Immunotherapy of colorectal cancer

Caroline L Smith, Nicolas Dulphy, Mariolina Salio and Vincenzo Cerundolo

Cancer Research UK Tumour Immunology Group, The Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, UK

Over the last decade, there has been a rapid expansion in the field of tumour immunology. There is now convincing evidence that both the cellular and humoral arms of the immune system are capable of interacting with tumour cells. The most significant advances have been in our understanding of cellular responses and the complex events that lead to T-lymphocyte activation, as well as in the identification of tumour antigens recognised by T-lymphocytes. This knowledge has led to the development of anticancer immunotherapies designed to produce tumour antigen-specific T-cell responses, adding to the earlier antibody or whole-cell vaccine approaches. In addition, new methods have been developed to quantify antigen-specific T-cell responses, and the emergent field of recombinant gene technology has led to an increasing number of novel methods for vaccine delivery. This review will explore these advances, as well as possible future directions, with an emphasis on colorectal cancer.

Anti-tumour immune responses

There is now definitive evidence that some patients with established tumours develop spontaneous anti-tumour immune responses. Although the identification of tumour antigens recognised by antibodies dates back to the 1950s, it is only over the last decade that techniques have been developed to identify antigens recognised by T-lymphocytes.

Melanoma is the tumour type that has been particularly studied by tumour immunologists. Evidence that melanoma is immunogenic is provided by the association of a lymphocytic infiltrate of the primary tumour with an improved prognosis\(^1\) and, more significantly, by the isolation of tumour antigen-specific T-cells from the blood, tumour-infiltrated lymph nodes and metastases of melanoma patients. These T-cells have been shown to be capable of killing melanoma cells \textit{in vitro}\(^2,3\).

Although a lymphocytic infiltration of primary colorectal carcinoma is similarly associated with improved overall and recurrence-free survival\(^4\), there is very little data about the antigen-specificity of these lymphocytes. Of interest, a high level of microsatellite instability in colorectal
carcinomas is associated with the presence of tumour-infiltrating lymphocytes, as well as an improved prognosis. It is possible that the increased number of lymphocytes are responding to large numbers of ‘neo-antigens’ created by the high rate of DNA mutation which is characteristic of this group of colorectal cancers, as a result of defects in DNA mismatch repair genes. It is unclear, however, whether tumour-infiltrating lymphocytes or other factors are contributing directly to this survival advantage. Functionally active T-cells, specific for several tumour antigens, have been demonstrated *ex vivo* from the blood of patients with colorectal cancer. These patients tended to have later stage disease, consistent with similar observations in melanoma patients. Antibody responses against tumour antigens have also been demonstrated in patients with colorectal cancer, and are also often higher in those with more advanced disease.

In the first part of this review, we shall briefly describe the mechanisms with which the immune system recognises tumour antigens. We shall then list the known tumour antigens relevant to colorectal cancer, and describe different vaccination strategies that are currently being pursued in patients with colorectal cancer.

### The immune system

Both innate and adaptive immune mechanisms are thought to play a role in anti-tumour immune responses. Cells involved in innate responses include macrophages and natural killer (NK) cells. Activation of these cells is not antigen-specific, and is thought to result from the recognition of receptors or antibodies on the surface of target cells. Activating and inhibitory receptors expressed on NK-cells are the focus of current research.

In contrast, the adaptive immune response involves the clonal expansion of antigen-specific lymphocytes, and consists of a cellular and a humoral arm.

#### The cellular arm

T-cells recognise antigens through their T-cell receptors (TCRs). Each T-cell expresses a TCR of a single specificity. T-cell antigens are expressed on cells (for instance tumour cells or infected cells) in the form of short peptide fragments (‘epitopes’) that have been digested from larger proteins. The peptides are ‘presented by’ human leukocyte antigen (HLA) molecules. While CD8+ T-cells recognise peptides derived from intracellular proteins in association with HLA class I molecules, CD4+ T-cells recognise peptides derived from extracellular proteins in association
with HLA class II molecules. Different processing pathways exist for the presentation of peptides by HLA class I and class II molecules.

CD8+ T-lymphocytes have a cytolytic function, releasing molecules such as perforin and granzyme on antigen recognition. CD4+ T-lymphocytes regulate the immune response towards either a cellular response (CD8+), or a humoral (antibody) response, by secreting different sets of cytokines on antigen recognition.

