumor antigens in the peripheral blood have been extensively studied. However, there has been limited information regarding tumor-infiltrating B cells, which often colocalize with T cells within tumor tissues [136]. Tumor-infiltrating B cells have been most extensively examined in breast cancer [137,138]. B cells were reported to infiltrate approximately 20% of breast cancer tissues, where they comprised approximately 60% of TILs. These tumor-infiltrating B cells were reported to be positively associated with survival in node-negative breast cancers and medullary breast cancers [137,139]. The prognostic significance of tumor-infiltrating B cells was also reported in other types of cancers [42,47,140–143]. For example, tumor-infiltrating B cells were detected in over 40% of high-grade serous ovarian cancers, and strongly correlated with improved survival [140]. In cervical cancers, the density of peritumoral $CD20^+$ cells as well as other types of TILs was significantly lower among patients with relapse [141]. In patients with soft tissue sarcomas, a high density of $CD20^+$ lymphocytes in tumors with wide resection margins was an independent positive prognostic indicator [142]. In NSCLC, $CD20^+$, $CD8^+$ and $CD4^+$ cells infiltrating tumors were associated with favorable clinical outcomes [42]. In head and neck cancer, Distel *et al.* found that higher numbers of intraepithelial $CD8^+$ T cells and $CD20^+$ B cells were associated with an improved survival in the low-risk group (early disease), whereas an increased number of $CD20^+$ B cells showed shorter survival in the high-risk group (inoperable disease) [144]. In addition, the same authors reported that increased numbers of intraepithelial $CD8^+$ T cells in metastatic tumors and peritumoural B cells in lymph node metastases were associated with favorable outcomes [47]. These findings suggest that the prognostic impact of infiltrating B cells may depend on the stage of diseases and/or the type of treatments.

Tumor-infiltrating B cells often form organized tertiary lymphoid structures, together with DCs, $CD8^+$ and $CD4^+$ T cells [136,143]. Recently, Dieu-Nosjean and colleague reported tertiary lymphoid structures, which were composed of mature DCs and T cell clusters adjacent to B cell follicles, within NSCLC specimens [143]. They demonstrated that the density of mature DCs that honed exclusively to the tertiary lymphoid structures was a positive predictor of long-term survival in patients with early-stage NSCLC [143].

Little is known about the mechanisms by which tumor-infiltrating B cells contribute to antitumor immunity. B cells infiltrating tumors were reported to express IgG on their surfaces and to show evidence of antigen-driven expansion and affinity maturation [137,145]. Therefore, they could potentially produce antibodies specific to tumor-associated self-antigens, which may promote antitumor immunity through various mechanisms, including opsonization of tumor antigens, antibody-mediated cytotoxicity, complement-mediated destruction of tumor cells, and direct modulation of target proteins. Indeed, similar antitumor effects via B cell-mediated immune responses were observed in cancer patients receiving tumor cell vaccines expressing GM-SCF, who showed favorable clinical outcomes through enhanced production of autoantibodies to self-antigens [146].

NK cells

NK cells can kill a wide range of tumors without the need for preactivation or education by antigen-presenting cells [147]. In particular, NK cells can recognize tumors that evade T cell-mediated killing via aberrant expression of HLA [147]. However, since NK cells cannot efficiently home to malignant tissues, they are not often detected in large numbers within advanced solid tumor tissues [148]. Thus, there has been limited information showing the prognostic values of tumor-infiltrating NK cells. In 157 patients with colorectal carcinomas, an extensive intratumoral infiltration of NK cells, evaluated by CD57 expression, was associated with a favorable clinical outcome [149]. Similarly, a higher level of $CD57^+$ NK cell infiltration within tumors was correlated with an improved prognosis in both gastric [150] and squamous-cell lung cancers [151]. In addition, the presence of tumor-infiltrating $CD56^+$ NK cells, which can kill target cells via antibody-dependent cell-mediated cytotoxicity, was found to be an independent predictor for survival in metastatic colorectal cancer patients treated with the first line cetuximab based-chemotherapy [152].

