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T-cell phosphokinome as a fingerprint of effective graft versus leukemia

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1. ABSTRACT

Bone marrow and hematopoietic stem cell transplants are given to leukemia patients after chemotherapy and ablative preconditioning, but a significant number will suffer from graft versus host disease (GvHD), where donor immune cells attack recipient tissues. Some graft versus leukemia (GvL) activity protects from leukemia relapse, but determining this balance requires multi-factorial consideration. Genetic and cytokine studies have attempted to improve patient outcome predictions, but there is still far to go. Here, we describe important considerations of the phosphokinome as a fingerprint for predicting GvHD and GvL with partial least squares regression (PLSR) multivariate analysis. Distinguishing factors of GvHD and GvL will first be highlighted to appropriately measure T cell responses to cues that stimulate opposite, orthogonal, and overlapping responses. We will also discuss important kinase signaling cascades predicting cellular responses of cytokine expression, proliferation, and death linked with GvHD or GvL. Higher throughput methods to characterize these signals and different model systems will be discussed, along with benefits and challenges of using the T cell phosphokinome as a fingerprint to predict GvHD and GvL.

2. INTRODUCTION

Even with improvements in tissue matching currently available in the clinic for allogeneic bone marrow and hematopoietic stem cell transplants, there is still an alarming rate of patients that will suffer from graft vs host disease (GvHD) (1), an attack of the recipient's tissues by the donor's immune cells. George Mathe presented the idea in the 1960s that immunologically active cells in the graft could be helpful in recognizing and destroying resident leukemia cells in the recipient and that this graft versus leukemia (GvL) effect would sustain leukemia-free survival (2, 3). Once the importance of GvL was realized, reduced intensity conditioning regimens were employed for graft preparation (4, 5) to maintain active T cells that eradicate residual leukemic cells, but not activated to initiate GvHD. Different types of leukemia have different survival responses to transplants. While T-cells were shown to lyse acute myelogenous leukemia and acute lymphoblastic leukaemia cells (6), greater number of the donor T cells was required to be effective against acute leukemia and myeloma. There has also been success for chronic myelogenous leukemia, chronic lymphoblastic leukaemia, acute leukaemia, multiple myeloma, and lymphoma (6). Even advanced stage diseases, considered incurable, have had long term leukemia free survival

attributed to GvL, evidenced by successful allogeneic transplants but not for autologous transplants (7). At times, a subsequent donor lymphocyte infusion after the initial transplant has been used to prime the GvL effect, but these patients are then at a greater risk of GvHD (8). Improving predictions of patient GvHD responses will help clinicians decide on best course of action and choice of donor prior to transplantation and engraftment to maximize GvL and minimize GvHD. Gene expression profiles of peripheral blood leukocytes have yielded successful discriminant signatures from patients with immune tolerance to allograft GvHD (or lack thereof) (9) and from those associated with GvL in mice (10). Gene expression arrays of donor T cells reveal a strong correlation between TGF-beta signaling pathway molecules and patient GvHD outcomes (11). Serum biomarkers and polymorphisms in cytokines and genes have also been investigated to improve clinical predictive power (12). This type of information, however, does not necessarily yield specific insight in the nature of the immune response processes underway. In contrast, measurements of the signal transduction events hold the promise of monitoring and directing interventions.

Although numerous families of molecules are implicated in signal transduction, kinases as a whole have proven to be one of the most frequently studied classes to monitor for several reasons: 1) the degree of phosphorylation is often correlative to protein activity and ample phosphor-specific antibodies are available for immuno-based detection; 2) the amplification steps in receptor-mediated signaling ensure abundance of molecules to measure; 3) the specificity of each protein allows for sampling within specific cascades or branches; 4) the duration/stability of post-translational modifications is generally long enough for facile detection. Immune cells respond to numerous soluble, matrix bound, mechanical, or cell produced signals that stimulate variant signaling cascades, some of which leading to GvHD and GvL. These downstream cascades converge at highly connected nodes, and examination of the phosphokinome activation state may be useful in predicting cell and tissue response. Some balance of donor T cell activation must be reached to maximize survival and minimize GvHD, and understanding T cell stimulation and responses in the recipients pre- and post-transplantation should provide quantitative cues that activate kinase networks to elicit cellular responses.