For naïve T-cells to become ‘activated’, they must receive two ‘signals’. Signal one is provided by ligation of the TCR with the HLA/peptide complex on the surface of the target cell. The second signal is provided by the ligation of ‘co-stimulatory molecules’, and can only be delivered by professional antigen presenting cells (APCs), the most important of which are now known to be dendritic cells (DCs). Immature DCs take up antigen in the periphery and, upon receiving appropriate inflammatory signals (for instance from bacteria or viruses), they ‘mature’ and migrate to draining lymph nodes. Mature DCs can present peptides through both the HLA class I and class II pathways, and also express co-stimulatory molecules. CD4+ and CD8+ T-cells are activated by DCs in the lymph nodes, proliferate, and then migrate to the inflammatory site (for instance infection or tumour), where antigen recognition results in triggering of their effector function, without the need for the second signal.

HLA class I and class II genes are highly polymorphic. The cells of individuals with different HLA alleles (tissue types) will present different arrays of peptides to their T-cells. In terms of immunotherapy design this has practical implications. A peptide vaccine, for example, may only be applicable to patients with a particular tissue type. Many class I epitopes identified to date are HLA-A*0201 ‘restricted’ because of the prevalence of this allele in 40–50% of the Caucasian population. The number of identified epitopes restricted by other HLA molecules is now increasing, making epitope-specific immunotherapies more broadly applicable.

The humoral arm

Antibodies constitute the soluble form of the B-lymphocyte antigen receptor, each B-cell bearing a receptor of single specificity. After a B-cell is activated by the ligation of this receptor with antigen, in the context of appropriate stimulatory signals (for instance from CD4+ cells), they mature into plasma cells which produce large amounts of monoclonal antibody. Antibodies bind to antigens based on the three-dimensional structures of the antigen, and are able to bind directly to molecules on the surface of target cells. Antibody binding to target cell can result in cell death by two mechanisms: the phagocytic cells of the innate system can recognise the antibody constant domain (Fc domain), and antibodies
can activate the complement cascade. There is now increasing evidence that antibody binding to tumour cells enhances uptake by APCs and augments cellular responses.

Many B- and T-lymphocytes expressing receptors that recognise self-derived molecules with high affinity are deleted during their development. There is not total deletion, however, and mechanisms exist in the periphery to down-regulate potentially auto-reactive lymphocytes. Tumour cells are derived from self, and many tumour antigens that have been identified are normal self-molecules. Therefore, for an anti-tumour immune response to be generated, tolerance to self must be broken. This may explain why spontaneous anti-tumour immune responses are often weak in comparison to those generated against infection, and it provides an additional hurdle for the tumour immunologist in the design of immunotherapeutic strategies.

**Tumour antigens**

Tumour antigens recognised by T-cells and antibodies are derived from a broad range of cellular proteins. Those relevant to colorectal cancer can be divided into 3 main groups. Differentiation and viral antigens that are relevant only to other tumour types will not be discussed.

**Cancer-testis antigens**

These are expressed on a broad range of tumour types, but not in normal tissues except spermatogonia, which do not express HLA class I molecules and, therefore, do not represent targets for class I-restricted immune responses. The mechanism for the expression of this group of antigens in tumours and testis is thought to result from the demethylation of genes that are silenced by methylation in non-germ cells. Members of this group that have been shown to be expressed on colon carcinoma cell lines include the MAGE family (with now over 20 members), as well as BAGE, GAGE, and SSX.

**Over-expressed antigens**

These are expressed to a variable degree in normal tissues, but are over-expressed in a broad range of tumour types. There are many possible mechanisms for over-expression, including demethylation, decreased protein degradation and multiplicity of genes. This group is particularly relevant to colorectal cancer.
Ep-Cam
Ep-Cam (EGP-2, 17-1A, GA733-2 or KSA), a glycoprotein that mediates cellular adhesion, is expressed on normal epithelial cells. It is over-expressed in small cell carcinoma and in many adenocarcinomas, particularly colorectal carcinoma. Several HLA A*0201-restricted epitopes have been described\textsuperscript{12,13}, and anti-Ep-Cam antibody responses have been demonstrated in approximately 15\% of patients with colorectal carcinoma\textsuperscript{14}.

