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INTRODUCTION

The Ludwig Institute for Cancer Research consists currently of 10 Branches that have been established at academic centers in Europe (Switzerland, Belgium, United Kingdom and Sweden), Australia, South America (Brazil) and North America (United States). Each Branch is focused on a program of research defined by the Branch Director in relation to the overall objectives of the Institute.

The Lausanne Branch, which was established in 1975, is involved in research in basic immunology and in immunology applied to cancer. The basic research program is focused on elucidating in molecular terms some of the key events that occur during an immune response, with current emphasis being on the regulation of T and Natural killer (NK) cell development, the generation of major histocompatibility complex (MHC) class I-presented peptides, the dynamics of T cell receptor-ligand interactions, the dynamics of cytolytic T lymphocyte (CTL) responses and the dissection of immunological interplays between virus and host. The patient oriented research program is aimed at applying the most recent progress in basic immunology to the development of cancer vaccines, with a special focus on the design of peptide-based vaccines that can trigger efficient tumor-specific CTL responses in patients with malignant melanoma or, in the future, other types of solid tumors.

The Branch is presently divided into five research groups, designated Developmental Immunology, Viral Immunology, Molecular Immunology, Molecular Tumor Immunology and Clinical Tumor Immunology. The first four groups share laboratories located at the Center for Biomedical Research in Epalinges and have close links with the Department of

Biochemistry (University of Lausanne), the Swiss Institute for Experimental Cancer Research (ISREC), and the Lausanne unit of the Swiss Institute for Bioinformatics. The fifth group has laboratories in close proximity to the Lausanne University Hospital (CHUV). The latter location allows direct access of our clinical investigators to the cancer clinic operated by the Multidisciplinary Oncology Center (CEPO), thus facilitating greatly the conduct of phase I clinical trials of cancer vaccines. Thus, through its close interactions with both research and clinical environments, the Branch is in a unique position to translate into clinical investigations and applications novel approaches emanating from recent progress in fundamental immunology.

Highlights of our research activities in the year 2000 are summarized below.

J.-C. CEROTTINI

RESEARCH REPORT

DEVELOPMENTAL IMMUNOLOGY GROUP

Lymphocytes, like myeloid and erythroid cells, are derived from a pluripotential hematopoietic stem cell. The three main lymphoid lineages (B cells, T cells and NK cells) are believed to be derived from a common lymphoid precursor (CLP). Whereas B cells and NK cells are produced primarily in the bone marrow, T cell development occurs almost exclusively in the thymus. Relatively little is known in molecular terms about the processes that determine commitment of a common precursor to the B, T or NK cell lineages. The developmental immunology group is particularly interested in the development and lineage commitment of T and NK cells. In addition we are studying an unusual lymphoid subset known as NKT cells, which combine properties of both the NK and T cell lineages.

Role of Notch-1 in T cell development and lineage commitment

During their differentiation from a CLP in the bone marrow T cells are confronted with 3 distinct cell fate specification events. First a CLP must decide whether to adopt a T or B cell fate. Once the T cell lineage is specified, pre-T cells in the thymus must choose between the T cell receptor (TCR) $\alpha\beta$ and $\gamma\delta$ lineages. Finally $\alpha\beta$ lineage-committed progenitor cells must differentiate into either CD4 (helper) or CD8 (cytolytic) mature T cells.

In addition to the role played by TCR and co-receptors, other inductive signals are believed to be critical for lineage commitment during T cell development. One signaling molecule that has received a great deal of attention in this respect is Notch-1, which has been reported to be involved in T/B, $\alpha\beta/\gamma\delta$ and CD4/CD8 lineage choices. Nevertheless these conclusions have

been based on overexpression of a dominant active form of Notch1 in transgenic mice and thymoma cell lines and hence need to be confirmed by loss-of-function approaches. Such studies have not been possible until recently since conventional gene targeting of Notch-1 results in early embryonic lethality.