Computational analysis of the dynamic changes in kinase activation has been useful to show that kinases serve as integrators of cue information to produce specific cellular responses (13-15). Partial least squares regression (PLSR) is a data-driven modeling technique able to weigh signaling metrics and rank the importance of their contributions to a measured cellular response. This PLSR model can be constructed from relating cellular kinase signals across multiple pathways to cell behavior responses to organize the information inherent in the signaling network downstream of stimulation with multiple cues, instead of trying to account for cell fate decisions from individual pathways in isolation. This has been shown with T cells, specifically, by integrating signaling information across multiple pathways in a dynamic manner to fully represent the information encoded into those pathways. *A priori* responses to novel peptide:MHC presentation could be predicted using the encoded network analysis (16). A different study used to predict cell differentiation with a multi-variate systems approach of multiple kinase pathway activities with computational analysis and derived quantitative combinations of kinase signal changes of adult stem cells (13). Such predictive models that incorporate cues, signals, and responses quantitatively may provide more robust predictions for multi-factorial diseases such as GvHD. Importantly, prediction of cell phenotype signaling pathway (13, 14, 16), and kinase cascades are central to many of these signal transduction networks.

3. COMPLICATIONS OF DISTINGUISHING GVHD FROM GVL RESPONSES

GvHD is characterized by cytotoxic T cells that damage skin, liver, and digestive tract epithelium and among many other tissues, occurring after hematopoeitic stem cell transplantation and bone marrow transplantation. The recipient immune system must be compromised (e.g. leukemia, sickle cell anemia), the donor and recipient must be mismatched antigenically, and the graft from the donor must contain active immune cells for GvHD to occur (12). Strategies to remove active immune cells prior to grafting reduce GvHD (17-19), but there is a greater chance of leukemia relapse (12) mitigating the beneficial effect, now known as GvL. Active immune cells are the complicating factor in these equations as they cause GvHD but also protect the recipient from leukemia. In fact, patients with a little GvHD see greater leukemia suppression, but some patients do survive the leukemia without any GvHD (20, 21).

3.1. Antigen specificity

The fundamental differences between GvHD and GvL are the antigen specificity and immune response, but the large overlap between the two, complicates strategic design to maximize GvL while minimizing GvHD. Signaling pathways are initiated to activate TCR kinases and cytokine pathways, changing the phosphokinome state of the immune cells and the subsequent responses. There is an increasing range of antigen specificity from chronic GvHD to acute GvHD to GvL. Chronic GvHD is the most nonspecific antigen response of the three; transplanted mature donor T cells recognize non-self histocompatibility antigens, which all cells in the recipient present an almost unlimited supply. In response, they employ a broad immunity strategy to attack the recipient tissues (12, 19, 22). Since there is no single pathogen in a specific tissue or location that is stimulating the inflammatory response and attracting the T cells; this broad response damages many different organs. In acute GvHD, there is a more directed antigen-specific response, directed at epithelial tissue cell epitopes and hematopoeitic cell markers of the recipient. GvL is considered to be antigen specific with donor T cells targeting hematopoietic cell markers exclusively which means targeted killing of residual leukemia cells in the recipient (12, 19).

3.2. Immune cell involvement

GvHD involves a number of immunologic cells including antigen presenting cells (APCs), CD8+ and

CD4+ T cells, dendritic cells, and others from both the recipient and the donor. Contribution of each to GvHD vs. GvL is varied. APCs play an initiation role in GvHD, and though they are not the focus of this review, it is important to briefly mention their role in activating and priming T cells for GvL or GvHD, which initiates kinase phosphorylation and cascades to use in predictive analysis. Donor APCs and recipient APCs prime the donor T cells in GvHD to stimulate donor T cell responses (19). Binding to APCs via integrins activates T cells and co-stimulation of T cell receptors promotes proliferation and other effector phenotypes (22). CD18 from the beta-2 integrin family forms the LFA-1 complex with CD11a. LFA-1 binds ICAM-1, 2, and 3 on the surface of endothelial and epithelial cells, targets of GvHD attack. Genetic knockdown of CD18 prevented stable contact between APCs and T cells and significantly reduced GvHD (22).

An improved understanding of the activation of T cells starts with elucidation of signal transduction and phosphokinome network changes downstream of antigen presentation, cytokine stimulation and APC binding. Investigation of these relationships with a cue-signal-response paradigm has been useful in other biological systems under different conditions and may be useful to make informative predictions of immune cell activation for GvHD or GvL in response to environmental stimuli.

4. CUE – SIGNAL – RESPONSE PHOSPHOKINOME ANALYSIS: CUES

Transient activation states and intracellular signaling dynamics can be captured using a cue-signal-response paradigm and partial least squares regression (PLSR). Partial least squares regression (PLSR) is a computational technique that reduces the dimensionality of multi-variate data sets by defining principle component (PC) axes containing the most important information and signals of the original data set with an algorithm to capture the maximal variance in the data. The algorithm uses a proposed relationship between the independent (signals; X) and dependent (responses; Y) variables, and then regresses the data with a linear solution in principal component space such that Y=**F**(X). With this method, the kinase signals with the greatest covariance contributing most to the dependent variable outputs (T cell responses here) are weighted more heavily in the solution/prediction function. This function can then be used to mathematically predict a response.