Carcino-embryonic antigen (CEA)
CEA is also an intercellular adhesion glycoprotein, expressed on normal colonic epithelium and fetal gastrointestinal epithelium. It is over-expressed in > 90\% of colorectal, gastric and pancreatic carcinomas\textsuperscript{15}, approximately 50\% of breast carcinomas\textsuperscript{16}, and > 70\% of non-small cell lung cancers. Anti-CEA antibodies are detectable at low titres in patients with colorectal carcinoma\textsuperscript{17}. Several HLA-A*0201-restricted epitopes\textsuperscript{18,19}, and an HLA-A*24-restricted epitope\textsuperscript{20} have been identified.

CEA is a member of the CD66 protein family, the members of which share approximately a 70\% sequence homology. Other CD66 proteins are expressed on granulocytes, epithelial cells, placenta, and fetal liver\textsuperscript{21}. Immune responses generated by anti-CEA immunotherapy could, therefore, result in toxicities in normal tissues expressing these homologous proteins.

HER-2/neu
HER-2/neu (erbB-2, p185) belongs to the epidermal growth factor receptor family. It is expressed on a wide variety of cancers and normal cells. It is expressed in up to 85\% of colorectal carcinomas\textsuperscript{22}, and antibody responses occur in approximately 14\% of patients with colorectal carcinoma\textsuperscript{23}. Many HLA class I- and class II-restricted epitopes have now been identified\textsuperscript{24}.

MUC-1
MUC-1 belongs to the family of human epithelial mucins, which are high molecular weight glycoproteins expressed on the luminal surface of glandular epithelium. They are characterised by a large number of O-glycosylated tandem repeat domains. In these domains, a 20 amino-acid polypeptide sequence is repeated between 25 and 100 times, hence the name ‘variable number of tandem repeat’ (VNTR) regions. Expression of MUC-1 is markedly increased in more than 90\% of breast, colon and lung cancers\textsuperscript{25}. Furthermore, on tumour cells, aberrant glycosylation occurs, such that underlying tandem repeat region peptide sequences are exposed. B-cells can recognise the exposed regions, and immune complexes containing MUC-1 have been detected in the blood of patients with colorectal and pancreatic carcinoma\textsuperscript{26}.
There is evidence that T-cells can recognise the revealed peptide sequences in an HLA non-restricted manner. In addition, an HLA A*0201- and an HLA A*1101-restricted epitope encoded within the tandem repeat region have been described, and 3 possible HLA A*0201-restricted epitopes encoded outside the tandem repeat region have been identified using transgenic mouse models.

p53
The p53 tumour suppressor gene product is a phosphoprotein that is barely detectable in the nucleus of normal cells. Upon cellular stress, however, particularly that causing DNA damage, it can arrest the cell cycle progression allowing DNA to be repaired, or direct the cell into apoptotic pathways. Mutations in p53 are present in 40–50% of cancers overall, and 60% of colorectal carcinomas. Most mutations result from point mutations. Mutated p53, as well as being unable to control cell proliferation, accumulates within cells due to increased stability. Antibodies specific for p53 have been found in the serum of 20% of patients with colorectal cancer. An HLA A*0201-restricted epitope has been identified that is not at the site of mutation.

Tumour cell-specific antigens
Genetic mutation, an inherent feature of carcinogenesis, potentially results in the translation of mutated proteins that have a different peptide sequence from that of the wild-type. These peptide sequences potentially encode new epitopes, ‘neo-epitopes’, that will not have previously been seen by the host’s immune system. A mutation that results in a frame shift will result in the translation of a truncated protein that is unique in the whole of its peptide sequence down-stream from the mutation, rather than just at the site of mutation, and that is, therefore, even more likely to encode new epitopes. Although these neo-epitopes may be recognised by T-cells, a random mutation that is only present in a particular tumour in a particular patient is not useful in terms of vaccine design. Mutations that occur predictably within a given tumour type, however, are potentially useful as targets for immunotherapy. Such mutations are more likely if they contribute to the molecular mechanisms of carcinogenesis, and hence provide tumour cell survival advantage.

Transforming growth factor-β receptor II (TGF-βR-II)
As previously discussed, colorectal cancers showing a high degree of microsatellite instability (MSI+) have large numbers of genetic mutations. Frame-shift mutations commonly occur in the microsatellite
sequences in several cancer related genes such as transforming growth factor-β receptor II (TGF-β-R-II) and Bax. An HLA A*0201-restricted epitope encoded within a TGF-β-R-II frame shift protein has been identified, as has a class II-restricted epitope. These epitopes are potential targets for the immunotherapy of MSI colorectal cancers, which comprise 15% of sporadic cancers, as well as those due to inherited disorders of mismatch repair genes.

