National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

Mechanisms of T cell immunosuppression during the graft-versus-host reaction

Julie Desbarats

Department of Physiology, McGill University, Montreal

August 1994

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the degree of Ph.D.

ISBN 0-612-05696-1

Canada

Abstract

The studies presented in this thesis examine the mechanisms of T cell immunosuppression during the graft-versus-host reaction (GVHR). GVHR was induced by the injection of parental lymphoid cells into F1 hybrid recipient mice. The ensuing acute reaction consisted of an initial immunoproliferative phase, followed by the development of profound immunosuppression and histopathological lesions of epithelial and lymphoid tissues. Survivors of the acute reaction developed the persistent immune abnormalities chacteristic of chronic GVHR.

We have found that the T cell protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$, involved in signal transduction through the T cell receptor (TCR), are downregulated in the T cells of mice during GVHR. The reduction of lck and fyn was prevented by adrenalectomy of the recipients, and a similar reduction could be induced in normal (non-GVH-reactive) mice by an injection of exogenous cortisone. These findings suggested that the GVHR-induced elevation in endogenous glucocorticoid levels could trigger the decrease of T cell lck and fyn, resulting in a T cell signalling defect during GVHR. In fact, we have demonstrated that glucocorticoids induced a decrease in lck and fyn in T cell clones *in vitro*. Thus, it appeared that the early GVHR-induced T cell unresponsiveness was due to the glucocorticoid-dependent downregulation of T cell lck and fyn.

We have investigated changes in T cell maturation and selection in the GVHR-dysplastic thymus, which may account for the persistent immune abnormalities of chronic GVHR. Thymocyte TCR expression and usage were aberrant during GVHR; changes included decreased expression of CD3 on $CD4^+8^-$ thymocytes, inconsistent TCR Vß usage, and appearance of phenotypically autoreactive mature thymocytes. These abnormalities, suggestive of defective positive and negative selection, are likely to result from

the GVHR-induced decrease in thymic class II MHC expression. Altered T cell education may lead to the long-term peripheral T cell defects observed in chronic GVHR. Lastly, we report that GVHR-induced cutaneous injury was exacerbated by irradiation of the target tissue, suggesting that in clinical GVHR, irradiation may intensify tissue damage, including thymic epithelial lesions; this could potentially lead to more serious alterations in thymic function, and thus to longer lasting, more severe peripheral T cell immune deficiency.

Résumé

Les études présentées dans cette thèse examinent les méchanismes de l'immunosuppression des cellules T pendant la réaction gu greffon-contre-l'hôte (RGCH). La RGCH à été induite par l'injection de cellules lymphoïdes parentales dans des hôtes hybrides F1. La réaction aïgue qui s'ensuit consiste en une phase initiale d'immunoprolifération, suivit du développement d'une immunosuppression profonde et de lésions histopathologiques des tissues épithéliaux et lymphoïdes. Les survivants de la réaction aïgue développent des anomalies immunitaires persistantes qui charactérisent la RGCH chronique.

Nous avons trouvé que le niveau des protéines-tyrosine-kinases $p56^{lck}$ et $p59^{fyn}$, impliquées dans la transduction des signaux du récépteur de la cellule T (RCT), est diminué dans les cellules T des souris pendant la RGCH. La diminution de lck et fyn a été empêcher par l'adrénalectomie des souris hôtes, et une diminution semblable pouvait être induite dans des souris normales (non-RGCH) par l'injection de cortisone exogène. Ces résultats suggèrent que l'augmentation de glucocorticoïdes endogènes pendant la RGCH peut provoquer la diminution du lck et fyn des cellules T, donnant lieu au défaut de transduction de siguaux des cellules T de la RCGH. De fait, nous avons démontré que les glucocorticoïdes induisent une diminution du lck et fyn des clones de cellules T *in vitro*. Il semble donc que l'immunosuppression des cellules T induite au début de la RGCH est dûe à la diminution de lck et fyn provoquée par les glucocorticoïdes.