In last years' report we described the use of Cre^{-loxP} targeting strategy to develop conditional knockout mice in which the Notch-1 gene can be inactivated in an inducible or tissue-specific fashion. By crossing these mice to transgenic mice expressing the Cre recombinase under the control of an interferon- α responsive Mx promoter, we were able to efficiently delete the Notch-1 gene in hematopoietic precursors in the adult bone marrow. Reconstitution of lethally irradiated hosts with genetically marked Notch-1^{-/-} bone marrow led to the generation of all hematopoietic lineages with the notable exception of T cells. Surprisingly, Notch-1-deficient precursors gave rise to immature B cells in the thymus rather than T cells, suggesting that Notch-1 plays an inductive role in directing bipotential T/B progenitors toward a T cell fate.

In order to investigate the possible role of Notch-1 at later stages of T cell development we crossed the conditional knockout mice with a transgenic mouse expressing the Cre recombinase under the control of a CD4 minigene (including promoter, enhancer and silencer elements). In these mice the Notch-1 gene was deleted in pre-T cells, prior to the expression of CD4 or CD8. Surprisingly the absence of Notch-1 at this early developmental stage had no effect on the subsequent lineage commitment, maturation or survival of CD4⁺ and CD8⁺ mature T cells. Thus in contrast to conclusions reached in gain-of-function studies we believe that Notch-1 plays no essential role in CD4/CD8 lineage commitment.

Regulation of T and NK cells by inhibitory receptors

The correct regulation of NK cell mediated effector functions requires a balance between activating and inhibitory signals. Specialized receptors that belong to the Ly49 family in the mouse prevent NK cell reactions upon the engagement of MHC ligands on target cells. The loss of MHC class I from target cells, which can occur upon transformation or infection, relieves NK cells from inhibition and consequently allows target cell lysis.

We are interested in elucidating the importance of inhibitory MHC receptors for the development, specificity and function of murine NK cells. These receptors are differentially distributed in developing NK cell clones thereby generating a diverse NK cell receptor repertoire. Following or perhaps superimposed on Ly49 receptor acquisition, NK cells adapt to the MHC class I environment in which they arise. We have obtained the first evidence that the expression of an inhibitory receptor endows NK cells with a significant developmental advantage over NK cells which lack this receptor. It seems as if inhibitory Ly49 receptors can also play a positive role and provide differentiation and/or maturation signals to developing NK cells.

The transduction of inhibitory signals in mature NK cells involves protein tyrosine phosphatases such as SHP-1. When this pathway was interrupted by over-expressing a catalytically inactive, dominant-negative form of SHP-1, inhibition via MHC receptors was impaired. However, these NK cells did not become auto-aggressive, which was explained by an inefficient activation. A reduction of inhibitory signals may thus be compensated by the concomitant down-modulation of NK cell triggering pathways.

Activating NK cell receptors and pathways are still poorly defined. However, since some T cells also express inhibitory MHC receptors, we have begun to investigate Ly49 receptor function in the context of the TCR as a well-defined activation receptor. Surprisingly, mouse T cells expressing a transgenic Ly49 receptor were unable to mount a significant antigen-specific CD8 T cell response to a well-defined tumor antigen *in vivo*. Consequently tumor rejection did not occur. This model system will enable us to further study the interplay between activating and inhibitory pathways in the regulation of T and NK cell function.

Development and function of NKT cells

NKT cells are an unusual subset of lymphoid cells expressing both an $\alpha\beta$ TCR and markers common to NK cells (including NK1.1 and members of the Ly49 inhibitory receptor family). Most mouse NKT cells express a semi-invariant TCR ($V\alpha 14/V\beta 8.2$) that is specific for glycolipid antigens presented by the monomorphic MHC class I-like molecule CD1d. The developmental origin of NKT cells, as well as their function under physiological conditions, remain poorly characterized.

Since NKT cells are normally present at very low frequency it is difficult to study their development and function. In an attempt to overcome this problem, we have generated transgenic mice expressing $V\alpha 24$ (the human homologue of mouse $V\alpha 14$) under the control of the T cell-specific CD2 minigene promoter. Human $V\alpha 24$ (rather than mouse $V\alpha 14$) was chosen for this purpose since monoclonal antibodies are available to the former (but not the latter) and *in vitro* studies have indicated that human $V\alpha 24^+$ NKT cells recognize mouse CD1d. As expected $V\alpha 24^+$ NKT cells

developed in a CD1d-dependent fashion in these transgenic mice. Somewhat surprisingly, however, the number of NKT cells was not increased in comparison with littermate controls. Whether this reflects a lower avidity of V α 24 (as compared to V α 14) for mouse CD1d or an independent homeostatic control of NKT cell development remains to be investigated.