Adenomatosis polyposis coli (APC)
The APC gene is mutated early in most cases of colorectal cancer. Although half of the mutations are point mutations that result in a stop codon, the other half arise by short deletions or insertions that alter the reading frame. These mutations cluster and, therefore, certain new reading frames are represented more frequently than others. Townsend et al. cloned the most commonly expressed new reading frame that they observed in patients, and expressed it in vaccinia virus. They demonstrated a vigorous cytotoxic T-lymphocyte response, specific for an epitope encoded within the sequence, in mice vaccinated with the recombinant virus.

Ras
Point mutations in the ras proto-oncogenes are commonly found in a diversity of human malignancies, occurring predominantly at codons 12, 13 and 61. In adenocarcinomas of the pancreas, colon and lung, codon 12 of the K-ras proto-oncogene is often mutated, resulting in an amino acid substitution of glycine to valine, aspartic acid or cysteine. An HLA A*0201-restricted neo-epitope that encodes the valine substitution has been identified.

Immunomonitoring

Until recently, progress in tumour immunology has been hampered by a lack of reliable and easily reproducible methods, with which spontaneous and vaccine-driven antigen-specific T-cell responses can be measured. Earlier assays relied on in vitro cell proliferation techniques and were indirect. Several assays have now been developed that allow direct enumeration of antigen-specific T-cells, even in ex vivo samples.

The most sensitive assay is based on the use of tetrameric HLA class I/peptide complexes, ‘tetramers’ (Fig. 1A). Fluorochrome-labelled tetramers can be used to stain cells, which are then analysed by flow cytometry (Fig. 1B). This technique also allows a detailed phenotypic analysis of antigen-specific T-cells when used in combination with the large panel of phenotypic markers that are available. Tetramer technology also
enables rapid and sensitive separation of homogeneous populations of antigen-specific T-cells by flow cytometry cell sorting. These lines or clones provide a unique source of cells for TCR-repertoire analysis and, potentially, for antigen-targeted adoptive therapy.

The other approach is to enumerate antigen-specific T-cells on the basis of cytokine release in response to antigen. Although indirect, it also provides a measurement of an effector function. Two experimental methods have been developed based on this approach. The ELISpot assay involves incubating lymphocytes with one or more peptides. Released cytokine is captured on a membrane coated with anti-cytokine antibody. The cells are then washed off, and a secondary antibody is added. After colour development, a visible ‘spot’ represents one antigen-specific cytokine-releasing cell (Fig. 1B). Alternatively, intracellular cytokine staining allows antigen-specific, cytokine-producing cells to be...
identified by flow cytometry. This technique involves the stimulation of T-cells with antigen, prior to intracellular staining with fluorochrome-labelled antibody specific for intracellular cytokines. This technique can be combined with tetramer staining\textsuperscript{39}.

**Therapeutic strategies**

We shall attempt to categorise tumour immunotherapeutic strategies according to modality, considering antigen-specific and non-specific modalities separately, and to discuss potential advantages and disadvantages of different approaches.

**Antigen non-specific immunotherapy**

**Cytokines**

Cytokines play a crucial role in the communication between cells and the subsequent activation of immune responses, and have been administered systemically as antigen non-specific immunotherapy. Due to their broad biological activities and the high doses that are often required, toxicities are often significant. Cytokines that have received particular attention include interleukin-2, the interferon family, and granulocyte macrophage-colony stimulating factor (GM-CSF).

Rather than using cytokines alone, newer approaches involve combining systemic cytokine treatment with many of the tumour antigen-specific therapeutic modalities described below\textsuperscript{40}. Alternatively, whole cell vaccines (tumour cells or dendritic cells) or viral delivery vectors can be genetically modified to express cytokines.

**Whole-cell vaccines**

Prior to the identification of tumour antigens, whole-cell vaccines were the mainstay of immunotherapeutic approaches. The advantage of whole-cell vaccines, especially those consisting of autologous tumour cells is that, in theory, they comprise all relevant tumour antigens, even if the antigen or epitopes have not previously been identified. Antibody responses may also be induced. Disadvantages include the technically difficult generation of autologous tumour cell lines. Immunomonitoring of these approaches is hampered by the fact that immune responses may be generated against tumour antigens and/or epitopes that are unknown and, therefore, not measurable.