Nous avons examinés les changements dans la maturation et la selection des cellules T dans le thymus dysplastique de la RGCH, qui pourraient donner lieu aux anomalies immunitaires persistantes de la RGCH chronique. L'expression et l'usage des RCTs des thymocytes sont anormaux pendant la RGCH; les altérations comprennent une diminution dans l'expression de CD3 sur les thymocytes $CD4^+8^-$, un usage non-consistant des segments Vß RCT, et

la présence de thymocytes matures de phénotypes autoréactifs. Ces anomalies, qui suggèrent un défaut de la selection positive et négative, pourraient être le résultat d'une diminution des antigènes du complexe majeur d'histocompatibilité classe II du thymus provoquée par la RGCH. Ces altérations dans l'éducation des cellules T pourraient provoquer les défauts à long terme des cellules T périphériques pendant la RGCH chronique. Finalement, nous avons constaté que les lésions cutanées induites par la RGCH sont exacerbées par l'irradiation du tissue cible, ce qui suggère que pendant la RGCH clinique, l'irradiation pourrait empirer le dommage tissulaire, y compris les lésions thymiques; ceci pourrait entrainer des altérations encore plus graves de la fonction thymique, et donc une déficience immunitaire des cellules T périphériques plus sévère et de plus longue durée.

Acknowledgments

I am deeply grateful to Dr. Wayne Lapp for having enabled me to be a gradute student in his laboratory. I wish to thank Dr. Lapp for his constant patience, understanding and kindness, and for all that he has taught me about immunology, and about so many other things. Dr. Lapp has given me a great amount of freedom to ask and answer my own questions, while providing excellent supervision and guidance for which I will always be grateful. It is an honour to be part of the family which constitutes "Lapp's lab".

I would like to thank Ailsa Lee Loy and Rosmarie Siegrist-Johnstone for their indispensable assistance during all my experiments; without them the lab would surely cease to function. Thank you, Ailsa and Rosy, for always being there when we need your help - for anything and everything, not only with the experiments.

I am grateful to have had the opportunity to interact with and learn from all the other students in Dr. Lapp's lab. I am particularly endebted to those who were already in the lab when I came, Anargyros Xenocostas, Fred Nestel, Murray Kornbluth, John Setrakien, Eric You-Ten, and Flemming Rasmussen, for their help and patience in teaching me and answering my unending questions; and I am also fortunate to have had the opportunity to work with and learn from Kursteen Price, Fred Bertley, Krikor Kichian, Koralia Kerketze, Annick Itié, John Ferrara, and Michel Emond.

I am indebted to Dr. Tom Seemayer for his collaboration with us in the histopathological studies, and for his time and enthusiasm in teaching me to interpret histological data. I would like to acknowledge Dr. André Veillette and his research assistant, Marielle Fournel, for reagents and assistance with Western immunoblotting. I would also like to thank Dr. Riaz Farookhi for allowing me to carry out Western blot experiments in his laboratory, and for his assistance, helpful suggestions and criticisms, and many interesting

discussions.

Finally, I am grateful for the constant support and encouragement of my parents, my sister Marie-Josée, my roommate and friend, Salimah Gillani, and my long-time friend Ruxandra Bunea, who have patiently put up with the erratic lifestyle of a lab-dweller over the past four years.

TABLE OF CONTENTS

University Guidelines for Thesis Preparation

Manuscripts and Authorship:

Candidates have the option, **subject to the approval of their Department,** of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis.

- If this option is chosen, **connecting texts, providing logical bridges between the different papers, are mandatory.**
- The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is more than a mere collection of manuscripts published or to be published. **The thesis must include, as separate chapters or sections:** (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion and/or summary.
- Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (eg. in appendices) to allow clear and precise judgement to be made of the importance and originality of the research reported in the thesis.
- In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent;** supervisors must attest to the accuracy of such claims at the Ph.D Oral Defense. Since the taks of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers.

Statement of Contribution to Original Knowledge

1. Levels of $p56^{lck}$ and $p59^{fyn}$ are decreased in T cells during GVHR.

2. The reduction in T cell lck and fyn during GVHR can be prevented by adrenalectomy of the recipients prior to GVHR induction.

3. The effect of adrenalectomy on the GVHR-induced reduction of lck and fyn is reversed by exogenous cortisone.

4. A decrease in T cell lck and fyn is induced in normal (non-GVHR) mice by an injection of cortisone.

5. Dexamethasone treatment of Jurkat cells (TH cell line) *in vitro* induces downregulation of lck and fyn.

6. Dexamethasone-induced downregulation of lck and fyn in Jurkat cells is reversible by RU 38486.

7. MHC class II expression is decreased in the GVHR-dysplastic thymus.

8. CD3 expression is decreased on $CD4^{+}8^{-}$ thymocytes during GVHR.

9. In GVH-reactive mice, the incidence of specific TCR-Vß segment usage becomes inconsistent, suggesting a GVHR-induced abnormality in positive selection.