External cues from the microenvironment may be from soluble factors, extracellular matrix, or juxtacrine contact with resident cells. All of this information is integrated and processed by the T cells with measureable signals (here, phosphorylation of kinases), to generate a response that, with proper use, is predictive of the cell behavior. This systems biology approach goes beyond measuring the fluctuation of some "master" transcription factor or one key enzyme, but instead examines changes at the kinase level, where integration of ubiquitous information is processed intelligently by the cell; kinases are up- and downstream of transcription factors, gene transcription, and protein translation. By sampling this nexus of inputs that precedes output, predictive information of the cell's terminal fate prior to synthesis of the cell-specific proteins and behavioral responses is provided.

4.1. Conditioning regimen and patient state

Patient condition pre-transplant provides a background level of stimuli that can alter the basal phosphokinome state. Patient to patient variability will be reflected in the treatments and pre-conditioning regimen of the recipient and donor and it is important to parse the effects. Irradiation of T cells stimulates cytokine release by T cells and high dose irradiation kills them. This has been correlated with severe GvHD, but it is important to distinguish the death of the T cells from the cytokines released (23). Ablating T cells suppresses GvL response, but cytokines released may have been the critical factor. Irradiation regimens also have been shown to transiently induce production of inflammatory chemokines from nonlymphoid tissues and extended maintenance of cell adhesion molecule upregulation (24-26), meaning that the recipient tissues and organs are primed to activate donor T cells after this pre-conditioning which may promote GvHD.

Patient infection is another factor that changes the baseline phosphokinome status. Infection not only changes baseline phosphorylation state through cytokine activation (27, 28), but also leads to activation of toll-like receptors and inflammation in the epithelial tissues. Increases in the number of T cells that traffic to those infected tissues and subsequent exacerbation of GvHD by this larger T cell population can now occur which must be considered in a cue-signal-response analysis as these are additional cues that will affect patient to patient variability and the predictive power.

4.2. Antigen initiation of cascades

T cell recognition of foreign antigen is an exquisitely sensitive response to MHC:peptide presentation that activates downstream signaling cascades through integrator kinases in response. Through the selection process in the thymus, the T cell population is narrowed to those cells that can bind to MHC (positive selection) but do not respond too strongly to self peptide (negative selection). While many of the molecular components of T cell activation have been known for decades, only recently has an optimal moderate half-life of peptide:MHC interaction with CD8+ cells and precise duration of signaling been shown to directly affect the cytolytic activity and *in vivo* cytotoxic T lymphocyte (CTL) tumor clearance (29). The strength of TCR activation in regulatory T cells (Tregs) is also directly related to their ability to suppress immune responses (30); however the necessity of activation has been recently questioned by observations of constitutive immune response suppression without APC peptide:MHC presentation (31). Upon presentation of peptide by MHC on antigen presenting cells, the T cell undergoes a series of rapid phosphorylation events that are timed to regulate the immune response according to the nature of the cue (32, 33) and if monitored, can yield insight in the phenotypic outcome of activation (16).