The physiological role of NKT cells is still controversial, although they have been implicated in control of autoimmunity as well as in protection against parasites and tumors. To investigate the immediate consequences of NKT cell activation *in vivo*, we have injected mice with α galactosyl ceramide (α Gal Cer), a CD1d-binding synthetic glycolipid that interacts with the semi-invariant TCR. Interestingly NKT cell activation by α Gal Cer led to a rapid and selective induction of NK cell proliferation and cytotoxicity, particularly in the liver and spleen. Bystander proliferation of T cells also occurred after NKT cell activation, but was restricted solely to cells with a memory (CD44 high) phenotype. Further analysis of bystander NK and memory T cell activation using mutant mice and neutralizing antibodies demonstrated a dependence upon interferon- γ production by NKT cells and IL-12 secretion by antigen-presenting cells. These studies suggest that CD1d-dependent NKT cells regulate immune responses indirectly by sampling blood-borne glycolipid antigens and rapidly activating NK cells and memory T cells.

**VIRAL
IMMUNOLOGY
GROUP**

The viral immunology group investigates interactions between viruses and the immune system. We use viruses and antigens as tools for a better understanding of the immune response occurring in the draining lymph node with special emphasis on T cell priming, T cell-B cell collaboration, germinal center formation, memory and effector cell differentiation, as well as the analysis of the fate of the interacting lymphocytes.

Immune responses induced with mouse mammary tumor virus (MMTV), vaccinia viruses, adenovirus as well as classical hapten-carrier conjugates are being used. MMTV-infected B cells express a superantigen on the cell surface. Superantigen presentation by dendritic cells and B cells leads to a classical immune response where dendritic cells prime superantigen-specific T cells and infected B cells receive cognate help via their expressed superantigen. Since superantigen-presenting B cells carry a retroviral integrated DNA marker and are not eliminated during the immune response, the fate of B cells that have interacted in the draining lymph node can be followed for prolonged times in the different compartments of the body.

Role of chemokines and integrins in plasmablast migration

Plasmablasts and plasma cells accumulate at the peak of the extrafollicular immune response in the medullary cords of the draining lymph node. We have shown previously that T cells and B cells having mutually interacted in the same lymph node environment acquire different migration patterns. The first wave of B cell emigration from the draining lymph node occurred around days 5-9 and consisted of virus-infected plasmablasts and plasma cells which had lost most chemokine receptors and/or chemokine responsiveness. CXCR4 was still expressed at the cell surface but did not induce migration towards its ligand.

Nevertheless these cells were able to immigrate into different peripheral tissues such as the mammary gland, skin, liver or lung. They expressed an activated form of $\alpha 4\beta 1$ integrin on the cell surface. Indeed, antibodies recognizing only the activated form of $\beta 1$ integrin blocked immigration into peripheral nonlymphoid tissues such as the mammary gland. It remains to

be determined whether other chemokine receptors are required for this migration, whether chemokine receptor responsiveness is re-induced in these cells upon migration or contact with endothelia expressing VCAM-1 or whether expression of the activated $\alpha 4\beta 1$ integrin alone is sufficient for cell entry into peripheral nonlymphoid organs.

Role of the draining lymph node in chronic virus neutralization

Using MMTV as a model system we observed a crucial role for the draining lymph node in inducing a long-lasting virus-neutralizing antibody response. Upon lymph node removal at different time points after virus injection this neutralizing activity was quickly lost and only slowly and partially recovered. The removal of the draining lymph node did result in normal viral spread and infection of lymphoid and nonlymphoid organs. As described in last year's report, secretory organs such as the salivary gland, the mammary gland and the pancreas were much better infected in the absence of the neutralizing immune response whereas other organs did not show this increase. Mammary carcinoma development is due to retroviral integration and activation of nearby proto-oncogenes. Therefore we speculated that lack of virus neutralization might lead to faster mammary tumor development. We observed that after lymph node removal reduced virus neutralization led to strongly accelerated cancer development. On the contrary induction of stronger neutralization resulted in lack of viral spread to the above cited organs, lack of tumor development and interruption of the viral life cycle.