10. Phenotypically autoreactive mature thymocytes are produced in thee thymus during GVHR, suggesting defective negative selection.

11. GVHR-induced cutaneous injury is exacerbated by local irradiation of the target tissue.

12. The formation of cutaneous lesions as a result of the interaction of systemic GVHR and local irradiation occurs specifically in tissues allogeneic to the donor, suggesting the involvement of donor T cells in the induction of tissue injury.

CHAPTER 1.

Introduction

1. The Graft-versus-host reaction: a brief history.

The graft-versus-host reaction (GVHR) has a rather convoluted early history. The idea of grafted tissue reacting against the host animal was first proposed in 1953 by Dempster (1) and Simonsen (2) as a result of their work in experimental renal transplantation. Ironically, their theory was based on a misinterpretation of their data, reached independently by both investigators (3). Proliferating cells observed in the grafted kidneys were erroneously assumed to be donor kidney graft mesenchymal cells responding to the host environment, giving rise to the notion of GVHR; actually these cells were most likely host-derived lymphoid elements reacting to the foreign graft in a standard host-versus-graft reaction. The first review of the newly described GVHR phenomenon observed that "we may eventually arrive at the interesting conclusion that the graft-versus-host concept in transplantation biology was perfectly sound, except for the experimental data on which it first had been based" (4).

GVHR was observed experimentally before its conceptualization as an immunological phenomenon. In 1916, grafts of adult splenic tissue onto the chorioallantoic membrane of embryonic chickens triggered GVHR-induced splenomegaly in the recipients (5). These findings were interpreted as an instance of "organ specific growth", an embryological theory in favor at the

time (5). In the early 1950s, experiments in transplantation tolerance involving the transfer of adult mouse splenocytes into allogeneic neonatal hosts mysteriously resulted in the death of up to 100% of recipients in certain strain combinations (4, 6). The term "runt disease" was coined to describe the syndrome of growth retardation, emaciation, splenomegaly, and diarrhea experienced by the transplanted neonates (7). Researchers using bone marrow transplantation (BMT) to rescue mice from lethal irradiation observed symptoms similar to those of runt disease in the bone marrow recipients; they termed the condition "homologous disease" (8) since it occured in response to a homologous graft, or "secondary disease" (9) since it followed radiation sickness. A reaction giving rise to the same pathology was induced in the absence of irradiation by transplanting parental strain cells into semi-allogeneic F1 hybrid recipients, and was designated "F1 hybrid disease", or "wasting disease" due to the marked cachexia of the recipient animals (10-13).

Simonsen and co-workers recognised that these experimentally induced disease states had a common immunological basis, and grouped them under the descriptive term of GVHR (4, 12). In 1962, he further formalized GVHR by defining the conditions under which it occurs and the parameters which influence its severity (4). He defined GVHR as occuring when 1) the graft contains immunocompetent cells; 2) there is an antigenic difference between donor and host; and 3) the recipient is unable to reject the graft. Thus, the

recipient may be immunoincompetent, as are neonates and irradiated recipients; or conversely, may be normal immunologically but blind to the donor antigens, as is the case of F1 recipients (12). The factors controlling the severity of the GVHR were considered to be 1) the strength of the antigenic stimulus, determined by the genetic differences between donor and host; 2) the number of grafted cells; and 3) the age of the recipient (4).

GVHR results in consistent, conspicuous pathological changes which historically were considered characteristic of the disease (4). Growth retardation, or runting, was the predominant gross characteristic noted in neonates and young animals. Splenomegaly, and to a lesser extent, hepatomegaly, was a predominant feature in all species examined, and in fact was used as an assay to grade the severity of GVHR (12). In the mouse, splenomegaly reached a maximum 8 to 10 days after grafting. Microscopically, the spleen lost its normal organisation and developed necrotic foci, which were also observed in the liver and in other organs such as the pancreas. Conversely, lymph nodes became involuted, although they may pass through a transient enlarged state. The thymus became atrophic by 12 to 14 days after grafting, and underwent profound histological changes. Histological changes in the bone marrow were accompanied by anemia, particularly in chickens. Finally, diarrhea and wasting were hallmarks of the reaction in all disease models. Although the pathological changes of GVHR have been recognised for

over 30 years, the challenge remains, now as then, to fully elucidate the cellular and molecular mechanisms producing these symptoms.