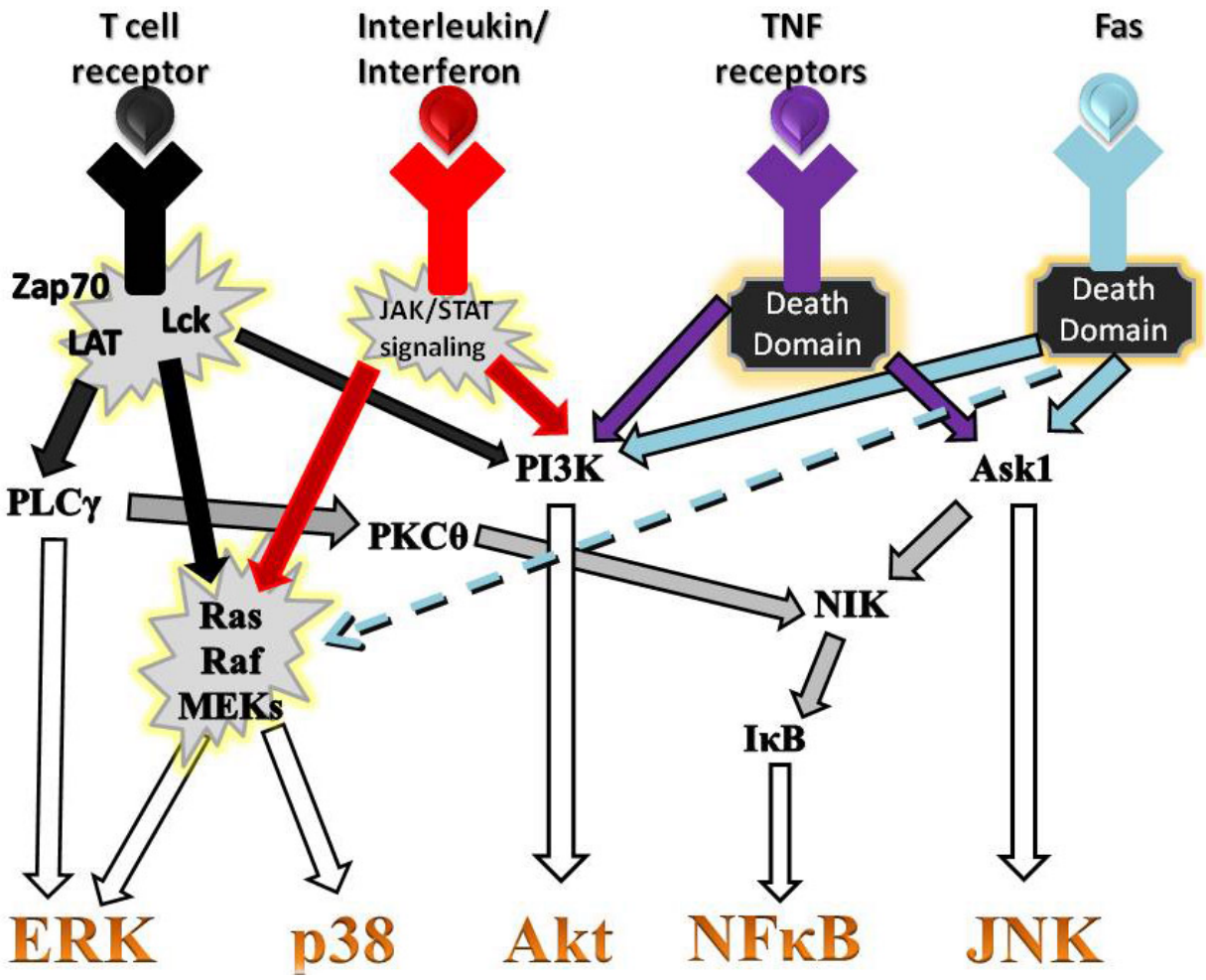

Figure 1. Integrator kinases are at the nexus of many signaling pathways initiated in T cells. Downstream of T cell receptor ligation, interferon and interleukin binding, TNF and Fas ligands, are convergent kinase signaling nodes that are top candidates to measure for simplifying phosphokinome analysis: ERK, p38, Akt, NF kappa B, JNK, PLC gamma, and PKC-theta.

5. PHOSPHOKINOME ANALYSIS: SIGNALS

Different receptors stimulate a number of pathways that integrate at ERK, p38 MAPK, Akt, NFkappaB, JNK, PKC-theta, and PLC-gamma. A brief overview of these pathways is given below (Figure 1).

5.1. T cell receptor signaling

Series of phosphorylation cascades are initiated by antigen ligation of the T cell receptor. Briefly, the ITAM regions of the transmembrane protein CD3 are phosphorylated by the Src-family kinases, Lck and Fyn, allowing for the kinase ZAP70 to be phosphorylated, which subsequently phosphorylates the lipid raft LAT. SLP76, ITK and PLC-gamma are recruited to the raft; the latter cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 initiates calcium release from the ER and the activation of NFAT through calcineurin, while DAG activates protein kinase C theta (PKCtheta), RasGRP, Ras, and downstream MAPKs. A secondary, slower route of Ras activation occurs via LAT recruitment of Sos and Grb2; both modes are needed for full activation (34). Erk phosphorylation is associated with a negative feedback loop to the phosphatase, SHP1, which dephosphorylates Lck to terminate the antigenic signal (35, 36). TCR ligation also induces PI3K and Akt activation; this signaling is reinforced through the CD28 co-receptor initiated cytoskeletal rearrangement. Akt-activated mTOR suppresses Treg development through repression of Foxp3 transcriptional activity (37, 38). Akt and PKCtheta interact with the CARMA1/MAT1 complex to initiate IKK activation (39, 40). The collective result of signaling events yields activation of NFAT, NFkappaB, Jun and Fos for initiation of transcriptional programs (41).

