Early in vivo signaling profiles in MUC1-specific CD4+ T cells responding to two different MUC1-targeting vaccines in two different microenvironments

Dawn K. Reichenbach and Olivera J. Finn*

University of Pittsburgh School of Medicine; Department of Immunology; Pittsburgh, PA USA

Vaccines are beginning to be explored for as measures to prevent cancer. Since determining the efficacy of vaccines by evaluating disease outcome requires a long time, there is an urgent need for early predictive biomarkers. To this end, immunological endpoints that can be assessed weeks or months post-vaccination are currently being evaluated. However, when multiple vaccines are available, waiting for the development of humoral and cellular immunity could still cause delays, whereas early assessments would allow for a timely shift to more effective prevention modalities. Applying the phospho-flow technique to primary T cells, we examined the phosphorylation status of various proteins that shape the activation, proliferation, and differentiation of mucin 1 (MUC1)-specific CD4+ T cells within the first 24 hours post-immunization. It is known that a vaccine composed of a MUC1-derived peptide loaded on dendritic cells is more effective in eliciting T-cell responses than a vaccine including the same peptide plus an adjuvant. Both these vaccines stimulate T cells more effectively in wild-type (WT) than in MUC1-transgenic mice. We examined if the signaling events downstream of the TCR or linked to various proliferative and survival pathways, monitored in two different hosts as early as 3, 6, 12 and 24 hours post-immunization, could predict the differential potential of these two MUC1-targeting vaccines. The signaling signatures that we obtained primarily reflect differences between the vaccines rather than between the hosts. We demonstrate the feasibility of using a phospho-flow-based approach to evaluate the potential of a given vaccine to elicit a desired immune response.

Introduction

Immunotherapy is gaining recognition not only as an important improvement to standard radio- and chemotherapeutic approaches against cancer but also as an effective form of anti-cancer monotherapy.[1] Vaccines are one form of immunotherapy that could provide benefits not only to advanced cancer patients, by boosting anticancer immune responses, but also to individuals who are at high risk for developing cancer, by eliciting immunological protection. Over the last three decades, great advances have been made in the characterization of immune responses in cancer patients and of the types of immunity that are required to control various tumors. In addition, numerous tumor-associated antigens recognized by tumor-specific T cells have been used to develop and test anticancer vaccines. Preclinical animal models, in particular genetically engineered mice, have been very useful in testing the immunogenicity and efficacy of anticancer vaccines. Several studies have demonstrated that, for being effective, a vaccine needs to elicit a vigorous effector T-cell response and a robust memory response. In turn, the ability of a vaccine to elicit these responses depends on the choice of tumor-associated antigen(s), the choice of adjuvant(s), and on status of the patient's immune system. The majority of well-characterized tumor-associated antigens[2] are closely related to self antigens and may be subjected to various degrees of self tolerance. The choice of adjuvant(s) and antigen-delivery systems (e.g., loaded on dendritic cells, DCs, coded by viral vectors, conjugated to DC-targeting antibodies) is a critical determinant of both the strength and the type of immune response elicited by anticancer vaccines. These and other variables eventually determine the efficacy of a vaccine, which moreover can vary in different patients.

The efficacy of anticancer vaccines can be assessed by two outcomes: (1) immunogenicity, measured as the production of new antigen-specific antibodies and T cells several weeks after vaccination, and (2) tumor control, which can be measured weeks after vaccination in mouse models but only months and years after vaccination in patients. Evaluating the efficacy of preventive anticancer vaccines would be even more delayed. According to preclinical and clinical studies, immunogenicity and tumor control are tightly correlated, that is, the more robust the antibody and T-cell responses induced by the vaccine are, the better long-term tumor control. The goal of our work was to evaluate in vivo a technique that has been successfully used to measure activation of T cells in vitro, in order to determine if an early T-cell activation signature can be obtained in primary T cells and might be developed as a predictive biomarker of vaccine efficacy.

CD4^{+} T cells play a central role in determining the intensity and quality of CD8^{+} cytotoxic T lymphocyte (CTL), antibody, and memory responses. In addition, CD4^{+} T cells participate in the activation and recruitment of innate effector cells to the tumor site.[3–5] Therefore, the ability of a vaccine to activate CD4^{+} T cells could be an important biomarker of its efficacy.

Mucin 1 (MUC1) is an O-linked glycosylated transmembrane protein normally expressed on the apical surface of ductal epithelial cells, but aberrantly expressed in a broad spectrum of adenocarcinomas. Upon malignant transformation, MUC1 loses polarity and becomes overexpressed and hypoglycosylated, revealing an immunogenic region of tandem repeats of 20 residues. Weak T-cell and antibody responses to MUC1 have been observed in adenocarcinoma patients, prompting the development of anticancer vaccines to boost this response.

We have previously shown that an anticancer vaccine based on DCs loaded with a MUC1-derived peptide elicits more effective antitumor responses than the same peptide combined with poly-inosinic-polycytidylic acid stabilized with polylysine and carboxymethylcellulose (poly:ICLC).[6] Furthermore, we have shown that MUC1-specific T cells proliferate less and produce less interferon γ (IFNγ) in response to both these vaccines in MUC1-transgenic mice than in their wild-type (WT) counterparts.[7,8] These findings provided a relevant model for investigating the potential of early biomarkers that are associated with different disease outcomes following the administration of anticancer vaccines.

The development of flow cytometry-based assays, a technique known as phospho-flow, has allowed for the activation of complex signaling networks to be precisely characterized within single cells.[9–12] Phospho-flow has already been used to investigate signal transducer and activator of transcription (STAT) activation in T cells from late stage cancer patients,[13] shifts in the signaling potential of leukocytes from acute myeloid leukemia patients,[14] and deficiencies of STAT signaling in monocytes from HIV-infected individuals,[15] as well as in attempts to predict the clinical responsiveness of patients affected by rheumatoid arthritis.[16] Here, we use the phospho-flow technique for the first time on tumor antigen-specific primary T cells to evaluate differences in the quality (which pathways are activated and with which kinetics) and quantity (strength of activation) of immune responses as elicited by two different MUC1-targeting vaccines in two different mouse strains. We hypothesized that the differences in the immunogenicity and efficacy of these vaccines in different hosts, as measured weeks and months post-vaccination, might be predicted by the state of CD4^{+} T-cell activation immediately after vaccination. We show that this is indeed the case and that the phosphorylation profile of MUC1-specific TCR-transgenic CD4^{+} T cells measured 3, 6, 12 and 24 hours post-vaccination differs in animals receiving soluble MUC1-derived peptides plus adjuvant or MUC-derived peptide-loaded DCs.

We propose that other vaccines can be similarly compared and their immunogenicity characterized, with the most robust T-cell activation signature indicating the best candidate(s) for further development.

Results

Kinetics of TCR signaling in MUC1-transgenic and WT mice in response to two different MUC1-targeting vaccines. We adoptively transferred MUC1-specific VFT T cells and control OT-II T cells into WT or MUC1-transgenic mice and immunized mice with either DCs loaded with a MUC1-derived peptide or the MUC1-derived peptide admixed with the adjuvant poly:ICLC. We then collected splenic T cells from 3 mice per group at each time point. Three hours after the administration of MUC1-derived peptide-loaded DCs, MUC1-specific T cells displayed high levels of (transient) Zap70 phosphorylation in MUC1-transgenic mice but not in WT mice. Six hours post-vaccination, the phosphorylation of Zap70 in MUC1-transgenic mice was still detectable but was much less intense, resembling that observed in WT mice (**Fig. 1A**). The percentage of MUC1-specific T cells manifesting Zap70 phosphorylation was relatively low (~2%) in both strains. In contrast, the phosphorylation of the inactive conformation of Lck was weak but measurable at all time points, and could be detected in a large percentage of T cells (**Fig. 1B**). In the case of the peptide vaccine, MUC1-specific T cells from WT and MUC1-transgenic mice had equivalent levels of phosphorylated Zap70 at 3 hours and a high number of activated T cells at early time points. As with the DC-based vaccine, Lck phosphorylation was measurable at 6 hours (**Fig. 1A**) and in a large number of T cells in both WT and MUC1-transgenic mice (**Fig. 1B**). The fact that these vaccines elicited similar signaling downstream the TCR suggests that their differential efficacy is not due to differences in the first steps of antigen recognition.