Role of neutralizing antibody in chronic MMTV

We generated a monoclonal rat IgG antibody that is highly efficient in MMTV neutralization. Systemic injection of mice with this antibody completely blocked the superantigen response and ensuing deletion of peripheral T cells. Surprisingly, despite the complete block of the superantigen response, antibody injection did not interfere with MMTV infection. Two days after virus injection similar levels of infection were observed in B cells. The superantigen-induced amplification of the infection, however, was blocked. We are currently investigating whether MMTV is taken up in a distinct compartment of B cells which does not allow superantigen presentation.

Role of B cells and dendritic cells in antigen priming in vivo

After superantigen challenge a significant proportion of superantigen reactive T cells remains undivided. In collaboration with H.R. MacDonald, we obtained evidence that the lymphoid environment limits T cell proliferation in the secondary lymphoid organs when the frequency of superantigen- or antigen-reactive T cells is unusually high. We developed a new method that allows precise determination of the percentage of antigen-reactive cells that enter into cell cycle and measurement of the number of cycles these cells undergo. This method is independent of cell death.

We monitored T cell proliferation and the percentage of undivided cells when the frequency of antigen-reactive T cells was low (1%), intermediate (15%) or high (30%-100%) by transferring fluorescently labeled cells into different recipients.

When the frequency was low practically all the reactive T cells entered cell cycle and proliferated maximally. At intermediate frequencies a large proportion of reactive T cells did not enter cell cycle and the whole population divided less. A further increase in reactive T cells did not alter the percentage of undivided cells but induced a further decrease in the number of cell divisions. Interestingly, the observations made with superantigens were confirmed with peptide antigen and T cell receptor transgenic mice. Moreover in vivo and in vitro data suggested that dendritic cells were the most likely candidates in limiting T cell proliferation in the lymphoid environment. The availability of APC in the lymphoid environment can therefore quantitatively limit T cell priming.

**MOLECULAR
IMMUNOLOGY
GROUP**

The main theme of work of this group concerns the analysis of the molecular interactions that elicit or inhibit activation of CD8⁺ CTL by defined MHC-peptide complexes. We mainly use as experimental model system the previously described K^d-restricted CD8⁺ CTL clones that are specific for a photoreactive PbCS peptide derivative (PbCS(ABA)). This system allows direct assessment of TCR-ligand interactions by TCR photoaffinity labeling.

CTL activation by defined soluble MHC-peptide complexes

To elucidate the minimal requirements for CTL activation, we constructed various well defined K^d-PbCS(ABA) complexes and evaluated their ability to bind to and activate cloned CTL. We found that the activation requirements were very different, depending upon whether the cells were in suspension or adherent to surfaces.

For activation of CTL in suspension, soluble K^d-PbCS(ABA) complexes needed to be tetrameric or larger and to co-engage TCR and CD8. Monomeric and dimeric complexes were unable to activate CTL and activation was limited to transient intracellular calcium mobilization and up-regulation of activation markers such as CD69 and CD25.

Tetrameric complexes in which K^d-PbCS(ABA) monomers were coupled to streptavidin via spacers of 18 to 80 Å bound to CTL with the same efficiency and blocking of CD8 gave the same reduction of binding. This is consistent with the observation that on cells TCR and CD8 form adducts that bind MHC-peptides one unit (see below). Remarkably, the ability of the tetramers to activate CTL decreased with the spacer length. The tetramers with the 80 Å spacer were over six-fold less effective in activating CTL as compared to the tetramers with the 18 Å spacer. Moreover, CTL activation was abolished by blocking of CD8 or by Src kinase inhibitors and the kinase activity of Lck in lipid rafts correlated with intracellular calcium mobilization. These findings indicate that activation of CTL in suspension is essentially driven by cross-linking induced activation of CD8-associated Lck in rafts, where TCR signaling is induced.

By contrast, surface adherent cells were fully activated, including release of esterases and cytokines, by monomeric MHC-peptide complexes. Adhesion of CTL induced phosphorylation of high molecular weight molecules and translocation of TCR/CD3 to lipid rafts, from which phosphatases were excluded.