5.2. Interferon and interleukin: signaling through JAK/STAT

Cytokine receptors use simpler biochemical cascades to initiate transcription in response to extracellular cues. A variety of cytokines ligands can specifically signal through a relatively small repertoire of proteins by sharing

components, interchanging isoforms, and limiting the scope of the biochemical cascade to relatively few steps. The common -gamma chain family, encompassing IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 receptors, consists of a ligand-specific subunit that creates a heterodimer with a shared transmembrane subunit, the gamma chain, to initiate signaling. Similarly, another family of interleukins (IL-10, IL-20, IL-22, IL-26) share the IL-10R2 component (42), and the IL-6 family (e.g. IL-6, IL-11, LIF) share the gp130 transmembrane protein (43). Type I interferons share a common receptor dimer whereas interferon -gamma (type II) is unique in its receptor specificity (44). Each of these cytokine families utilize a two-step process of Janus kinase phosphorylation (JAK) leading to signal transducer and activator of transcription (STAT) dimerization, activation, and nuclear import. A suppressor of cytokine signaling (SOCS) is upregulated upon STAT-induced transcription to terminate the pathway activation in a subset of receptor systems. With 7 STAT isoforms and 4 JAK isoforms (including the related Tyk) (45), the specificity lies in which combination of the modular components are engaged through receptor ligation (46). Furthermore, additional crosstalk with other signaling pathways, such as Ras/MAPKs and/or PI3K/Akt can occur.

5.3. Cytotoxic pathway signaling

Major cytotoxic pathways implicated in GvHD and GvL are TNF-alpha and TNF-related apoptosis-inducing ligand (TRAIL), FasL, and perforin/granzyme among others. *In vitro* and *in vivo* data suggest that donor T cells use different cytolytic pathways in GvHD and GvL. Signaling cascade responses after ligand binding reveal a number of kinases that may be predictive of T cell activation in GvHD and GvL. Manipulation, perturbation, and isolation of these pathways have elucidated their respective contributions to GvHD and GvL and even suggest that the GvL effect can be maintained while reducing acute GvHD by modulating specific cytotoxic mechanisms (47, 48). The two cytotoxic pathways that deserve more detailed discussion are TNF/TNFR and FasL/Fas (23, 49-56), that all interact with the same integrator kinase nodes downstream of T cell receptor activation and interleukin/ interferon binding but also stimulate release of factors that activate these pathways. They should therefore be included in phosphokinome analysis to discern GvL outcomes from GvHD.

5.3.1. TNF/TNFR

The TNF family of ligands bind homotrimeric receptors and induce cell death and stress responses. Elevated TNF has been measured in patients with GvHD (57). TNF-alpha signaling through TNFR1 causes IL-1 and IL-6 release (58, 59) as well as contributing to cytotoxic effects (60, 61). Ligation of this receptor also activates JNK and PI3K cascades that may contribute to either of these outcomes. Inhibition of this signaling axis with monoclonal antibodies against TNF-alpha impaired GvL by diminishing the cytotoxic effects and T cell activation (62, 63). TNF signaling is further complicated by the different downstream pathways initiated by membrane bound TNF-alpha and the soluble ligand binding to TNFR1 or TNFR2; the membrane bound form can bind TNFR2 and induce apoptosis in the target cell but the both membrane and soluble TNF-alpha can use TNFR1 for this purpose (64, 65). *In vivo* experiments using TNFRp55knockout recipient mice were protected from mortality after allogeneic bone marrow transplant of T cells implicating TNF in the GvHD outcomes (23).

5.3.2. FasL/Fas

Fas is a ligand that binds its receptor Fas and initiates the extrinsic apoptotic pathways and caspases. A number of studies implicate FasL in GvHD but not for GvL activity (48). Of course, the main role for Fas is cytotoxicity; when FasL on the cytotoxic T cell surface binds Fas on the cell to be killed, downstream cascades lead to caspase activation(66). T cells from FasL knockout mice were still cytotoxic and involved in GvL activity (52, 67). Although canonically known to activate these death pathways, there is also crosstalk with PI3K pathway after tyrosine phosphorylation of Fas exposes SH2 protein binding domains(68), and activation of p38 MAPK (69), JNK (70), ERK (71), and NFkappaB (72) pathways to elicit responses in a variety of cells and phenotypes.