Activation of cell cycle-related and pro-survival pathways following MUC1-targeting vaccines in WT and MUC1-transgenic mice. The phosphorylation of ERK1/2 has been shown to be critical for T-cell activation and proliferation in response to alloantigens in vivo[17] and was hence of interest for MUC1-specific responses. We investigated MUC1-specific T cells responding to two MUC1-targeting vaccines in WT and MUC1-transgenic mice for differences in the phosphorylation status of ERK1/2 (laying downstream of TCR signaling in the RAS/RAF signaling pathway) and of two other tyrosine kinases, p38[MAPK] and Akt, both of which are involved in cell survival and proliferation. Both Akt and p38[MAPK] have also been shown to play
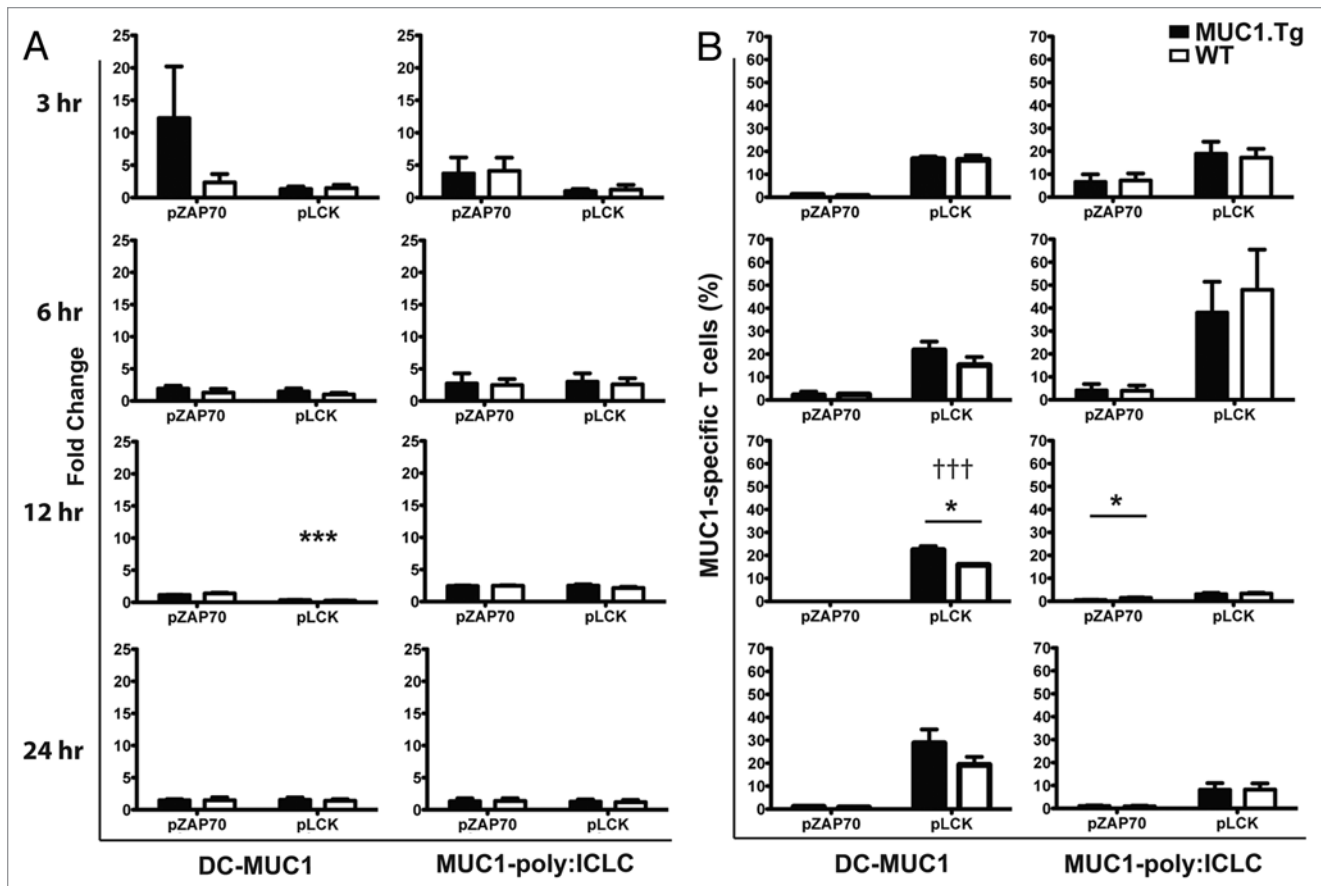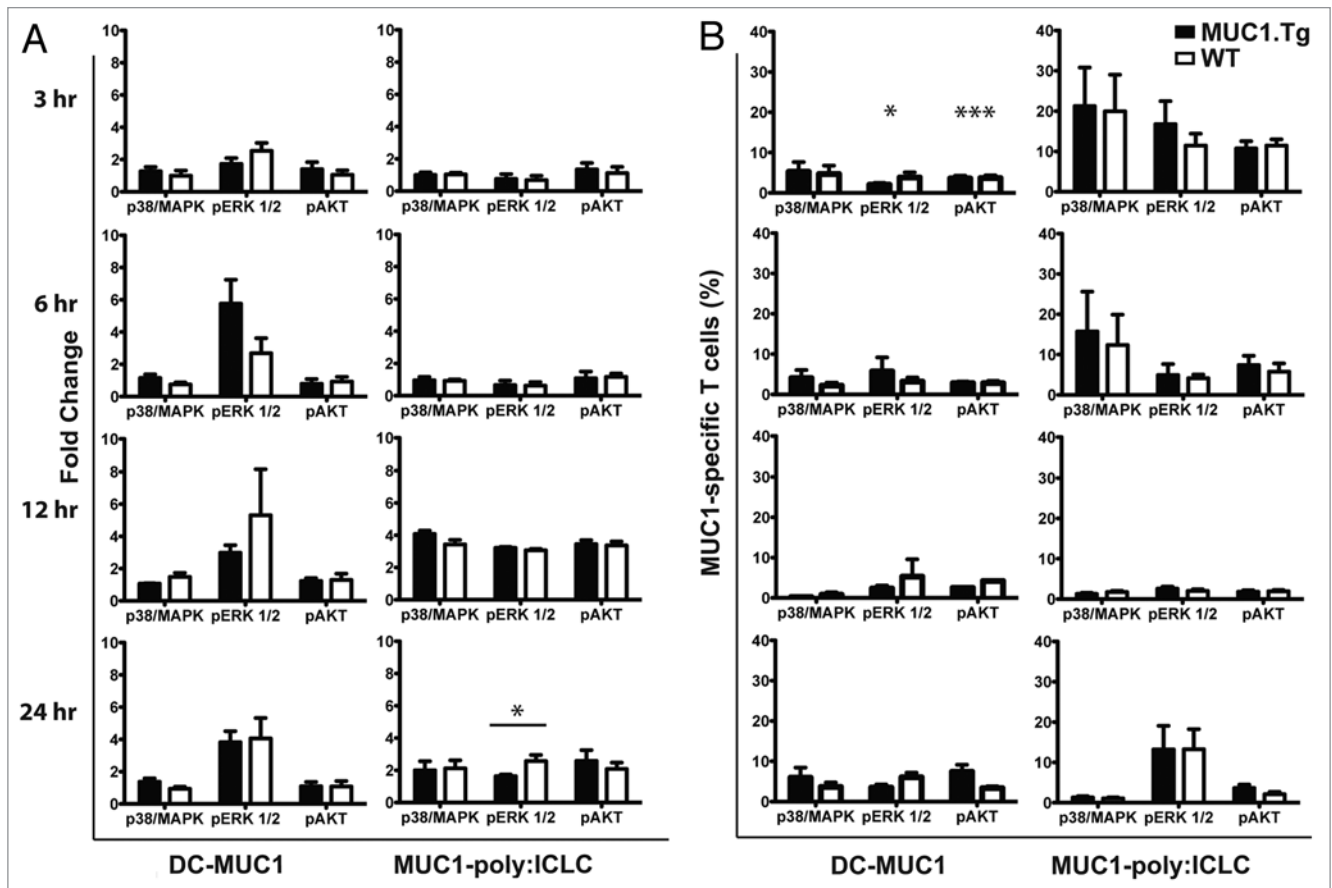
Figure 1. TCR-elicited signaling. (**A**) The levels of phosphorylated Zap70 and Lck were examined in MUC1-transgenic or wild-type (WT) mice 3, 6, 12 and 24 h following the administration of either MUC1-derived peptide-loaded dendritic cells (DC-MUC1, n = 6 per strain per time point, except 12 h, when n = 3 per strain per time point) or a MUC1-derived peptide combined with polyinosinic-polycytidylic acid stabilized with polylysine and carboxymethylcellulose (MUC1-poly:ICLC, n = 6 per strain per time point, except 6 h, when n = 5 per strain per time point). ***$p < 0.001$ (unpaired Student's t-test), as compared with mice receiving the peptide-based vaccine. (**B**) The percentage of THY1.1+ cells manifesting the phosphorylation of Zap70 and Lck in response to vaccination was determined at the indicated time points. *$p < 0.05$; †††$p < 0.001$ (unpaired Student's t-test), as compared with mice receiving the peptide-based vaccine.