2. Experimental graft-versus-host reaction

GVHR has been studied in the laboratory both as a model for clinical GVHR, and for the insight it provides into basic immunological phenomena. Three principal types of experimental models have been used: neonatal or fetal recipients of adult allogeneic lymphoid cells; irradiated recipients of allogeneic or semi-allogeneic lymphoid cells; and F1 hybrid recipients of semi-allogeneic parental lymphoid cells. All three models correspond to the classical rules for producing GVHR, since the recipients are in each case unable to reject the donor cells, because of immunological immaturity, induced immunosuppression, or congenital tolerance to shared antigens, respectively. Neonatal GVHR was induced in chickens by placing the adult cells directly onto the chorioallantoic membrane (14), and in mice by injecting fetuses through the uterine wall or neonates into the head-vein (6, 15 - 17). This model system is no longer frequently used. Instead, investigators use irradiated hosts as an analogue of the clinical situation, in which the patient is often irradiated as part of the anti-neoplastic treatment or the pre-transplant conditioning regimen, to induce bone marrow aplasia which facilitates engraftment (18). In addition, if the host is lethally irradiated, bone marrow must be infused to rescue the animal; this system is used to study bone marrow engraftment and chimerism (19). In this laboratory, we induce GVHR by the transfer of parental lymphoid cells into unirradiated F1 hybrid

recipients, the so-called "P --> F1" model. The P --> F1 system allows the analysis of the reaction in its "pure" form, and is probably the most useful model for answering basic questions about the immune response.

What, then, *is* the P --> F1 GVHR? Essentially, it consists of an *in vivo* alloresponse by the donor T cells against the recipient histocompatibility antigens inherited from the second parent; it is an *in vivo* analogue of the *in vitro* mixed lymphocyte culture. *In vivo*, however, matters cannot remain quite so simple. As Simonsen commented in the first review written about GVHR, "...it is almost obvious that the pathological changes must be complicated, since the host cannot possibly be a passive medium in which the grafted cells are just stimulated and sustained for their own immune reaction" (4). The reaction can be considered in three main phases, distinct functionally though overlapping temporally, and of course linked causally:

1) the lymphoproliferative phase, consisting of a massive burst of T cell proliferation and cytokine production, followed by T cell anergy and death; 2) the phase of tissue injury, culminating in death from acute GVHR, or subsiding into chronic GVHR; and 3) the chronic phase, characterized by persistent immunosuppression and histopathology, and systemic autoimmune symptoms.

2.1 The lymphoproliferative phase and T cell anergy

2.1.1 The cellular requirements for GVHR induction

It is well established that T cells are necessary and sufficient to induce GVHR, although the requirement for T cells in the subsequent stage of histopathology are less well understood (20, 21). When T cells are depleted from the graft, GVHR does not occur (22). Further investigations have examined the requirements for various T cell subsets in GVHR induction. CD45 (T200, the common leukocyte antigen), a leukocyte cell surface molecule with tyrosine phosphatase activity, is expressed on T cells in a variety of isoforms distinguishable by monoclonal antibodies. CD45 isoform expression seems to correlate, to a certain extent, with a cell's antigenic experience or state of activation; in the rat, CD45RC is associated with naive or resting T cells, while CD45RO is expressed on memory or activated cells. Only cells of the naive or resting phenotype ($CD45RC^+$) are efficient at inducing GVHR (23).

The role of the $CD4^+$ and $CD8^+$ T cell subsets in GVHR induction is largely dependant on the antigenic differences between donor and host. CD4 binds to class II MHC, and thus restricts CD4-expressing T cells to reacting to MHC class II-peptide complexes; CD8 plays a parallel role in class I binding and restriction. Thus, in reactions limited to a class II MHC difference between

donor and host, $CD4^+$ cells are necessary and sufficient to induce a strong reaction (24). Many $CD4^+$ T cell clones have also been reported to induce GVHR when injected into mice expressing an appropriate MHC class II haplotype, and to produce GVHR tissue lesions, whether or not they displayed cytotoxic effector function *in vitro* (25 - 27). In reactions where the antigenic difference is exclusive to class I MHC, $CD8^+$ cells play the pivotal role (28). In fact, purified $CD8^+$ cells are able to induce GVHR even when the recipient is depleted of $CD4^+$ T cells, provided a class I antigen difference exists, indicating that $CD4^+$ cell-derived help is not essential for GVHR induction (29). The presence of donor and/or host $CD4^+$ cells, however, dramatically augments the reaction, despite the lack of class II MHC differences (29). In the case of boosting by host $CD4^+$ cells, a role for viruses has been postulated (30 - 34). In mice, the induction of endogenous retroviruses or the reactivation of latent viruses by immune activation may further activate host $CD4^+$ cells to provide help to the anti-host reactive, donor derived $CD8^+$ cells. Indeed, when infectious mouse cytomegalovirus (CMV) is injected into the host together with the donor lymphoid cells, the frequency of anti-host CTL precursors increases by 10-fold compared to recipients treated with lymphoid cells alone (32). Clinically, the reactivation of latent viruses, especially herpes viruses such as herpes simplex, varicella zoster, and CMV, has been well documented during GVHR, and correlates with increased GVHR severity (35). In reactions across a full major histocompatibility complex (MHC) difference, where the donor and