One of the earliest immunotherapies developed for colorectal cancer has involved using autologous whole cell vaccines combined with bacillus Calmette-Guérin (BCG). Three large randomised trials have been performed comparing whole tumour cell and BCG with no therapy,
after surgery for colorectal cancer. Two trials showed no difference in clinical outcome\textsuperscript{41,42}. The third showed a 44\% reduction in the relative risk of recurrence in the vaccine arm\textsuperscript{43}.

**Antigen-specific immunotherapy**

**Peptides**
The ability to synthesise large quantities of well-defined antigenic peptides has led to large numbers of clinical trials of peptides in combination with various immunological adjuvants (which function to augment the immune response). Many of these trials have been performed in the field of melanoma, and have shown that this is a safe and practical approach. Clinical responses have been seen\textsuperscript{44}, most commonly in skin rather than visceral metastases, but have tended not to match the immunological responses demonstrated using older monitoring techniques. Convincing evidence of peptide-induced antigen-specific immunological responses has now been demonstrated with tetramer and ELISpot assays\textsuperscript{45}. A disadvantage of peptide-based approaches is that they are limited by the HLA-type of the patient.

Several phase I trials have been performed in patients with metastatic carcinoma whose tumours have been shown to contain mutations in the K-ras proto-oncogene, with peptides that span the common mutations\textsuperscript{46,47}.

**Soluble proteins**
Full-length proteins potentially harbour HLA class I- and class II-restricted T-cell epitopes, as well as antibody epitopes. Furthermore, patient HLA-type is not a limitation. Even with strong immunological adjuvants, however, targeting these molecules to the class I processing and presentation pathway remains a challenge, potentially limiting the generation of CD8\(^+\) responses.

Recombinant CEA protein has been used in phase I studies in patients with Dukes’ A, B and C colon cancer. Cellular and antibody responses against CEA were demonstrated and these were shown to be enhanced when GM-CSF was co-administered\textsuperscript{40}.

**Recombinant plasmid DNA and viral vectors**
Advances in gene technology have resulted in the development of new antigen delivery strategies. Viruses or plasmid DNA can be genetically engineered to contain genes or parts of genes of tumour antigens, cytokines or co-stimulatory molecules. Poly-epitope vaccines, in which multiple epitopes are expressed as a ‘string of beads’ are also under development.
Plasmid DNA has the advantages that it is readily deliverable, molecularly-defined, and can be readily constructed and produced in large quantities. It is neither infectious, nor capable of replication. Immunisation with plasmid DNA has been shown to result in both antibody and cellular immune responses in murine models. Preclinical studies have demonstrated that DNA-based vaccines against model tumour antigens can result in potent immune responses, leading to tumour rejection.

Viral vectors have the advantage that by mimicking natural infection, potent cell-mediated responses may be induced. Several viral vectors are established in clinical trials. The pox viruses have received particular attention. Their safety, particularly that of attenuated strains such as modified vaccinia Ankara (MVA), was demonstrated in the large numbers of individuals vaccinated in smallpox eradication programmes. Their efficacy in generating immune responses against foreign encoded antigens is established in animal models. Other members of the pox virus family, such as canary pox and fowl pox have been developed as vectors in an attempt to circumvent the potential problem of pre-existing immunity to vaccinia, as a result of childhood vaccination. Heterologous ‘prime-boost’ vaccination protocols, in which the ‘priming’ delivery system is different to the ‘boosting’ delivery system (for instance different viral vectors, or DNA followed by virus) have been developed. These protocols result in increased levels of specific immunity against infectious agents in murine models, and this approach has obvious applicability to cancer vaccines.

Many clinical trials have now been performed using recombinant pox viruses encoding full-length CEA or truncated CEA. The first clinical trials involved the use of recombinant vaccinia virus encoding CEA. Although no T-cell proliferative responses were observed in the first trial, T-cells specific for an HLA-A*0201 epitope were generated from post-vaccination blood samples after in vitro culture with peptide.

More recently, a series of promising clinical trials has been undertaken using a canary pox vector encoding full-length CEA. The co-stimulatory molecule B7.1 has also been encoded within this recombinant vector, and adjuvant GM-CSF has been studied, as well as a prime-boost regimen involving recombinant vaccinia-CEA and canary pox-CEA. Immunomonitoring with ELISpot has demonstrated CEA-specific T-cells in post-vaccination blood samples. These trials have shown that CEA is safe to use as an immunotherapeutic target.

Clinical trials are currently underway involving vaccinia and fowl pox vectors encoding the CEA-TRICOM® construct which includes CEA as well as three co-stimulatory molecules.