a role in T-cell differentiation and function,[18,19] and their activation is known to rely on co-stimulatory signaling.[20,21]

We found differences in the kinetics of ERK1/2 phosphorylation in MUC1-transgenic mice as compared with WT mice following the administration of MUC1-derived peptide-loaded DCs. In MUC1-transgenic mice, six hours post-vaccination, we observed a small percentage of MUC1-specific T cells experiencing a peak in ERK1/2 phosphorylation that quickly decreased by 12 hours (**Fig. 2A and B**). The signal phopsho-ERK1/2 peaked in WT mice only 12 hours post-vaccination. The activation of p38[MAPK] and Akt in response to the DC-based vaccine was similar. MUC1-transgenic and WT mice exhibited low levels of p38[MAPK] and Akt phosphorylation in a small percentage of MUC1-specific T cells. The phosphorylation of ERK1/2, p38[MAPK] and Akt in response to the peptide-based vaccine was minimal but equivalent in MUC1-transgenic and WT mice. Although the activation of cell cycle-related and pro-survival signaling pathways in MUC1-transgenic and WT mice following the administration of the peptide-based vaccine had a similar trend in terms of intensity and duration, there was an initial

increase in the subpopulation of MUC1-specific T cells manifesting p38[MAPK] and ERK1/2 phosphorylation that could not be observed in response to MUC1-derived peptide-loaded DCs (**Fig. 2B**). In summary, MUC1-specific T cells responding to DC-based and peptide-based MUC1-targeting vaccines differ in terms of ERK1/2, but not p38[MAPK] and Akt, phosphorylation.

Activation of T$_H$1-associated signaling molecules in response to MUC1-targeting vaccines in WT and MUC1-transgenic mice. We have previously demonstrated that MUC1-derived peptide-loaded DCs elicit -specific T$_H$1 responses in both MUC1-transgenic and WT mice, although a decrease in IFNγ production by MUC1-specific cells occurs in MUC1-transgenic mice as compared with their WT counterparts.[8] Expecting this to be linked to differences in the percentage of cells or the degree of phosphorylation of T$_H$1-associated signaling molecules,[7] we examined the phosphorylation status of T$_H$1-associated signaling molecules operating downstream of the IFNγ receptor and the interleukin (IL)-12 receptor (i.e., STAT1 and STAT4, respectively), as well as the expression of the transcription factor Tbet. MUC1-specific T cells displayed a much more intense phosphorylation

Figure 2. Activation of cell cycle-associated and pro-survival signaling pathways. (**A**) The phosphorylation status of signaling molecules in the mitogen-activated protein kinase (MAPK), RAS/RAF, and phosphoinositide-3-kinase (PI3K) pathways (p38^{MAPK}, ERK1/2 and Akt, respectively) was examined in MUC1-transgenic or wild-type (WT) mice 3, 6, 12 and 24 h following the administration of either MUC1-derived peptide-loaded dendritic cells (DC-MUC1, n = 6 per strain per time point, except 12 h, when n = 3 per strain per time point) or a MUC1-derived peptide combined with polyinosinic-polycytidylic acid stabilized with polylysine and carboxymethylcellulose (MUC1-poly:ICLC, n = 6 per strain per time point, except 6 h, when n = 5 per strain per time point). *p < 0.05; (unpaired Student's t-test), as compared with mice receiving the peptide-based vaccine. (**B**) The percentage of MUC1-specific cells manifesting the phosphorylation of p38^{MAPK}, ERK1/2, and Akt in response to vaccination was determined at the indicated time points. *p < 0.05; ***p < 0.001 (unpaired Student's t-test), as compared with mice receiving the peptide-based vaccine.

of STAT1 than of STAT4, in both MUC1-transgenic and WT mice, 3 and 6 hours post-vaccination (**Fig. 3A**). Although STATs were equivalently activated in both groups of mice responding to the peptide-based vaccine, MUC1-specific T cells from MUC1-transgenic mice collected 3 hours after the administration of the DC-based vaccine exhibited a more robust and more widespread activation of STAT1 than MUC1-specific T cells obtained from WT mice (**Fig. 3A and B**). In addition, from 6 to 24 hours post-immunization, the percentage of MUC1-specific T cells exhibiting phosphorylated STAT1 was consistently higher in mice receiving MUC1-derived peptide-loaded DCs than in their counterparts treated with the peptide-based vaccine (**Fig. 3B**). Thus MUC1-derived peptide-loaded DCs activate a more robust T$_H$1 signaling than MUC1-derived peptides combined with poly:ICLC, in both WT and MUC1-transgenic mice.

T$_H$2 and T$_H$17-associated signaling responses to MUC1-targeting vaccines in MUC1-specific T cells. We also examined the phosphorylation status of STAT6 and STAT3, in order to potentially predict if our vaccines might be activating T$_H$2 or