Inhibition of CTL activation by prolonged or permanent TCR engagement by MHC-peptide complexes

We have previously observed that peptide variants that exhibit slow TCR-ligand complex dissociation are recognized by CD8⁺

CTL less efficiently than the wild type peptide. To substantiate this finding, we studied T cell hybridomas that express CD8 and mutants of the N30 TCR, which recognizes the VSV peptide RGYVYQGL in the context of K^b. The TCR mutants were all localized in the CDR3 β loop, which previous studies showed to be particularly critical for antigen recognition in this system.

The genes for wild type and variant N30 TCR were transfected in the T cell hybridomas. The ability of the transfectants to recognize the VSV peptide was assessed by using target cells pulsed with graded concentrations of VSV peptide. Several CDR3 β mutations reduced antigen-induced IL2 production by the transfectants to various degrees. Measurement of TCR-ligand complex dissociation by fluorescent labeled K^b-VSV tetramers revealed several cases where antigen recognition was inefficient and TCR-ligand complex dissociation remarkably slow. This inefficient antigen recognition was improved when the VSV was presented in a less efficient mode, such as on target cells expressing a K^b mutant that binds the N30 TCR less well or plate bound K^b. Similar results were obtained with PbCS(ABA)-specific CTL clones.

These results indicate that efficient activation of CD8⁺ T cells is limited to a specific stability range (dwell time) for the TCR/MHC-peptide interaction and that excessively long half-lives for the TCR/MHC-peptide interaction inhibit T cell activation. Currently ongoing studies indicate that this effect results from an active inhibition and is caused by the recruitment of a phosphatase to hyperphosphorylated LAT, an adaptor protein that is involved in the early steps of T cell activation. Indeed, dephosphorylation of LAT prevents activation of down-stream signaling cascades.

Molecular basis of CD8 coreceptor function

Our previous studies have shown that homodimeric CD8 α/α is a much less effective coreceptor than heterodimeric CD8 α/β and that on cells CD8 α/β , but not CD8 α/α , substantially increases TCR-ligand binding. To further investigate the basis of this difference, we prepared soluble CD8 α/β , soluble T1 TCR and soluble K^d-PbCS(ABA) and assessed their interactions by plasmon surface resonance. We found that K^d-PbCS(ABA) bound T1 TCR and CD8 α/β with dissociation constants of 10 and 75 μ M, respectively. The results clearly demonstrated that the two bimolecular reactions were independent, i.e. that CD8 α/β had no effect of the interaction of T1 TCR with K^d-PbCS(ABA). Essentially the same findings were obtained when soluble CD8 α/α was used.

To investigate how CD8 β endows CD8 with superior coreceptor function, we examined T cell hybridomas expressing the T1 TCR, CD8 α and various CD8 β variants. These studies revealed that the cytoplasmic portion of CD8 β , in part because it is palmitoylated, enables CD8 to partition in lipid rafts and that this is essential for efficient association of CD8 with lck, which is also palmitoylated and raft-associated. The importance of these findings is underscored by the fact that the initiation of CTL activation is driven by cross-linking induced activation of lck in rafts as mentioned above. Moreover our studies showed that both cytoplasmic and transmembrane portions of CD8 β mediate association of CD8 with TCR/CD3. Formation of this adduct is constitutive and only marginally strengthened by MHC-peptide monomers, which explains the high avidity of ligand binding observed previously on CD8⁺ T cells.

Interestingly adduct formation and hence high avidity ligand binding is lost when the connecting peptide and transmembrane sequences of the TCR α chain are replaced by the corresponding sequences of the TCR δ chain. As this variant TCR has been shown not to associate with CD3 δ , our results suggest that TCR-CD8 adduct formation involves CD3 δ . These findings are in line with the demonstration that positive selection and activation of CD8⁺ T cells is dramatically impaired in mice expressing either this mutant TCR α chain or tailless CD8 β , thus suggesting that coupling of the TCR with the CD8 coreceptor is crucial for efficient TCR signaling.

**MOLECULAR
TUMOR
IMMUNOLOGY
GROUP**

CTL-defined peptide tumor antigens, like most antigenic peptides, are the product of intracellular degradation. Although the general features of antigenic peptide production have been described, a detailed analysis of peptide antigen processing in tumor cells is lacking. Our group has focused its effort in identifying and characterizing the factors affecting the production of peptide tumor antigens as well as the intracellular localization of their precursor polypeptides. The clinical relevance of these studies is highlighted by the finding that several tumor-associated proteins may be differentially processed depending on their intracellular localization and on the tumor environment.