Dendritic cells

Despite the technical difficulties associated with the generation of autologous DCs for clinical application, there is an increasing number of
promising clinical trials for a broad range of tumour types in the literature. These trials have examined methods for generating DCs, the maturation status of DCs, the route of injection of DCs, and the co-administration of cytokines as adjuvants. Various methods for ‘loading’ tumour antigens onto DCs have also been examined. They include ‘pulsing’ DCs with peptides, loading DCs with tumour cell lysates, and the fusion of DCs with tumour cells. DCs have also been manipulated to express tumour antigens, co-stimulatory molecules and cytokines, using recombinant gene technology.

A clinical trial of DCs pulsed with MAGE-3 peptides has been performed in patients with gastrointestinal carcinomas. Similarly, studies have been performed in patients with CEA-expressing tumours, involving DCs pulsed with the HLA-A*0201-restricted or HLA-A*24-restricted CEA epitopes. Another trial in patients with colon or non-small cell lung cancers, involved DCs pulsed with a peptide analogue of the HLA-A*0201-restricted CEA epitope. The substitution of aspartate to asparagine in this epitope, has been shown to be more potent in inducing anti-CEA T-cells in vitro. Five of seven patients demonstrated significant expansions in CEA antigen-specific T-cells measured by tetramers, and two patients had disease regression.

Antibodies
As well as the considerations necessary with regards choice of antigen for T-cell-based approaches, additional attention must be given to the fate of antigen at the cell surface after therapeutic antibody binding. When antibody–antigen complexes are internalised, a theoretical advantage exists for antibodies labelled with radioisotopes or immunotoxins. Unlabelled antibodies, however, must remain on the surface for activation of immune effector functions. Antigens that are shed from the cell surface and then circulate are less optimal as targets, as the administered antibody may bind to this circulating antigen, preventing it from reaching tumour cells.

The first clinical studies were performed using murine, rabbit, or rat antibodies purified following immunisation of the animal with the antigen preparation. This approach can result in the development of antibodies to these foreign antibodies, and is also associated with immune-complex related adverse events such as serum sickness and anaphylaxis. Furthermore, murine, rabbit and rat antibodies may not always able to recruit human immune responses. To overcome these obstacles, DNA technology has been used to construct hybrids composed of human antibody regions linked with murine or primate backbone.

Edrocolomab (Panorex®) is a murine IgG2a monoclonal antibody (17-1A) that recognises Ep-Cam. It has been shown to improve overall survival in patients with Dukes’ C colorectal cancer. In a study involving...
189 patients, subjects were randomised to receive 500 mg edrecolomab at the time of surgery followed by 4-monthly doses of 100 mg, or observation alone. At 7-year follow-up, edrecolomab resulted in an improvement in absolute overall survival of 20%, from 37% to 57%61. Large phase II studies aiming to compare standard chemotherapy with edrecolomab alone, or edrecolomab in combination with standard chemotherapy, have now finished recruiting, and an interim analysis of safety data has indicated a near total lack of additive toxicity when it is used in combination with 5-fluorouracil.

Humanised antibodies against HER-2/neu have received much attention, particularly in the field of breast cancer. A large phase III trial has demonstrated survival benefit with chemotherapy plus antibody compared to chemotherapy alone in breast cancer patients whose tumours over-express HER-2/neu and who have not previously received chemotherapy62. HER-2/neu as a target has obvious applicability to other tumour types, including colorectal cancer.

The mouse monoclonal antibody mAb A33 recognises a cell surface glycoprotein (A33) that is expressed in human colonic epithelium and colon cancer, but which is absent from most other normal tissues. In patients, mAbA33 localises with high specificity but is cleared rapidly from normal colon in 5–6 days. Phase I/II trials of this antibody labelled with $^{131}$iodine and $^{125}$iodine have been performed in patients with advanced colon cancer, demonstrating safety and localisation of the radioisotope to sites of disease. Repeated dosing is, however, limited by the development of human anti-mouse antibodies. More recently, a humanised version has been constructed. In clinical trials, this maintained full affinity and specificity, but was associated with the development of human anti-human antibodies65.

Contrary to the idea that recipient antibody responses against the therapeutic antibody produce a negative effect, is the concept that they generate a beneficial anti-idiotypic network66. Some secondary antibodies may recognise the antigen-specific binding site of the primary antibody. The antigen-binding sites of these secondary antibodies constitute a three-dimensional structure, mimicking the tumour antigen epitope recognised by the therapeutic antibody. These secondary antibodies can, themselves, be used as immunotherapy, as an antibody response generated against the site of structural mimicry will also potentially recognise the original tumour epitope. A potential advantage of an anti-idiotype approach is that these antibodies also act as functional mimics of tumour antigens: they can be taken up and presented by APC in a class I- or class II-restricted manner, potentially inducing T-cell responses.