T$_H$17 cells, which are not routinely assessed in response to anti-cancer vaccines. The activation of STAT3 promotes indeed T$_H$17 differentiation,[22,23] while T$_H$2 responses require STAT6 and STAT3 signaling.[24] Following the administration of both DC- and peptide-based vaccines, MUC1-specific T cells from MUC1-transgenic and WT mice contained an equivalent percentage of cells exhibiting STAT6 and STAT3 phosphorylation, developing with similar kinetics. In general, the DC-based vaccine elicited robust STAT3 signaling in > 50% of MUC1-specific T cells in the first 24 hours (**Fig. 4A and B**), with little to no signs of STAT6 activation. Twenty-four hours after vaccination, the STAT3 phosphorylation was more intense in MUC1-transgenic mice than in their WT counterparts. Conversely, the peptide-based vaccine induced greater levels of STAT6 phosphorylation than of STAT3 phosphorylation at all time points (**Fig. 4A**). Although we observed an initially larger percentage of cells exhibiting STAT6 phosphorylation, as time progressed the population of MUC1-specific T cells bearing activated STAT6 decreased to barely detectable levels, while the population of MUC1-specific
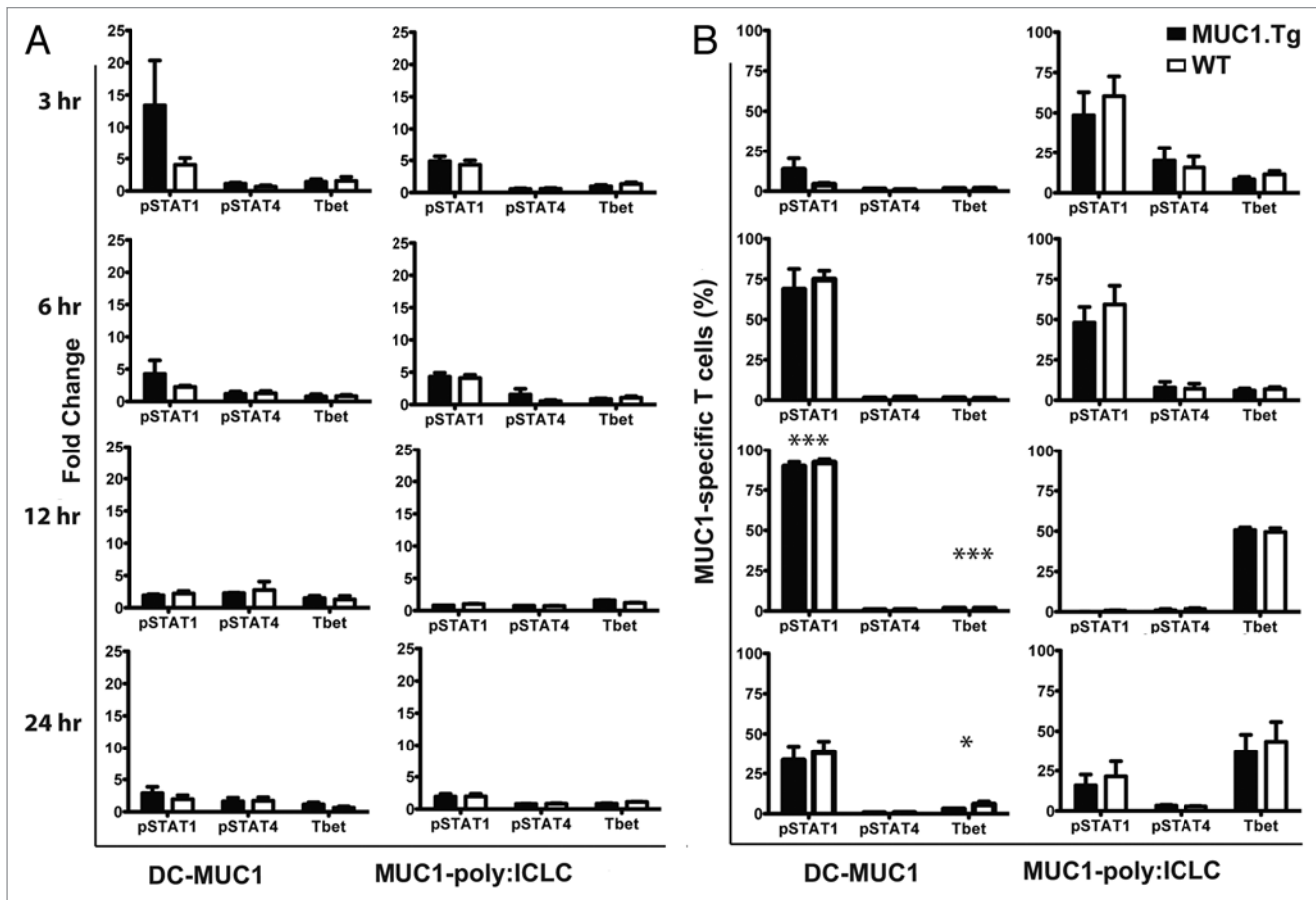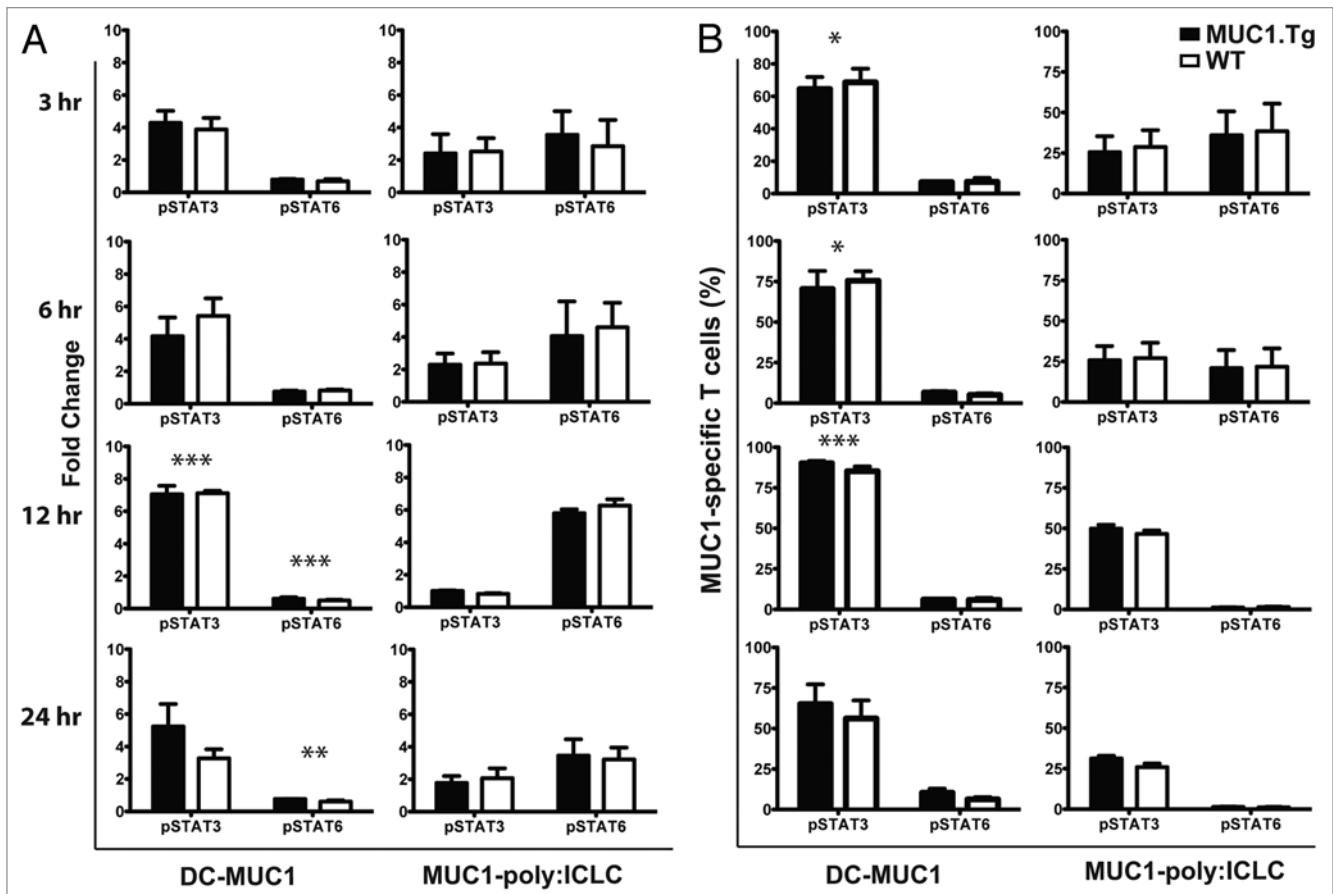
Figure 3. $T_H 1$-associated signaling. (**A**) The phosphorylation of signal transducer and activator of transcription 1 (STAT1) and STAT4, as well as the expression levels of the transcription factor Tbet were examined in MUC1-transgenic or wild-type (WT) mice 3, 6, 12 and 24 h following the administration of either MUC1-derived peptide-loaded dendritic cells (DC-MUC1, n = 6 per strain per time point, except 12 h, when n = 3 per strain per time point) or a MUC1-derived peptide combined with polyinosinic-polycytidylic acid stabilized with polylysine and carboxymethylcellulose (MUC1-poly:ICLC, n = 6 per strain per time point, except 6 h, when n = 5 per strain per time point). (**B**) The percentage of MUC1-specific cells manifesting the phosphorylation of STAT1 and STAT4 or expressing Tbet above baseline in response to vaccination was determined at the indicated time points. *p < 0.05; ***p < 0.001 (unpaired Student's t-test), as compared with mice receiving the peptide-based vaccine.

T cells exhibiting STAT3 phosphorylation remained constantly elevated (**Fig. 4B**).

Fewer MUC1-specific T cells show S6 activation in response to MUC1-derived peptide-loaded DCs than following the administration of MUC1-derived peptides plus poly:ICLC. We have previously demonstrated that regulatory T cells (Treg) affect MUC1-specific CD4+ T cell responses in MUC1-transgenic mice.[7] To assess if the activation of Tregs can be used to predict the efficacy of different vaccines in different hosts, we examined the phosphorylation status of the S6 kinase (operating downstream of the Akt/mTOR pathway), which inversely correlates with Treg development,[25] and STAT5 (the main transducer of IL-2-driven signals) as well as the expression of the Treg-associated transcription factor FOXP3. These markers did not differ in T cells obtained from vaccinated MUC1-transgenic and WT mice, with the exception of FOXP3 expression, being higher in MUC1-transgenic mice 6 h after the administration of MUC1-derived peptide-loaded DCs (**Fig. 5A**). There was a difference, however, between the two vaccines. Indeed,

MUC1-derived peptides combined with poly:ICLC elicited a much greater percentage of cells manifesting S6 phosphorylation and significantly fewer cells exhibiting STAT5 phosphorylation than MUC1-derived peptide-loaded DCs (**Fig. 5B**).