Factors affecting the production of CTL-defined peptide tumor antigens

One of the crucial elements in the specific recognition of tumor cells by CTL are peptide tumor antigens presented by HLA class I molecules at the cell surface. The central protease implicated in the production of peptide tumor antigens is the proteasome, present in the cytosol and nucleus of eukaryotic cells. The

proteasome is composed of at least 28 subunits and we recently identified a chaperone specifically required for its assembly and maturation.

In mammalian cells, two main populations of proteasome have been identified: the standard proteasome, expressed constitutively in most cell types, and the immunoproteasome, expressed in dendritic cells and cells treated with IFN- γ . The immunoproteasome distinguishes itself from the standard proteasome by the presence of three different catalytic subunits, resulting in an altered cleavage specificity. Until recently, the presence of immunoproteasomes was assumed to result in a more efficient production of antigenic peptides. However, this notion was challenged by our results, obtained in collaboration with the groups of B. Van den Eynde of the Brussels Branch and of J. E. Gairin (CNRS, Toulouse), which demonstrated that some peptide tumor antigens are not efficiently produced by immunoproteasomes *in vitro*. The implications of these findings for tumor immunity *in vivo* remain to be analyzed.

Prediction of putative HLA class I ligands within a tumor-associated protein currently relies on the identification of appropriate pairs of amino acids, termed anchor residues, which can form direct contacts with the HLA class I molecule. Unfortunately, this method appears not to be sufficient to accurately predict whether a particular peptide will be presented by HLA class I molecules at the surface of tumor cells. We and others have shown that several CTL-defined peptide tumor antigens, originally identified by the presence of appropriate anchor residues, are either not generated or not presented by tumor cells. Closer analysis of the processing of one of these peptide tumor antigens (MAGE-3₂₇₁₋₂₇₉/HLA-A2) allowed us to demonstrate that proximal amino acids play an important role

in determining whether particular CTL-defined epitopes can be produced by the proteasome.

Whereas certain peptide tumor antigens are directly produced by the proteasome, other carry N-terminal extensions. Several aminopeptidases have been postulated to trim longer precursors to the size fitting the groove of HLA class I molecules. We are in the process of characterizing two aminopeptidases, which are required for the final trimming of a particular antigenic peptide.

Biochemical characterization of melanoma-associated antigens

The molecular identification of CTL-defined peptide melanoma antigens by functional screening of cDNA libraries has led to the discovery of gene families such as MAGE, GAGE, and NY-ESO-1. The relationship between expression of these gene products and the process of tumorigenesis is not fully understood, and their physiological role is yet to be determined. We have undertaken the study of the cellular localization of MAGE-A proteins using previously derived antibodies that cross-react with most members of this family. While most of the MAGE-A proteins expressed by melanoma cells are predominantly localized in the cytoplasm, MAGE-A10 is located in the nucleus. We have identified a short sequence at the amino-terminus as the nuclear localization signal for this protein. Moreover, we have also shown that the expression of MAGE-A10, under the control of an inducible promoter, does not affect cell proliferation or apoptosis mediated by death receptors.

CTL responses to the peptide antigen derived from Melan-A are extensively investigated at our Branch and elsewhere. However, little is known about the subcellular localization and function of the protein Melan-A. Because its expression is restricted to melanocytes and melanoma, Melan-A has been assigned to the

category of melanocyte differentiation antigens even though it bears no sequence homology to other well characterized melanocyte-differentiation antigens, such as tyrosinase, tyrosinase related proteins 1 and 2, and Pmel-17/gp100. Moreover, results of our study aimed at defining the subcellular localization of Melan-A by immunofluorescence and electron microscopy (in collaboration with J. Davoust, Paris, and A. de Maziere, Univ. of Utrecht) demonstrated that, unlike the other melanocyte differentiation antigens (which are predominantly localized in the melanosomes) Melan-A appears to localize, in pigmented melanoma cells, in or near the Golgi complex. This particular localization suggests a possible involvement of Melan-A in the formation of melanosomes.