5.4. Signal measurement methodologies

Proteomic tools have been widely employed for investigating basic T cell biology for a number of years. Patterns of activation on population averaged lysates on protein arrays (73-75) or mass spectrometry (76) provide rich datasets that warrant multivariate modeling techniques in order to interpret the results for pertinence to desired outcomes. Alternatively, single cell analysis of phosphorylation profiles is constrained by the limits on flow cytometry channels, but can distinguish prognostic indicators through hierarchical clustering (77) and is readily adaptable to clinical samples and technologies. Sophisticated network reconstruction methods can be used to extract entire pathway structures from multivariate relationships (78). Luminex technology which allows for quanititaive bead-based immunoprecipitation and fluorescent tagging has added high throughput capabilities to phosphokinome analysis. Commercially prepared kits are now available from Millipore, Bio-Rad, and EMD Biosciences to study up to 17 kinases in one lysate.

6. PHOSPHOKINOME ANALYSIS: RESPONSES

6.1. Cytokine profiling

Immune system dysregulation characteristic of acute GvHD is accompanied by what has been characterized as a “cytokine storm” with the production of IL-1, IL-2, IL-4, IL-6, IL-7, IL-10, IFN-y, macrophage inhibitory protein-la, and others (63). Profiling of acute GvHD and chronic GvHD has identified cytokines important for each of these outcomes; IL-1, IL-6 and TNF-alpha, IFN-gamma, and IL-2 with acute GvHD and Il-4, IL-5 and IL-10 with chronic GvHD (79, 80). Serum levels of the cytokine receptors TNF-alphaR1, IL-2R, IL-8, HGF have even been used to predict acute GvHD (12). Cytokine production is one metric that may be useful as both a measure of T cell activation since they are characteristics of the immune response, but the upregulation of the cytokines also serve as signals to induce more responses. This is

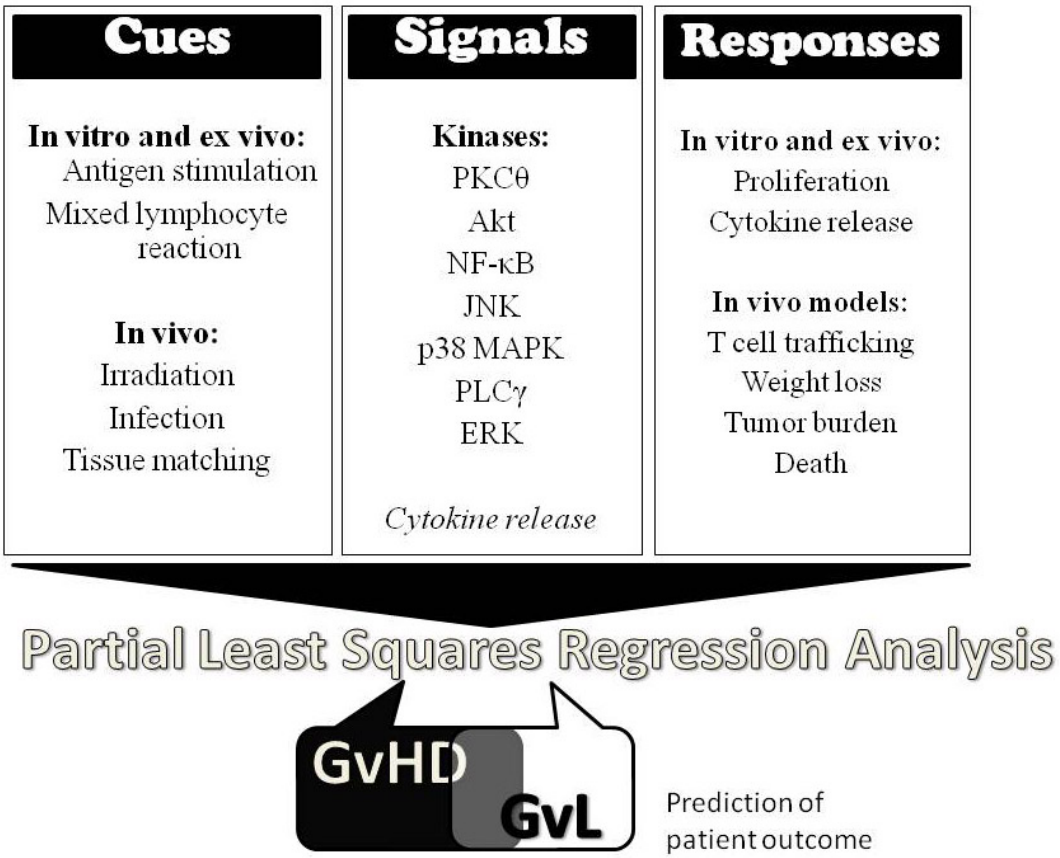

Figure 2. Cue-signal-response paradigm for using phosphokinome as a fingerprint of GvL or GvHD. A schematic of the plan to use multiple cues, multivariate kinase pathways, and responses with PLSR analysis to predict GvL or GvHD is given. There is also a gray area of overlap in between.