Differences in signaling profiles as assessed a few hours post-vaccination correlates with the composition of the T-cell response. We analyzed cell surface activation markers on VFT T cells obtained from WT or MUC1-transgenic mice vaccinated with MUC1-derived peptide-loaded DCs or MUC1-derived peptides combined with poly:ICLC, whose signaling events were determined above. We found that a large percentage of these cells expressed CD25 for the first 12 hours post DC-based immunization, regardless of their origin (MUC1-transgenic vs. WT mice). By 24 hours, however, the percentage of cells expressing CD25 dropped significantly in WT mice. Conversely, CD69 expression was low for the first 6 hours post-vaccination, peaked at 12 hours and was more prominent on cells from WT mice than on cells from MUC1-transgenic animals (**Fig. 6A**).

Figure 4. T_H2 and T_H17 signaling. (**A**) The phosphorylation of signal transducer and activator of transcription 3 (STAT3) and STAT6, as well as the expression levels of the transcription factor Tbet were examined in MUC1-transgenic or wild-type (WT) mice 3, 6, 12 and 24 h following the administration of either MUC1-derived peptide-loaded dendritic cells (DC-MUC1, n = 6 per strain per time point, except 12 h, when n = 3 per strain per time point) or a MUC1-derived peptide combined with polyinosinic-polycytidylic acid stabilized with polylysine and carboxymethylcellulose (MUC1-poly:ICLC, n = 6 per strain per time point, except 6 h, when n = 5 per strain per time point). **p < 0.01; ***p < 0.001 (unpaired Student's t-test), as compared with mice receiving the peptide-based vaccine. (**B**) The percent of MUC1-specific cells manifesting the phosphorylation of STAT3 and STAT6 or expressing Tbet above baseline levels in response to vaccination was determined at the indicated time points. *p < 0.05; ***p < 0.001 (unpaired Student's t-test), as compared with mice receiving the peptide-based vaccine.

Since S6 inversely correlates with the development of Tregs, we determined the percentage of FOXP3$^+$CD4$^+$ T cells in MUC1-transgenic and WT mice following a secondary boost with either the DC- or the peptide-based vaccine. Approximately 3% of CD4$^+$ T cells were FOXP3$^+$ in MUC1-transgenic and WT mice receiving MUC1-derived peptides combined with poly:ICLC. In contrast, MUC1-derived peptide-loaded DCs were more effective at stimulating the development of Tregs, yielding twice as many FOXP3$^+$ T cells (**Fig. 6B**).

A quantitative data set representing the early activation of signaling pathways that are important for the development of anti-cancer immune response allowed us to compile one activation fingerprint. **Figure** 7 represents signaling signatures of MUC1-specific CD4$^+$ T cells responding to MUC1-targeting vaccines in MUC1-transgenic mice, where MUC1 is a self antigen, and in WT mice, where MUC1 is a foreign antigen. This is an example of the potential of this approach, which will need to be further validated by comparing other vaccines and relating phopho-flow data with disease outcome.

Discussion

The phospho-flow technique has proven reliable and reproducible in vitro.[14] In addition, phospho-flow has been successfully used in vivo, in models of stimulation in which a bolus of adjuvant[26] or a soluble antigen that can directly bind to the cleft of MHC molecules were administrated,[27] but many hurdles still preclude its use for antigen-specific events. The time it takes for antigens to be processed and for the TCR:MHC synapse between DCs and T cells to form in the spleen or lymph nodes can vary to considerable extents for different vaccines and in different hosts. Furthermore, only a small fraction of cells is being activated at a given moment, manifesting transient phosphorylation events, implying that the signal is easily diluted, especially at very early time points. In spite of these theoretical hurdles, we demonstrate that the phosphorylation signatures of MUC1-specific T cells reacting to MUC1-derived peptides or MUC1-derived peptide-loaded DCs can be compared, allowing for the identification of differences in the strength and quality of signaling events
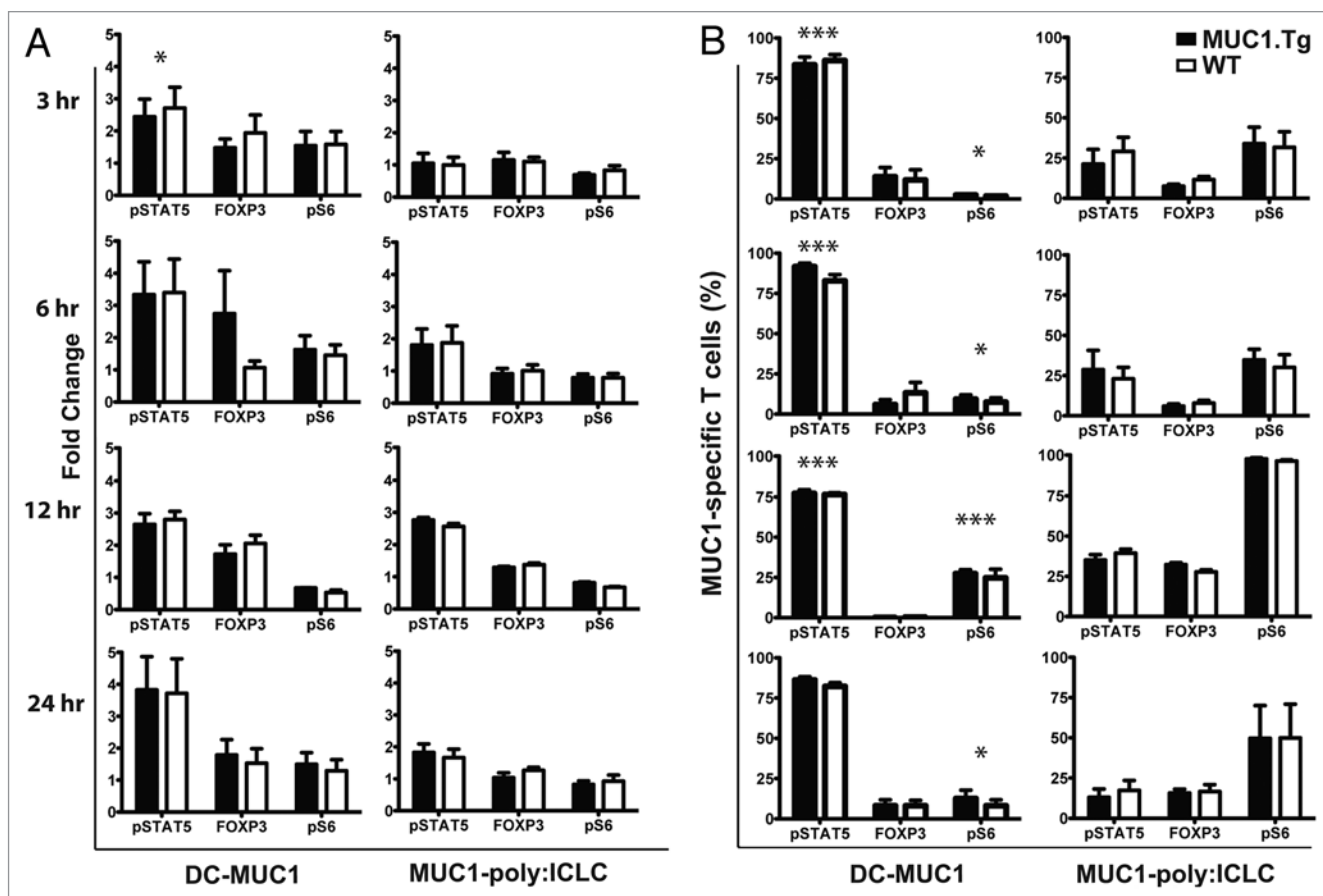
Figure 5. Signaling through regulatory T cells-associated pathways. (**A**) The expression levels of FOXP3 and phosphorylation status of signal transducer and activator of transcription 5 (STAT5) and S6 examined in MUC1-transgenic or wild-type (WT) mice 3, 6, 12 and 24 h following the administration of either MUC1-derived peptide-loaded dendritic cells (DC-MUC1, n = 6 per strain per time point, except 12 h, when n = 3 per strain per time point) or a MUC1-derived peptide combined with polyinosinic-polycytidylic acid stabilized with polylysine and carboxymethylcellulose (MUC1-poly:ICLC, n = 6 per strain per time point, except 6 h, when n = 5 per strain per time point). *$p < 0.05$ (unpaired Student's t-test), as compared with mice receiving the peptide-based vaccine. (**B**) The percent of MUC1-specific cells manifesting the phosphorylation of STAT5 and S6 or expressing FOXP3 above baseline levels in response to vaccination was determined at the indicated time points. *$p < 0.05$; ***$p < 0.001$ (unpaired Student's t-test), as compared with mice receiving the peptide-based vaccine.

associated with T$_H$1, T$_H$2, T$_H$17 and Treg responses as well as with cell survival and proliferation.