Recently, the phenotype and properties of tumor-infiltrating NK cells have been characterized in detail. In renal cell carcinoma, higher levels of NK cell infiltration were associated with the presence of the $CD16^{bright}$ NK cell subset, which shows better cytolytic potential than other NK cell subsets [153]. In NSCLC, $CD56^{bright}$ $CD16^-$ cells, which were mainly capable of producing cytokines rather than killing cancer cells directly, were enriched within tumors [154].

The activation of NK cells remains an attractive modality for cancer immunotherapy [147]. However, a fundamental problem with adoptive transfer of IL-2-stimulated NK cells or activation of endogenous NK cells could be that they fail to infiltrate tumors. Since the migration and accumulation of NK cells and T cells are shown to be differentially regulated in the tumor microenvironment [155], the complex processes of migration and tissue homeostasis of NK cells remains to be clarified.

NKT cells

NKT cells, which share the characteristics of both T and NK cells, constitute a unique class of T-cell lineage [156,157]. Invariant NKT (iNKT) cells, or type I NKT cells, bear a semi-invariant αβ TCR (Vα24Jα18 chain paired with diverse Vβ11 chains), which is restricted by the nonpolymorphic MHC class I-like molecule CD1d. They display reactivity to synthetic α-galactosylceramide (α-GalCer) as well as self-derived glycolipid antigens presented by CD1d. Despite homogeneous Vα24Vβ11 TCR segment usage and α-GarCer/CD1d specificity, human iNKT cells are phenotypically and functionally heterogeneous. Both $CD4^+$ and $CD4^-$ iNKT cell subsets produce Th1 cytokines (IFN-γ and TNF-α), but the $CD4^+$ subset displays an enhanced ability to produce Th2 cytokines (IL-4, IL-10 and IL-13) and possesses regulatory activity. In addition, different iNKT cell subsets express different arrays of NK and honing/chemokine receptors. Although NKT cells can directly kill $CD1d^+$ tumor cells, the majority of human tumors do not express this molecule. Therefore, the roles of NKT cells in antitumor immunity remain largely unknown. Interestingly, CD1d-restricted killing of tumor-associated monocytes/macrophages by NKT cells was recently reported to be associated with suppression of tumor growth in a human glioblastoma xenograft model [158].

Various abnormalities of iNKT cells in the peripheral blood have been documented in cancer patients [159]. Decreased frequencies and/or impaired functions in peripheral iNKT cells were observed in patients with various types of advanced cancers [160–162]. Although iNKT cells in the peripheral blood have been well studied in cancer patients, very few studies have analyzed those within tumors. This may be a critical issue since biological aspects of iNKT cells, such as homing and function, may be altered in tumor microenvironments. Most of the currently available studies showed an increase of the frequencies of tumor-infiltrating iNKT cells in primary and metastatic tumors [163–165]. In colorectal cancers, an increase in intratumoral infiltration of $Vα24^+$ NKT cells was reported to be a favorable prognostic factor [163]. Dhodapkar *et al.* showed that $Vα24^+Vβ11^+$ iNKT cells in the blood and tumor bed had a marked functional defect in patients with progressive multiple myeloma, but not in those with nonprogressive myeloma or premalignant gammopathy, suggesting that iNKT cells play an important role in preventing malignant progression [164]. In primary and metastatic liver tumors, the iNKT cell repertoire was skewed by the enrichment of $CD4^+$ iNKT cells, a subset producing Th2 cytokines, which may promote tumor growth and recurrence [165]. In patients with gastric and colorectal cancers, the numbers of $CD3^-CD56^+$ NK cells and $CD3^+CD56^+$ NKT cells were decreased in metastatic livers, compared with normal livers without metastases [166].

Only a few studies have examined the mechanism of NKT cell localization to tumor sites. Metelitsa *et al.* found that NKT cells migrate toward neuroblastoma cells in a CCL2-dependent manner and infiltrate tumors that express high levels of CCL2 [167]. Although NKT cell-based immunotherapy has been clinically tried as a potentially promising strategy for the treatment of cancer patients [159], more phenotypical and functional data of NKT cells in the peripheral blood as well as within tumors would be required.