challenging as there are so many cytokines and different combinations must have some weighted contribution towards a GvHD or GvL outcome. Cytokine profiling may be input first into PLSR to regress phosphokinome state to a sub-response of cytokine release, and then repeated to link the cytokine profile to GvHD or GvL outcomes. For example, IL-2 and IL-15 promote T-cell proliferation and survival (81, 82) via kinase activation. TNF-alpha and IL-1 stimulate CD4+ T cell and CD8+ T cell induced GvHD (83). IL-4 secretion by the donor T cells means decreased GvHD (84). IL-2 is an early marker of GvHD (85). Layering of the PLSR algorithm may be useful to make sense of cytokines as responses and signals.

6.2. Immune cell behaviors

Predictive outputs of the cue-signal-response paradigm will differ depending on the use of *in vitro* and *in vivo* experimental model systems, but some may be applicable to both. Cytokine secretion (IL-4, TNF, IFN-gamma, IL-10) (86), T cell anergy (adaptive tolerance) (87), proliferative response (86), and T cell tracking (22) change pre- and post-transplantation. It is also important to note which responses will be associated with a GvHD or GvL response. A metadata analysis of *in vitro* data may be necessary to incorporate the cellular response with the potential activation and response in the full animal or human model. GvL responses include CD8+ T cell proliferation (88) and CD4+ effector memory T cell proliferation (89). GvHD responses include IL-4 cytokine release (84, 90-92), T cell trafficking to GvHD target organs (47, 89), death in animal models (post-transplant with or without weight loss and tumor growth) (22).

Inconclusive responses from cues that are partially associated with both outcomes are those that benefit most from multivariate analysis to better categorize or grade their contributions to GvL or GvHD. IL-10 is a ubiquitous cytokine that suppresses the production of IL-12, IL-1, IL-6 and TNF-alpha production by activated monocytes/macrophages (93-96) and other proinflammatory chemokines (Mip-1-alpha, -1beta, -3-alpha, -3beta, IL-8, IP-10 and Mip-2) by activated monocytes (97-101) but also has been shown to be important in chronic GvHD (80, 100).

6.3. Pre-clinical and clinical response measurements

Responses indicative of GvHD and GvL include death, weight loss, tumor burden, T cell trafficking, and cytoxicity of T cells (Figure 2). These outcomes are not measurable in immune cells alone and require the use of pre-clinical and clinical animal and human experimental models to quantify these responses to link them to

phosphokinome network state. Mouse models are open to genetic manipulation to completely remove a cytokine, receptor, or other biomolecule from the progression of GvHD or initiation of GvL. A GvL mouse model has been developed by transferring bone marrow and spleen cells from genetic knockout donors into irradiated recipients that had been previously inoculated with leukemia or mastcytoma cells. This parent (C57BL/6) to F1 (C57BL/6 × DBA/2) bone marrow transplant model reflects human GvL effect as it recapitulates human outcomes of leukemia relapse after T cell depletion and syngeneic transplant (47). Animal models provide an experimental system in which multicellular and multi-organ perturbations can be handled, but one issue with animal models is that many of the novel findings that have worked to minimize GvHD while maximizing GvL have not translated to humans due to the differences in biology (12).

Immune cell trafficking has been observed with dynamic two-photon *in vivo* microscopy (47) and *in vivo* bioluminescence imaging (102). *In vitro* imaging of hematopoietic subpopulations and ex vivo imaging and analysis has also been useful in measuring these outcomes (89). Some GVH reactive T cells do not traffic to GvHD target organs but stay in the peripheral circulation; the recipient's environment must be playing a role in determining the extent of GvHD and preventing T cell trafficking and homing. This was corroborated when these same T cells that did not home to an organ were implanted into a different, irradiated recipient and were able to cause GvHD (47).

Mixed lymphocyte reactions are useful *in vitro* to observe donor and recipient interactions and cellular responses of proliferation with thymidine (89) and carboxyfluorescein diacetate succinimidyl ester (CFSE) (103), T cell receptor expression (flow cytometry) (89). Surgical or cell based interventions can also be tested in murine models prior to moving them large animal, non-human primates such as rhesus monkeys (104, 105), or human clinical trials. T cell depletion studies (106, 107), heterogeneous cell to cell synergisms and interactions (89), and timing of events in disease progression (89) may be relevant responses that can be quantified for use in computational and PLSR models to analyze phosphokinome predictive power of GvHD and GvL.