In future studies, in order to obtain an even more precise vaccine-induced T-cell activation signature, it may be necessary to expand the panel of signaling molecules that are assessed. Many of the signaling molecules that we evaluated have numerous downstream targets and multiple upstream modifiers. This could hinder the interpretation of the activation and differentiation of the T-cell response and hence affect the reliability of the prediction of efficacy. For instance, an increased phosphorylation of Akt could be indicative of cell growth, proliferation, or autophagy inhibition, while decreased Akt activation might suggest cell cycle blockade as well as the activation of immune functions, as it has been shown for the immunosuppressive activity of Tregs.[28] Signal transducers may have various upstream activators. For instance, the IL-6 receptor, the IL-21 receptor and CD40L all signal through STAT3. The role of STAT3 signaling in the differentiation of T helper subsets has been well defined. However, STAT3 has also been shown to play a role in the development

and maintenance of immunological memory.[29] In addition, the synthesis of IL-17 is dependent on downstream transducers of MAPK signaling and—in CD4$^+$ T cells—it requires the activation of p38^{MAPK}.[30] Increases in the phosphorylation of MAPKs in response to anticancer vaccines may therefore be indicative of increased cell survival or a skewing toward a T$_H$17 response. These instances of crosstalk demonstrate the importance of understanding the context and cell type in which signaling pathways are activated in order to predict later effects on the immune response.

All caveats acknowledged, we were able to demonstrate differences in the kinetics of activation of signaling pathways and in the intensity of specific phosphorylation events that depended on whether a foreign (MUC1 in WT mice) or abnormal self (MUC1 in MUC1-transgenic mice) antigen was being recognized by the immune system. These responses changed with the design of the vaccine (peptide-loaded DCs or peptides combined with an adjuvant), generating unique signaling signatures. This suggests that—with additional optimization and proper validation—the signaling fingerprint developing within a few days
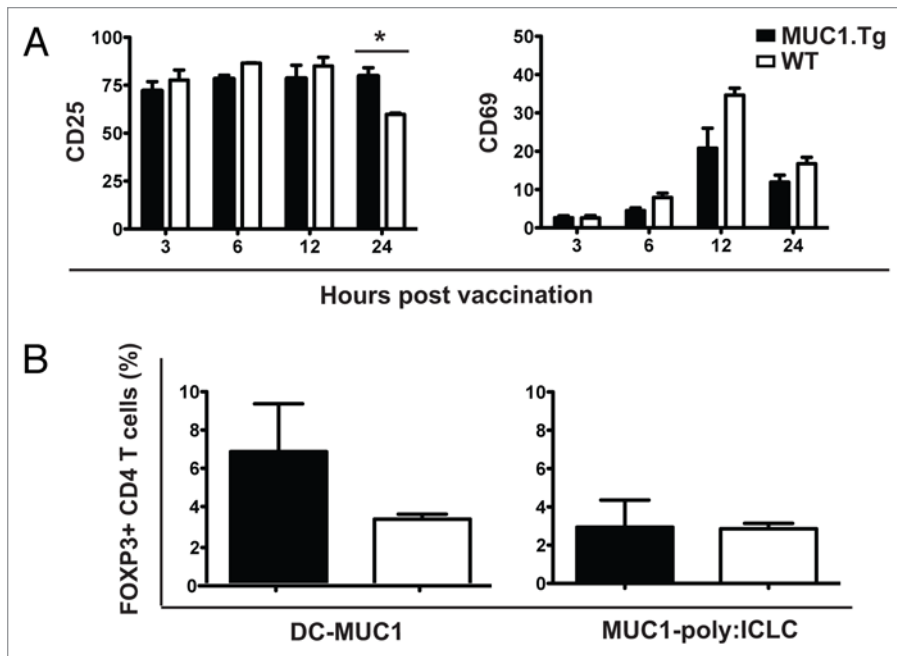
Figure 6. Small differences in signaling yield differences in T-cell outcome. (**A**) The expression of the cell surface activation markers CD25 and CD69 was examined on MUC1-specific T cells from MUC1-transgenic or wild-type (WT) mice (n = 3 per strain per time point) 3, 6, 12 and 24 h following the administration of either MUC1-derived peptide-loaded dendritic cells (DC-MUC1) or a MUC1-derived peptide combined with polyinosinic-polycytidylic acid stabilized with polylysine and carboxymethylcellulose (MUC1-poly:ICLC). *p = 0.05 (unpaired Student's t-test), as compared to WT mice. (**B**) MUC1-transgenic and WT mice (n = 3 per strain) were vaccinated as indicated in panel (**A**) and boosted twice at four-week intervals. The endogenous CD4$^+$ T cell populations were analyzed 24 h after the secondary boost for FOXP3 expression levels.

post-vaccination can serve as a biomarker of vaccine efficacy. Moreover, this approach can represent a valuable strategy to rapidly assess the influence of vaccine design, including changes in the active components, adjuvants and delivery vehicle.

Materials and Methods

Mice. C57BL/6 (WT) and C57BL/6-Tg (TcraTcrb)42SCbn/J (OT-II TCR-transgenic) mice were purchased from The Jackson Laboratory, MUC1-transgenic mice were originally obtained from Dr. S. Gendler (Mayo Clinic), and VFT TCR-transgenic mice were generated at the University of Pittsburgh transgenic mouse facility, as previously described.[8] All mice were housed in a specific pathogen-free environment. All breeding and experimental procedures were approved by and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Generation of bone marrow dendritic cells (BMDCs). BMDCs were generated as previously described.[31] In brief, bone marrow cells were harvested from the tibia and femur of WT mice. Following red blood cell lysis, bone marrow cells were plated at 1.5×10^6 cells/mL in AIM-V media (Invitrogen) containing sodium pyruvate, 2-mercaptoethanol, non-essential amino acids, and supplemented with 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; Miltenyi Biotec). On day 3,

half the medium was replaced with fresh AIM-V and 20 ng/mL GM-CSF. On day 6, BMDCs were harvested, counted, and loaded with MUC1-derived peptides overnight in the presence of 50 μg/mL poly:ICLC (Hiltonol) (Oncovir, Inc.). The 100-mer MUC1-derived peptide represents 5 repeats of the 20 residue sequence HGV TSA PDT RPA PGS TAP PA found in the VNTR region of MUC1. The synthesis and quality control of this peptide was performed at the University of Pittsburgh Genomics and Proteomics Core Laboratories.[7]

Adoptive T-cell transfers. Spleens were harvested from VFT and OT-II TCR transgenic mice, mechanically processed into single-cell suspensions, and subjected to red blood cell lysis. CD4$^+$ T cells were negatively isolated by magnetic bead separation, per manufacturer's instructions (Miltenyi Biotec). VFT TCR-transgenic CD4$^+$ T cells were distinguishable from endogenous cells via Thy1.1 congenic background, therefore only OT-II TCR-transgenic CD4$^+$ T cells were labeled with 2 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) prior to transfer. Equivalent numbers of CD4$^+$ VFT and OT-II T cells ($3–5 \times 10^6$/strain) were injected via the lateral tail vein 1–2 d pre-vaccination.

In vivo stimulation. For immunization, each mouse received either a DC-based vaccination of 3×10^6 MUC1-derived peptide-loaded BMDCs or a MUC1-derived peptide plus poly:ICLC vaccination comprising 100 μg MUC1 peptide and 50 μg of poly:ICLC by lateral tail vein injection. Splenocytes were harvested and processed for phospho-flow analysis as described previously.[26,27,32,33] In brief, spleens were harvested at various time points post-immunization and fixed immediately while processing them into a single-cell suspension in 1.6% paraformaldehyde (Electron Microscopy Services). After fixation for 10 min at room temperature, cells were permeabilized with the addition of ice cold methanol to a final concentration of 80%. Samples were incubated for 30 min at 4°C before being stored at -80°C.