Current issues & future perspective

Assessment of localization of tumor-infiltrating immune cells

Various types of immune cell subsets infiltrate tumor tissues in variable quantities and interact with each other. Recent detailed analyses suggest that TILs are not randomly distributed, but appear to be organized to accumulate more or less densely in different regions within tumors. It now seems to be clear that not only the overall quantity, but also the localization of the immune cells in the tumor microenvironment has a major impact on the prognosis of patients [44,52,62,63]. However, in most of the currently available studies, the quantity of TILs was the only variable typically considered. More detailed analyses regarding where and how actively the immune cells infiltrate within tumors would be informative.

Simultaneous detection of different immune cell subsets

Recent data have revealed that the composition of tumor-infiltrating immune cells is not homogenous, but rather represents varying contributions from many different cell subsets. Not only lymphocytes, but also other immune cells, such as macrophages, granulocytes, DCs and myeloid-derive suppressor cell, infiltrate tumors and affect tumor growth via their mutual interaction. The functions of TILs are often compromised as a result of the accumulation of immunoregulatory cells and various tumor escape mechanisms. To understand the mechanisms of infiltration to tumors and the biological significance of infiltrating cells, the spatial distribution and relationship of different immune cells need to be clarified by assessing and analyzing multiple cell subsets simultaneously. Currently, IHC remains the most important diagnostic technique to evaluate protein expression in tissue specimens. However, current IHC analyses have some limitations. In general, IHC has not been quantitative, owing to the low specificity of the antibodies used to capture the analytes and instability of fluorescence dyes used as the detecting agents. In addition, because of the limited availability of different fluorescence dyes, simultaneous, multiplexed detection of several markers is quite difficult. Figure 1 shows an example of a multiplexed IHC with quantum dots (QDs), which have recently emerged as a novel class of fluorophores that show promise for many biomedical imaging applications [168]. Fluorescence from QDs is significantly brighter and more photostable than organic dyes. In addition, QDs offer the capacity for multiplexed detection of several analytes simultaneously. These novel techniques need to be developed to allow for simultaneous detection and quantitation of multiple cells within tumors.

Appropriate reagents for assessing immune cells

Appropriate reagents for assessing immune cells within tumors may be the primary critical consideration. However, the currently available reagents are quite restricted. In particular, only limited numbers of antibodies are suitable for IHC. Most of antibodies used for IHC in recent studies were developed and provided by commercial suppliers, but not all commercial antibodies perform as expected [169,170]. Different commercial suppliers offer antibodies of varying quality and are sometimes unable to provide expert technical support for IHC use. For example, in the IHC staining for FoxP3 on formalin-fixed paraffin-embedded sections, two commonly used monoclonal antibodies from different commercial sources are reported to show significantly different staining patterns [171]. Development and confirmation of reliable reagents thus may be critical.

In addition, selection of markers for identifying specific cell subsets may be important. For example, NK-markers, such as CD56 and CD57, have frequently been used to identify NK cells. However, these molecules are known to also be expressed in other cell subsets, such as terminally-differentiated T cells and NKT cells [172]. Therefore, the simple use of these markers may overestimate the frequency/density of NK cells by detecting not only NK cells but also other cell subsets. To precisely identity NK cells, other markers should be employed in combination. In addition, as discussed above, the simple use of FoxP3 may also overestimate the frequency/density of Treg cells, although most studies use FoxP3 alone to identify Treg cells. Because FoxP3 is known to also be expressed in other types of cells, including activated $CD4^+$ and $CD8^+$ T cells and tumor cells [93–98], Treg cells should be defined by combined staining with FoxP3 and