We are also investigating the relationship between the intracellular localization of melanocyte-differentiation antigens and their processing and presentation by HLA class I molecules. Using the integral membrane protein Melan-A as a model, we were able to show that melanoma cells expressing a mislocalized form of Melan-A are much more efficiently recognized by specific CTL. This enhanced recognition correlates with the amount of antigenic peptide produced by the intracellular proteolytic machinery. Whether this applies to other melanocyte-differentiation antigens remains to be demonstrated.

**CLINICAL
TUMOR
IMMUNOLOGY
GROUP**

It is now well established that cancer patients may develop tumor specific T- and B-cell responses. Various types of human tumor cells often express multiple CTL-defined tumor antigens that are shared among tumors, providing the rationale for generic vaccines applicable to large subsets of cancer patients. The patient oriented research program carried out by the clinical

tumor immunology group is focused on understanding the generation and maintenance of spontaneous or induced CTL responses to tumor associated antigens and applying this knowledge to the development of new strategies in cancer immunotherapy.

Monitoring of spontaneous tumor-specific CTL responses

Whereas many of the CTL-defined antigens have been identified using CTL clones derived from cancer patients, information concerning the magnitude of CTL responses to these antigens in cancer patients is still lacking. We have used recently developed techniques to measure the frequency of antigen-specific MHC-class I restricted T lymphocytes in *ex vivo* (non-cultured) samples of peripheral blood mononuclear cells. These include (i) a functional assay based on the release of the cytokine IFN- γ upon a short stimulation with the antigenic peptide and (ii) a flow cytometry-based method that allows the direct enumeration of single antigen-specific T lymphocytes with fluorescent tetramers. In last year's annual report we summarized the findings on monitoring Melan-A reactive T lymphocytes in different immune compartments including peripheral blood, metastatic and normal lymph nodes as well as tumor infiltrating lymphocyte populations. We have now extended these studies to the assessment of quantitative and functional HLA-A2 restricted CTL responses to cancer/testis antigens, such as NY-ESO-1, CAMEL and MAGE-A10 tumor antigens. Our results clearly indicate that more than 50% of melanoma patients have detectable CTL responses to these tumor antigens. Of note, the Melan-A CTL response is unusually strong so that antigen-specific CD8⁺ T cells are detectable *ex vivo* with fluorescent tetramers in a high proportion of melanoma patients.

Monitoring of micrometastases in melanoma sentinel lymph nodes

The sentinel lymph node (SLN) is the first lymph node draining a given tumor site. Analysis of SLNs allow the early identification of melanoma patients bearing micrometastases and thus at high risk of disease progression. In the past few years we have assembled a multidisciplinary team to implement lymph node mapping for patients with primary melanoma. Whereas microscopy-based techniques still represent the gold standard for micrometastasis detection in SLNs, molecular assays may increase the sensitivity of tumor cell detection. We have undertaken a prospective study to directly compare immunopathological and molecular methods for the detection of micrometastases in SLNs from patients with ≥ 1 mm primary melanomas. A detailed parallel analysis by microscopy (H&E and immunohistochemistry for HMB-45/gp100, S-100, tyrosinase and Melan-A/MART-1) was performed in 71 SLNs from 57 patients. While concordant results were obtained in the majority of samples, approximately a third of the SLNs were positive by PCR only. A significant number of the latter represented false-positives because of capsular nevi. Nevertheless, on clinical follow up three regional recurrences occurred in patients with a SLN positive by PCR only, suggesting that even sensitive immunochemistry techniques may underestimate the presence of micrometastases. Molecular analysis therefore appears an undoubtedly more sensitive and promising method. However, it needs further improvement in order to attain acceptable specificity before it can be applied diagnostically. From an immunology standpoint, the SLN offers the opportunity to monitor spontaneous tumor specific T cell responses in the lymphoid organs draining the tumor site at a very early stage of tumor progression.