Optimization of the phospho-flow technique for analysis of primary T cells. Intracellular phospho-specific staining was done in conjunction with staining for surface markers, as previously described.[9] Briefly, cells were washed twice in staining buffer (0.5% bovine serum albumin and 0.02% sodium azide in PBS) before blocking with an anti-CD16/32 antibody for 20 min at room temperature. Cells were stained for 45 min at room temperature with a mixture of surface marker-specific antibodies including CD4 V500, B220 PeCy7, CD90.1 APC-eFluor 780 (eBioscience), and an exclusion panel of biotin—streptavidin PE-Texas Red labeled antibodies (CD49b, TER119, CD11c, CD8a, F4/80 and CD11b) for an improved resolution of the populations of interest. Two intracellular or phospho-specific antibodies among
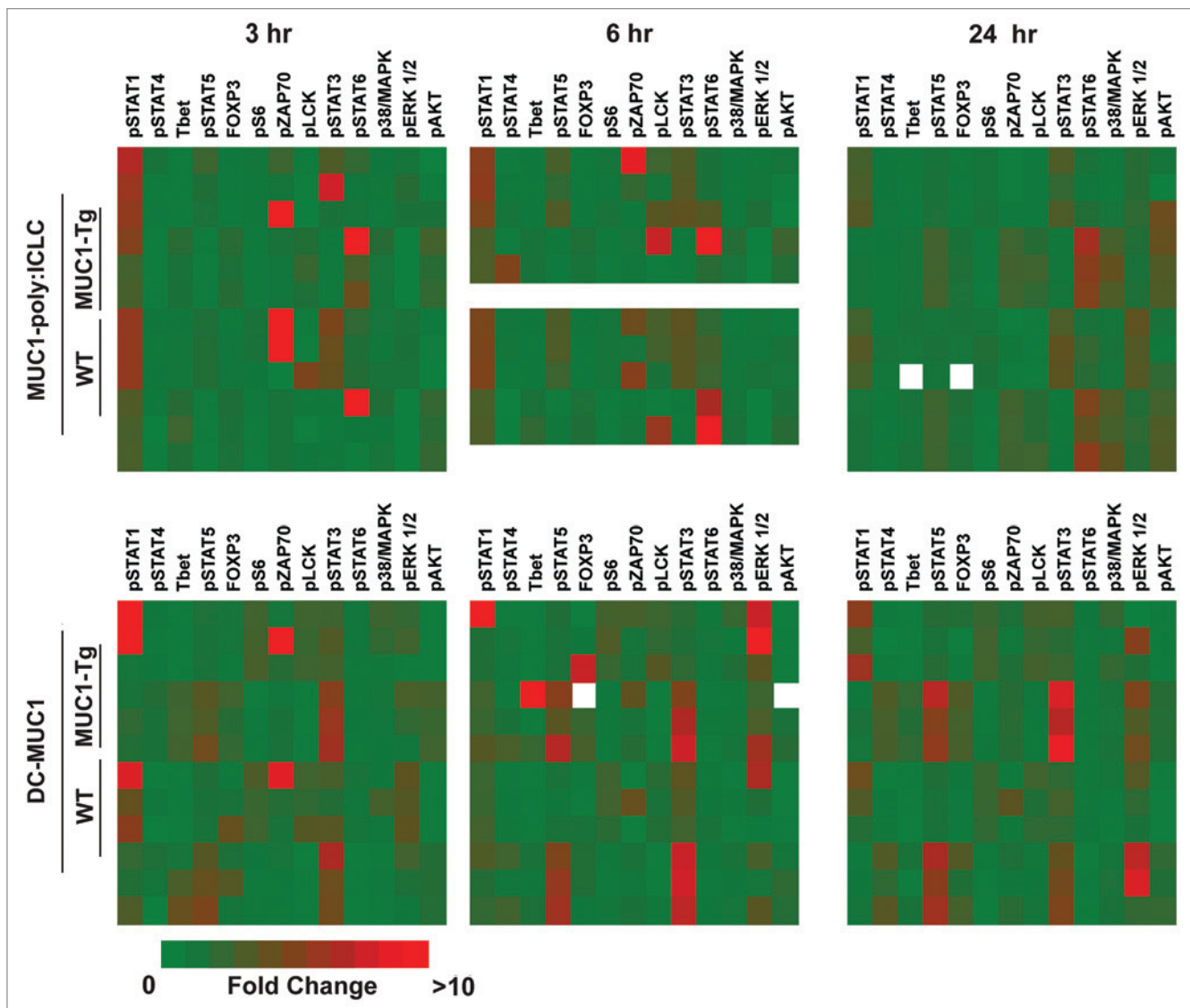
Figure 7. MUC1-specific CD4^{+} T cells activation "fingerprint" in vivo. Signaling profile of the CD4^{+} T-cell population in MUC1-transgenic and wild-type (WT) mice responding to the administration of either MUC1-derived peptide-loaded dendritic cells (DC-MUC1) or a MUC1-derived peptide combined with polyinosinic-polycytidylic acid stabilized with polylysine and carboxymethylcellulose (MUC1-poly:ICLC). The profile encompasses TCR-associated, cell cycle-associated, pro-survival, T helper-associated and regulatory T cell-associated signaling pathways.

the following were used per sample: Tbet Alexa Fluor 647, Akt Alexa Fluor 647 (pS473), ERK1/2 PE (pT202/pY204), Lck Alexa Fluor 647(pY505), p38 MAPK Alexa Fluor 647 (pT180/pY182), Stat1 PE (pY701), Stat3 PE (pY705), Stat4 PE (pY693), Stat5 PE (pY694), Stat6 Alexa Fluor 647 (pY641), S6 Alexa Fluor 647 (pS244), Zap70 PE (Y319). After staining, all samples were washed and resuspended in staining buffer. All antibodies were obtained from BD Biosciences, unless otherwise noted.

All samples were acquired on the LSR Fortessa and analyzed with FlowJo software (Treestar). The transferred MUC1-stimulated VFT CD4^{+} T cell population (exclusion [-]CD4^{+}B220^{-}CD90.1^{+}CFSE^{-}) as well as the co-transferred control OT-II population, which does not respond to MUC1-derived peptides (exclusion [-]CD4^{+}B220^{-}CD90.1^{-}CFSE^{+}), were identified. Median Fluorescent Intensity (MFI) was calculated for each

population within the sample and used to calculate fold changes upon stimulation, after normalization to the corresponding fluorescence minus one (FMO) control: fold change = absolute value $(MFI_{stimulated}/MFI_{stimulated\ FMO})/(MFI_{unstimulated}/MFI_{unstimulated\ FMO})$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Weiner LM, Surana R, Wang S. Monoclonal antibodies: versatile platforms for cancer immunotherapy. Nat Rev Immunol 2010; 10:317-27; PMID:20414205; http://dx.doi.org/10.1038/nri2744.

2. Cheever MA, Allison JP, Ferris AS, Finn OJ, Hastings BM, Hecht TT, et al. The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. Clin Cancer Res 2009; 15:5323-37; PMID:19723653; http://dx.doi.org/10.1158/1078-0432.CCR-09-0737.

3. Pardoll DM, Topalian SL. The role of CD4+ T cell responses in antitumor immunity. Curr Opin Immunol 1998; 10:588-94; PMID:9794842; http://dx.doi.org/10.1016/S0952-7915(98)80228-8.

4. Quezada SA, Simpson TR, Peggs KS, Merghoub T, Vider J, Fan X, et al. Tumor-reactive CD4(+) T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. J Exp Med 2010; 207:637-50; PMID:20156971; http://dx.doi.org/10.1084/jem.20091918.

5. Corthay A, Skovseth DK, Lundin KU, Røsjø E, Omholt H, Hofgaard PO, et al. Primary antitumor immune response mediated by CD4+ T cells. Immunity 2005; 22:371-83; PMID:15780993; http://dx.doi.org/10.1016/j.immuni.2005.02.003.