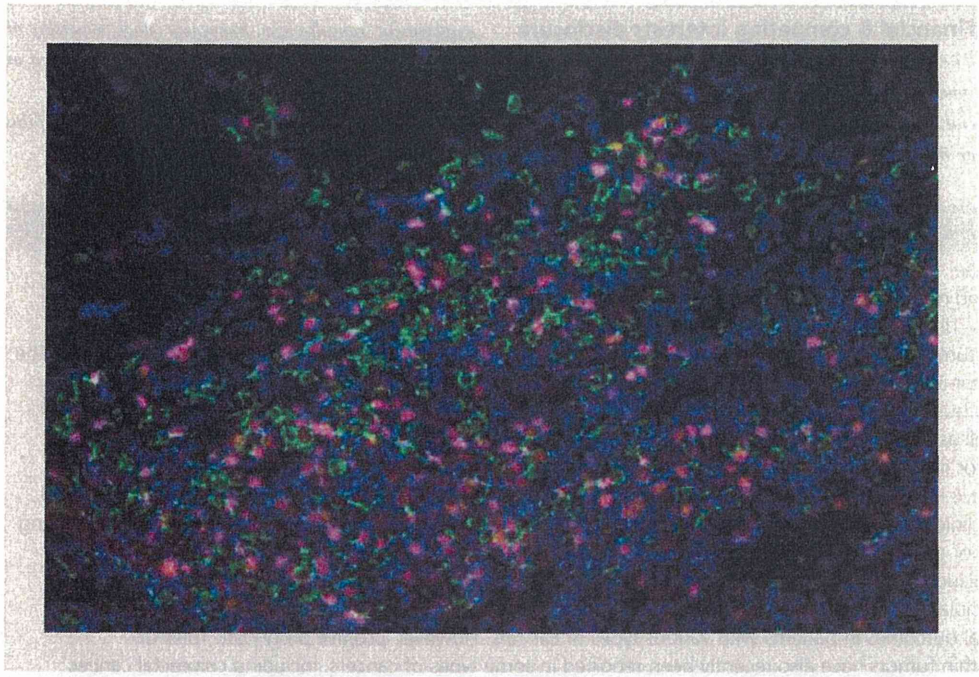

Figure 1. Multicolor, multiplexed fluorescence detection of CD4, CD8 and FoxP3 antigens with quantum dot nanocrystals. A paraffin-embedded tumor tissue was simultaneously stained with quantum dot-labeled anti-CD8 (green), anti-CD4 (blue) and anti-FoxP3 (red) antibodies.

other markers, such as LAG-3 [99]. In addition, functional analysis of TILs directly isolated from tumor tissues may be quite important to prevent misinterpretation of data [100,101].

Assessment of antigen-specificity of TILs

The assessment of antigen-specificity of infiltrating T cells can provide important information to understand the dynamics and magnitude of T-cell responses specific to tumors. Currently, staining of antigen-specific T cells with fluorescently labeled MHC–peptide multimers has provided a powerful experimental approach to characterizing immune responses. However, this technique has been limited to *ex vivo* studies to analyze the cells isolated from blood or tissues by flow cytometry. In order to monitor antigen-specific T cells *in situ*, staining of tissue sections with MHC–peptide multimers has been developed, although this technology is still challenging and requires optimization for individual MHC–peptide multimers [173,174]. To obtain the best possible results, it is necessary to optimize the staining protocols; including the amount of MHC–peptide complexes and CD8 antibody, amplification with secondary antibodies, and incubation temperature and time; but once optimized this technique would allow researchers to directly assess the dynamics and magnitude of antitumor T-cell responses within tumors.

Assessment of functionality of tumor-infiltrating immune cells

Recent technical developments have allowed the refinement of phenotypical and functional analyses of human T-cell subsets, which has cast light on their heterogeneity and plasticity. The capacity of single T cells to exert several effector functions, so-called polyfunctionality, has been shown to correlate with protective immunity against infectious diseases [175] and cancers [176–178]. Therefore, functional properties, in particular polyfunctionality of TILs, may also be important considerations. Since it is conceivable that the long-term culture used for generating clones *in vitro* may contribute to significant phenotypical and functional changes, bulk T cells, directly isolated from tumor sites after mechanical dissociation without *in vitro* culture, will provide useful confirmatory information (Box 1).