Mouse models for preclinical testing of cancer vaccines

Whereas clinical trials are required to definitively test the safety and efficacy of cancer vaccines, variables such as peptide dose, regimen of immunization, route and immunological adjuvants ought to be rapidly determined. To gather preclinical data on optimal vaccination schedules or novel adjuvants, we have set up mouse models. Two strains of transgenic mice are used. Both strains express a transgene encoding a chimeric HLA-A2/H-2^b molecule specifying the α -1 and α -2 domains of HLA-A2 and the α -3, transmembrane and cytoplasmic domains of the mouse H-2^b class I molecule. The former domains contain the peptide binding site that is recognized by the TCR and the latter the domain interacting with the mouse CD8 coreceptor. We have shown that these mouse strains can respond to vaccination with synthetic peptides corresponding to Melan-A and NY-ESO-1 tumor antigens.

Using these mouse models we have documented the effect of two new adjuvants that can efficiently enhance the induction of specific CTL by peptide-based vaccines. One of these is a bacterial outer membrane protein from *Klebsiella pneumoniae*. The second adjuvant is CpG containing oligodeoxynucleotides (CpG-ODN). Bacterial DNA contains unmethylated CpG dinucleotides that can interact with TLR-9 receptors in macrophages and dendritic cells leading to their activation and release of inflammatory cytokines. Synthetic CpG-ODNs with stabilized backbones have been identified that incorporate CpG motifs and activate B, NK and dendritic cells as well as macrophages. We have found that vaccination of mice with peptide mixed with CpG-ODNs leads to the induction of strong CTL responses that are superior in magnitude to those induced by peptides emulsified in incomplete Freund's adjuvant.

These findings suggest the potential of these bacterial molecules as immunological adjuvants. We intend to test them in future phase I clinical trials of peptide vaccination in cancer patients.

Monitoring of vaccine-induced tumor-specific CTL responses

A primary focus of our research program is on the optimization of techniques to monitor molecular and cellular events occurring during tumor-specific immune responses. We are conducting phase I clinical trials of cancer vaccines in collaboration with the Multidisciplinary Oncology Center at the Lausanne University Hospital, with participation of the Brussels branch and the Oncology Division of the Geneva University Hospital. HLA-A2 patients are treated with Melan-A and influenza peptides together with adjuvants given intramuscularly or subcutaneously at monthly intervals. The immune response to vaccination is being monitored by *ex vivo* analysis of peptide specific CD8⁺ T lymphocytes, using fluorescent HLA-A2 tetramer/peptide complexes and IFN- γ ELISPOT assays.

We have included 34 patients in the clinical trial LUD-96-010 (January 2001). Administration of peptide alone caused no significant side effects. The immunological adjuvant SB AS-2 containing the saponin QS21 and detoxified lipid A caused no or only grade 1-2 systemic side effects, and mild to moderate local pain resolving within a few days. Five out of 19 patients evaluated thus far were tumor free, of which 4 remained so during the study (observation period: 11 - 22 months). The other 14 patients were tumor bearing at study entry. Eleven of them showed unaltered progressive disease, whilst two had stable disease during a remarkably long period (19 and 23 months) and one patient had a minor clinical response with regressing metastases in skin, lymph nodes and lung. We found significant

expansion of Melan-A specific CD8⁺ T lymphocytes in 3 patients. In addition, we observed that vaccination induced a shift towards an activated phenotype among these cells that was accompanied by an increased ability to release specifically IFN- γ but not IL-4.

In a search for optimal vaccination strategies, we have developed a novel approach to assess vaccine efficiency (clinical trial LUD 98-009). Melanoma patients were injected twice with two different antigenic peptides (derived from Melan-A and influenza matrix protein) and an adjuvant. Two to 4 weeks later, the lymph node draining the site of vaccination (which was identified by applying recent techniques of lymph node mapping by injection of Evans patent blue and lymphoscintigraphy) was removed and analyzed for antigen-specific CD8⁺ T cell responses. Remarkably, in all three patients tested thus far, we found increased frequencies of activated antigen-specific lymphocytes, thus allowing detailed characterization of the vaccine induced CD8⁺ T cell responses. In contrast to the draining lymph node, no or only weak T lymphocyte activation was observed in peripheral blood.

Our program aims at translating results from the laboratory bench into experimental immune therapy in carefully planned phase I clinical trials. In turn, the results from clinically oriented research continue to yield new insights concerning the dynamics of tumor antigen-specific T cell responses in humans. We very much hope that this constant interplay between patients, clinicians and basic science will ultimately provide solid foundations for effective cancer vaccine development.