6. Soares MM, Mehta V, Finn OJ. Three different vaccines based on the 140-amino acid MUC1 peptide with seven tandemly repeated tumor-specific epitopes elicit distinct immune effector mechanisms in wild-type versus MUC1-transgenic mice with different potential for tumor rejection. J Immunol 2001; 166:6555-63; PMID:11359807.

7. Turner MS, Cohen PA, Finn OJ. Lack of effective MUC1 tumor antigen-specific immunity in MUC1-transgenic mice results from a Th/T regulatory cell imbalance that can be corrected by adoptive transfer of wild-type Th cells. J Immunol 2007; 178:2787-93; PMID:17312122.

8. Ryan SO, Turner MS, Gariépy J, Finn OJ. Tumor antigen epitopes interpreted by the immune system as self or abnormal-self differentially affect cancer vaccine responses. Cancer Res 2010; 70:5788-96; PMID:20587526; http://dx.doi.org/10.1158/0008-5472.CAN-09-4519.

9. Krutzik PO, Nolan GP. Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events. Cytometry A 2003; 55:61-70; PMID:14505311; http://dx.doi.org/10.1002/cyto.a.10072.

10. Krutzik PO, Irish JM, Nolan GP, Perez OD. Analysis of protein phosphorylation and cellular signaling events by flow cytometry: techniques and clinical applications. Clin Immunol 2004; 110:206-21; PMID:15047199; http://dx.doi.org/10.1016/j.clim.2003.11.009.

11. Krutzik PO, Nolan GP. Fluorescent cell barcoding in flow cytometry allows high-throughput drug screening and signaling profiling. Nat Methods 2006; 3:361-8; PMID:16628206; http://dx.doi.org/10.1038/nmeth872.

12. Perez OD, Krutzik PO, Nolan GP. Flow cytometric analysis of kinase signaling cascades. Methods Mol Biol 2004; 263:67-94; PMID:14976361.

13. Galligan CL, Siebert JC, Siminovitch KA, Keystone EC, Bykerk V, Perez OD, et al. Multiparameter phospho-flow analysis of lymphocytes in early rheumatoid arthritis: implications for diagnosis and monitoring drug therapy. PLoS One 2009; 4:e6703; PMID:19693272; http://dx.doi.org/10.1371/journal.pone.0006703.

14. Irish JM, Hovland R, Krutzik PO, Perez OD, Bruserud Ø, Gjertsen BT, et al. Single cell profiling of potentiated phospho-protein networks in cancer cells. Cell 2004; 118:217-28; PMID:15260991; http://dx.doi.org/10.1016/j.cell.2004.06.028.

15. Lee AW, Sharp ER, O'Mahony A, Rosenberg MG, Israelski DM, Nolan GP, et al. Single-cell, phosphoepitope-specific analysis demonstrates cell type- and pathway-specific dysregulation of Jak/STAT and MAPK signaling associated with in vivo human immunodeficiency virus type 1 infection. J Virol 2008; 82:3702-12; PMID:18216116; http://dx.doi.org/10.1128/JVI.01582-07.

16. Mortarini R, Vegetti C, Molla A, Arienti F, Ravagnani F, Maurichi A, et al. Impaired STAT phosphorylation in T cells from melanoma patients in response to IL-2: association with clinical stage. Clin Cancer Res 2009; 15:4085-94; PMID:19509154; http://dx.doi.org/10.1158/1078-0432.CCR-08-3323.

17. Lu SX, Alpdogan O, Lin J, Balderas R, Campos-Gonzalez R, Wang X, et al. STAT-3 and ERK 1/2 phosphorylation are critical for T-cell alloactivation and graft-versus-host disease. Blood 2008; 112:5254-8; PMID:18838616; http://dx.doi.org/10.1182/blood-2008-03-147322.

18. Gamper CJ, Powell JD. All PI3Kinase signaling is not mTOR: dissecting mTOR-dependent and independent signaling pathways in T cells. Front Immunol 2012; 3:312; PMID:23087689; http://dx.doi.org/10.3389/fimmu.2012.00312.

19. Shao Q, Ning H, Lv J, Liu Y, Zhao X, Ren G, et al. Regulation of Th1/Th2 polarization by tissue inhibitor of metalloproteinase-3 via modulating dendritic cells. Blood 2012; 119:4636-44; PMID:22415751; http://dx.doi.org/10.1182/blood-2011-08-376418.

20. Perkey E, Miller RA, Garcia GG. Ex vivo enzymatic treatment of aged CD4 T cells restores cognate T cell helper function and enhances antibody production in mice. J Immunol 2012; 189:5582-9; PMID:23136198; http://dx.doi.org/10.4049/jimmunol.1200487.

21. Awasthi A, Mathur R, Khan A, Joshi BN, Jain N, Sawant S, et al. CD40 signaling is impaired in L. major-infected macrophages and is rescued by a p38MAPK activator establishing a host-protective memory T cell response. J Exp Med 2003; 197:1037-43; PMID:12695487; http://dx.doi.org/10.1084/jem.20022033.

22. Yang XO, Panopoulos AD, Nurieva R, Chang SH, Wang D, Watowich SS, et al. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. J Biol Chem 2007; 282:9358-63; PMID:17277312; http://dx.doi.org/10.1074/jbc.C600321200.

23. Harris TJ, Grosso JF, Yen HR, Xin H, Kortylewski M, Albesiano E, et al. Cutting edge: An in vivo requirement for STAT3 signaling in TH17 development and TH17-dependent autoimmunity. J Immunol 2007; 179:4313-7; PMID:17878325.

24. Stritesky GL, Muthukrishnan R, Sehra S, Goswami R, Pham D, Travers J, et al. The transcription factor STAT3 is required for T helper 2 cell development. Immunity 2011; 34:39-49; PMID:21215659; http://dx.doi.org/10.1016/j.immuni.2010.12.013.

25. Turner MS, Kane LP, Morel PA. Dominant role of antigen dose in CD4+Foxp3+ regulatory T cell induction and expansion. J Immunol 2009; 183:4895-903; PMID:19801514; http://dx.doi.org/10.4049/jimmunol.0901459.

26. Krutzik PO, Hale MB, Nolan GP. Characterization of the murine immunological signaling network with phosphospecific flow cytometry. J Immunol 2005; 175:2366-73; PMID:16081807.

27. Zell T, Khoruts A, Ingulli E, Bonnevier JL, Mueller DL, Jenkins MK. Single-cell analysis of signal transduction in CD4 T cells stimulated with antigen in vivo. Proc Natl Acad Sci U S A 2001; 98:10805-10; PMID:11535838; http://dx.doi.org/10.1073/pnas.191567898.

28. Crellin NK, Garcia RV, Levings MK. Altered activation of AKT is required for the suppressive function of human CD4+CD25+ T regulatory cells. Blood 2007; 109:2014-22; PMID:17062729; http://dx.doi.org/10.1182/blood-2006-07-035279.

29. Siegel AM, Heimall J, Freeman AF, Hsu AP, Brittain E, Brenchley JM, et al. A critical role for STAT3 transcription factor signaling in the development and maintenance of human T cell memory. Immunity 2011; 35:806-18; PMID:22118528; http://dx.doi.org/10.1016/j.immuni.2011.09.016.

30. Noubade R, Krementsov DN, Del Rio R, Thornton T, Nagaleekar V, Saligrama N, et al. Activation of p38 MAPK in CD4 T cells controls IL-17 production and autoimmune encephalomyelitis. Blood 2011; 118:3290-18; PMID:21791428; http://dx.doi.org/10.1182/blood-2011-02-336552.

31. Vlad AM, Muller S, Cudic M, Paulsen H, Otvos L Jr., Hanisch FG, et al. Complex carbohydrates are not removed during processing of glycoproteins by dendritic cells: processing of tumor antigen MUC1 glycopeptides for presentation to major histocompatibility complex class II-restricted T cells. J Exp Med 2002; 196:1435-46; PMID:12461079; http://dx.doi.org/10.1084/jem.20020493.

32. Schulz KR, Danna EA, Krutzik PO, Nolan GP. Single-cell phospho-protein analysis by flow cytometry. Current protocols in immunology / edited by John E Coligan [et al], 2012:Unit 8.17.1-20.

33. Krutzik PO, Clutter MR, Nolan GP. Coordinate analysis of murine immune cell surface markers and intracellular phosphoproteins by flow cytometry. J Immunol 2005; 175:2357-65; PMID:16081806.