7. BENEFITS AND CHALLENGES

7.1. Using PLSR for phosphokinome analysis

In comparison to other multivariate regression techniques, PLSR is amenable to datasets where the number of predictor variables exceeds the number of observations, and the algorithm does not require a full-rank matrix. Furthermore, in a recent study directly comparing it to other multivariate regression techniques such as principal component regression and Lasso regression, PLSR was found to handle multicollinearity better - as prevalent in high-dimensional, proteomic datasets (108). As a proof of principle of this approach for understanding immune function, a murine T cell hybridoma system was used to investigate avidity-encoding within an altered peptide ligand system (16). After training on a series of hierarchical cues associated with IL-2 magnitude, a PLSR model could numerically predict T cell responses in new conditions from a key set of dynamic signals (Erk, Akt, IKK, p38, MK2, NFAT, JNK). The model provided a conceptual understanding of how complex signaling networks are integrated to translate peptide:MHC stimuli into functional responses via cytokine production, one of the relevant responses described herein to parse GvHD from GvL. A new molecular mechanism, Akt/Erk cooperativity, involved in T cell signaling was identified through the correlative relationships extracted by the multivariate analysis.

More recently, a PLSR model was used to predict (R^2=0.96) the number of ex vivo population doublings that had occurred within a primary CD8+ population based upon early TCR signal transduction events and surface marker expression (109). Changes in the coefficients of variation associated with biomarkers were used as additional metrics in the model and proved to be highly informative predictors. The model was only trained on three donor datasets with large noise in the measurements, yet could capture patterns of a grossly under-defined cellular process, immunosenescence, in which prior univariate or hierarchical clustering attempts of "aging biomarkers" had fallen short. This speaks to the ability of the algorithm to extract subtle patterns of co-variance with low number of donor sets.

7.2. Data interpretation considerations

The pertinent kinases to monitor in GvHD and GvL depend on the subtype of T cells used in immunotherapy, and while many of the promising signal transducers that have emerged in the past years have only been characterized in murine models of GvL it is expected that most of the molecular mechanisms controlling adaptive immunity will be conserved in humans. A number of studies have been devoted to understanding the differences in signaling between Tregs and other peripheral T cell types. Allogeneic hematopoietic cell transplantation in mice showed a synergistic effect of combining Tregs and rapamycin therapy (110), suggesting a possible use of phosphorylation events to distinguish pathway responsiveness in different T cell subpopulations. Tregs preferentially responded to IL-2 signaling as measured by phospho-STAT5, while conventional T cells showed higher p70S6K (indicative of mTOR pathway). In comparison to other T cells, Tregs also show sequestration of PKCθ away from the immunological synapse (111) and deficient LAT:PLC-gamma binding (112). These nuances in differential signaling become more important when T cell populations do not differ in surface marker classification/differentiation, but only by location. CD26+CD45RA+ peripheral T cells showed more Lck, LAT, ZAP70 phosphorylation with CD3 crosslinked T cell activation than CD26+CD45RA+ cord blood T cells due to altered molecular recruitment to lipid rafts; this differential signaling is proposed to elicit a lower incidence of GvHD from cord blood (113). In another study, splenic Tregs from tumor-injected mice showed lower basal and activated ITK phosphorylation compared to the tumor infiltrating Tregs

(114). Furthermore, the tumor-infiltrating Tregs and effector CD8+ T cells had impaired calcium flux associated with exposure to the tumor microenvironment.

7.3. Timing and other patient specific influences

Timing will be another important consideration but it can be studied now with reduced intensity regimens. In humans, detection of leukemia reactive cytotoxic lymphocytes can be as early as 14 days (115), but as long as one year after the transplant (7). This broad range of timescales for measuring the responses of GvL or GvHD may be a complicating factor in using those outcomes with phosphokinome prediction analysis.

8. SUMMARY AND PERSPECTIVE

Patient specific influences and cell types involved will always complicate the predictive power and use of computational analytical methods. Use of this new modality to analyze patient-tissue matching incorporates another class of biomolecules to be used by clinicians to predict cell responses. Kinases, particularly, the integrator kinases suggested here, offer a way to identify the cell from the inside out and link this internal look to pathways that have previously been mechanistically linked to a GvHD or GvL outcome. Additionally, mathematical analysis provides an unbiased examination that, once validated and its robustness has been determined, offers a new strategy to predict GvHD prior to the hematopoietic stem cell and bone marrow transplantation.

9. ACKNOWLEDGEMENTS

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