One of the best-characterised anti-idiotypic antibody vaccines is CeaVac®. This was produced by immunising mice with a monoclonal
antibody that recognises a CEA epitope. Anti-CEA antibody responses, as well as T-cell responses have been demonstrated in colorectal cancer patients in a phase II trial of CeaVac® in the adjuvant setting. Phase III trials are on-going. Anti-idiotypic antibody approaches have also been studied against Ep-Cam and other tumour antigens expressed on colorectal carcinoma.

‘Tumour escape’: an obstacle to immunotherapy?

Multiple mechanisms by which tumour cells can escape the immune system have been identified. One mechanism is the development of ‘antigen-loss variants’, cells that no longer express tumour antigen, through down-regulation, mutation, or loss of genes. Loss of HLA expression has been shown to be relatively common in colorectal carcinomas. It can occur via a variety of independent mechanisms: loss or mutation of β₂-microglobulin genes, loss of HLA heavy chain genes, selective lack of expression of HLA alleles, and regulatory defects in HLA expression. Furthermore, tumour cells can express molecules or secrete cytokines that potentially inhibit immune cells. Ep-Cam has recently been shown to be a ligand for LAIR-1 (human leukocyte-associated immunoglobulin-like receptor) which is expressed on many cells of the immune system. Ligation of the LAIR-1 receptor results in the inhibition of cell-mediated cytotoxicity. It is, therefore, possible that Ep-Cam expression on tumours can down-regulate anti-tumour immune responses.

Ways of circumventing these hurdles include treating in the adjuvant setting when disease burden is low, using poly-epitope strategies to minimise the out-growth of antigen-loss variant clones, combining immunotherapeutic strategies, and combining with chemotherapy.

Future directions

Several other aspects of anti-tumour immune responses particularly relevant to colorectal carcinoma are the focus of current interest and research.

CD4⁺CD25⁺ regulatory T-cells

As discussed previously, the mechanisms that maintain immunological tolerance to self may also impede immune responses against autologous tumour cells. The mechanisms of maintenance of self-tolerance include deletion in thymus, ‘anergy’ in the periphery, and ‘ignorance’ of self-antigens. Self-reactive T-cells may also be inhibited by regulatory T-cells.
such as the CD4⁺CD25⁺ T-cell population. The elimination of CD4⁺CD25⁺ T-cells in murine models has been shown to allow the development of anti-tumour immune responses mediated by tumour-specific T-lymphocytes and NK-cells. These cells have also been shown to regulate intestinal inflammation. The transfer of regulatory T-cells into mice with inflammatory bowel disease has been shown to prevent the development of pathological changes in the bowel\textsuperscript{71}. There is, therefore, interest in manipulating regulatory T-cells for the treatment of autoimmune diseases. Conversely, their deletion could potentially augment anti-tumour vaccine therapies, autoimmunity being an obvious potential hazard.

*NK receptors and anti-tumour activity*

Over the last decade, there has been a great expansion in our understanding of the surface receptors and molecular mechanisms involved in NK-cell function. NK-cell receptors are divided into two structural families: immunoglobulin-like receptors and C-lectin-like molecules. The immunoglobulin-like receptor family consists of killer immunoglobulin-like receptors (KIRs) and leukocyte immunoglobulin-like receptors (LIRs) also called immunoglobulin-like transcripts (ILTs). These receptors recognise some HLA class I alleles and produce either stimulatory or inhibitory signals, depending on the sequence of their intracellular domain. C-type lectin-like receptors are heterodimers consisting of an invariant chain CD94 and an NKG2 chain, which provides an inhibitory (NKG2A) or an activatory (NKG2C, NKG2E) signal to the cell. CD94/NKG2 molecules recognise specifically the non-classical HLA class I molecule HLA-E, loaded with peptide derived from the signal sequence of the classical HLA class I molecules expressed by the target cell. Recognition and lysis of target cells by NK-cells is the result of a balance of activating and inhibitory signals delivered through the NK-cell receptors. More recently antigen-specific T-cells have also been shown to express inhibitory NK-cell receptors.