Box 1. Current issues and future perspectives.

- Assessment of localization of tumor-infiltrating immune cells
- Simultaneous detection of multiple cell subsets (i.e., multiplexed Immunohistochemistry with quantum dots)
- Development or confirmation of reliable reagents
- Selection of appropriate markers (i.e., combined use of multiple markers)
- Assessment of antigen-specificity of tumor-infiltrating lymphocytes (i.e., *in situ* staining with MHC-peptide multimers)
- Assessment of functions of tumor-infiltrating immune cells (i.e., polyfunctionality of tumor-infiltrating lymphocytes)

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Executive summary

- Tumors develop and progress under the influence of a microenvironment comprising various types of immune cells and their products, such as cytokines, chemokines and metabolites. A unique microenvironment of each tumor may shape the composition and function of tumor-infiltrating lymphocytes (TILs) in a unique way. A variety of immune cell subsets, belonging to both the innate and adaptive immune systems, accumulate at tumor sites and play critical roles in promoting or inhibiting tumor development and/or progression. The diversity and composition of tumor-infiltrating immune cells may significantly affect clinical outcomes in cancer patients.
- The clinical significance of TILs in human cancers remains unclear, and is a subject of considerable controversy, largely owing to the lack of functional data for TILs, as well as owing to the enormous variability of TILs in different tumors. A great deal more functional data about TILs need to be obtained for individual tumors before TILs can be considered as a prognostic parameter in human cancers.
- Tumor-infiltrating $CD8^+$ T cells have been most extensively studied. An abundance of tumor-infiltrating $CD8^+$ T cells has been reported to be associated with improved clinical outcomes in various types of cancers. For example, in colorectal cancers, a novel immune scoring system that reflects the densities of tumor-infiltrating $CD8^+$ and $CD45RO^+$ T cells, has been proposed to be superior to the standard prognostic clinical parameters, including the TNM staging system. Further studies will be required to validate its clinical utility.
- Considerable evidence has accumulated suggesting that an increased frequency of $CD4^+FoxP3^+$ regulatory T cells within tumors is associated with negative clinical outcomes in patients with various types of cancers. However, positive prognostic roles of $CD4^+FoxP3^+$ regulatory T cells within tumors have also recently been reported in some types of cancers, including colorectal cancer, lymphoma, and head and neck cancer.
- Accumulating evidence has suggested that not only $CD8^+$ and/or $CD4^+$ $\alpha\beta$ T cells, but also other lymphocyte subsets, including $\gamma\delta$ T cells, B cells, NK cells, and NKT cells, infiltrate tumor tissues in variable quantities and play key roles in the regulation of antitumor immunity. More data need to be accumulated before their precise roles within tumors can be determined.
- TILs are not randomly distributed, but appear to be organized to accumulate more or less densely in different regions within tumors. Not only the overall quantity, but also the localization of the immune cells in the tumor microenvironment may have a major impact on prognosis of patients. In future studies, it is critical to precisely analyze both their localization and phenotypes.
- The spatial distribution and relationship of different immune cell subsets remains to be determined, in order to understand the mechanisms of tumor infiltration and biological significance of each cell subset. Novel techniques need to be developed in order to allow for simultaneous detection and quantitation of multiple cells within tumors.
- Appropriate reagents for assessing immune cells within tumors may be the primary critical consideration. Different commercial suppliers may offer reagents of varying quality. Development and confirmation of reliable reagents may thus be critical. In addition, selection of appropriate markers for identifying specific cell subsets may be important to prevent over- or underestimation.
- The assessment of antigen specificity of TILs can provide important information to understand the dynamics and magnitude of T cell responses specific to tumors. In addition, functional properties, in particular the polyfunctionality of TILs, may be also important considerations. Bulk T cells directly isolated from tumor sites without *in vitro* culture will provide useful information.

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