It has been shown that an NK-cell infiltrate is a good prognostic indicator in patients with colorectal carcinoma\textsuperscript{72}. The expression of ligands for the activating receptors, together with the high frequency of loss of expression of HLA class I molecules on colorectal carcinoma cells, potentially results in a susceptibility to NK-cell lysis.

Recent experiments have shown a cross-talk between NK-cells and DCs\textsuperscript{73,74}, opening a new area of investigation of the interplay between adaptive and innate immunity. DCs as immunotherapy may, therefore, be manipulated to produce both antigen-specific T-cell and NK-cell responses.
**NKG2D/MICA**

NKG2D is an activating receptor expressed on NK-cells and on most CD8^+ αβ T-cells and γδ T-cells\(^75\). NKG2D binds MIC-A and MIC-B which are human non-classical class I molecules. MIC molecules are stress-inducible and are expressed in many epithelial tissues (such as the gastrointestinal epithelium) on malignant transformation\(^76\). It has been shown that Vδ1 T-cells, present at high frequencies in intestinal tumours, specifically recognise MIC-expressing tumour cells, in an NKG2D- and TCR-dependent manner. Conversely, NK-cell recognition of MIC-positive targets is solely dependent on NKG2D and over-rides any inhibitory signal delivered at the same time\(^77\). In addition, NKG2D exerts a co-stimulatory role for antigen-specific CD8^+ cells when the density of HLA/peptide complexes on target cells is reduced\(^78\), a situation that often occurs in tumour cells.

Other ligands for NKG2D include the human ULBP molecules and their distant mouse homologues, Rae1 and H60. In mouse models, it has been recently shown that Rae1 and H60 are potent stimulators of anti-tumour responses\(^79\)–\(^81\). There is, therefore, an increasing interest in understanding the role of NKG2D ligands expressed upon malignant transformation, in eliciting innate and adaptive anti-tumour immune responses, with the aim of designing novel immunotherapeutic strategies.

**CD1-restricted NKT-cells**

As discussed above, T-cells usually recognise protein antigens in the form of peptides bound to HLA molecules. Nevertheless, a particular subset of T-cells (NKT-cells) are capable of recognising lipid ‘epitopes’ on the surface of target cells. These lipid antigens are presented by the non-MHC encoded β₂-microglobulin-associated CD1d molecule. It has been shown in mice that NKT-cells are able to modulate the immune response by either preventing autoimmune diseases or promoting tumour rejection\(^82\). The key effector molecules involved in NKT-mediated tumour rejection are IL-12, IFN-γ and, in some models, perforin\(^83\). Classical NKT-cells have a constant TCR, common to all individuals. We have, however, recently reported the existence of a CD1d-specific response mediated by the more usual adaptive T-lymphocytes\(^84\). CD1d is expressed on gastrointestinal epithelium\(^85\); therefore, it is of great interest to investigate lipid-specific T-cell responses, both classical NKT and adaptive T-cell, in the context of colorectal tumours.
References

5. Michael-Robinson JM, Biemer-Huttmann A, Purdie DM et al. Tumour infiltrating lymphocytes and apoptosis are independent features in colorectal cancer stratified according to microsatellite instability status. Gut 2001; 48: 360–6
15. Wagener C, Petzold P, Kohler W, Totovic V. Binding of five monoclonal anti-CEA antibodies with different epitope specificities to various carcinoma tissues. Int J Cancer 1984; 33: 469–75
Immunotherapy of colorectal cancer


Gjertsen MK, Bjorheim J, Saeterdal I, Myklebust J, Gaudernack G. Cytotoxic CD4+ and CD8+ T-lymphocytes, generated by mutant p21-ras (12Val) peptide vaccination of a patient, recognize 12Val-dependent nested epitopes present within the vaccine peptide and kill autologous tumour cells carrying this mutation. *Int J Cancer* 1997; 72: 784–90


Hamilton JM, Chen AP, Nguyen B et al. Phase I study of recombinant vaccinia virus (rV) that expresses human carcinoembryonic antigen (CEA) in adult patients with adenocarcinomas [abstract]. *Proc ASCO* 1994; 961


Sadana N, Nagashima H, Mashino K et al. Dendritic cell vaccination with MAGE peptide is a novel therapeutic approach for gastrointestinal carcinomas. *Clin Cancer Res* 2001; 7: 2277–84


80 Diefenbach A, Jensen E, Jameson A, Raulet D. Rae1 and H60 ligands of the NKG2D receptor stimulate tumor immunity. Nature 2001; 413: 165–71