host differ at both class I and class II loci, the two subsets exert a synergistic effect; the severity of the reaction increases dramatically when both subsets are present (28).

GVHR can also be induced when the donor and host differ only by minor histocompatibility antigens (mHA) (36). Clinically, in related donor marrow transplants where complete HLA matching is possible, this is generally the case. Experimentally, mHA GVHR is often used as a model for chronic GVHR since it usually results in a comparatively mild reaction with little or no mortality. Most reports indicate that $CD8^+$ cells are required for inducing a GVHR to mHA (28, 29, 37). Since most mHA are probably self-peptides bound to class I MHC molecules, acquired intracellularly in the endoplasmic reticulum and then expressed on the cell surface (38), the response would be expected to be class I restricted. There are some cases of anti-mHA GVHR mediated predominantly by $CD4^+$ T cells (39 - 41); these may represent the superantigen products of endogenous viruses (39), which bind to MHC class II (42), or extracellular proteins such as serum components acquired by the host antigen presenting cell by endocytosis and presented in association with host MHC class II on the cell surface.

2.1.2. Proliferation

T cells, then, are the effectors in GVHR induction. Once they enter the host, they encounter allo-MHC antigens: host MHC associated with a vast array of peptides, generating enough antigenic diversity to activate a very high proportion of the donor T cell inoculum (43). It is commonly accepted that a massive T cell proliferation ensues. Clinical reports have extensively documented the frequency of donor anti-host CTL (44 - 46) and IL-2-producing (47 - 50) precursors in the graft, and have described a correlation between these frequencies and the incidence and severity of GVHR. However, very little experimental data documenting the actual frequency of activated cells *in vivo* has been published. An interesting series of studies has examined the frequency of cells activated *in vivo* by sub-optimally restimulating them *in vitro* in limiting dilution analysis, and assaying their cytokine production (51 - 55). In normal mice, only 0.03 - 1% of T cells responded to the sub-optimal activation stimulus by secreting cytokines, indicating prior activation; in contrast, up to 70% of the T cells from the proliferative stage of GVHR responded in their assay (51). This frequency of responder cells is higher than would be expected on the basis of alloreactivity alone (52), suggesting that *in vitro* estimates of alloreactive precursors represent an underestimate, or that the T cells are responding to additional activation stimuli during early GVHR. In fact, host cells are also believed to undergo some degree of proliferation, although the impetus for host cell activation is poorly understood (56, 57). Two possibilities have been suggested. Endogenous virus may be reactivated as

discussed earlier, thus stimulating a host anti-viral memory response (30 - 34). The donor cells themselves may also provide some degree of stimulation to the host cells; during donor T cell recognition of a host T cell, the host's MHC antigens, as well as as various co-stimulatory molecules such as adhesion molecules, would be ligated. Together with the proximate secretion of IL-2 by the activated donor T cell, these combined stimuli might prove sufficient for at least partial activation of host cells. In addition, some peptides bound by donor class I MHC may be absent from the same MHC antigens on F1 cells, due to competition from parent-2 derived peptides in the F1 cells. This would create a mHA antigen on the donor cells to which the recipients' cells could react (38).

In any case, most of the proliferating cells in the early GVH-reactive spleen are of donor origin (58). Although the kinetics vary depending on the strain combination (which determines the strength of the antigenic stimulus), generally the T cell proliferation begins within 48 hours of grafting, and peaks 5 to 7 days after induction. At the peak of donor cell proliferation, host T cells begin to die, probably as a result of direct donor cell-mediated anti-host cytotoxicity. Then, around 9 days after GVHR induction, the donor cells gradually become anergic and begin to die (58). The ultimate extent of T cell death is related to the severity of the reaction, determined by the number of T cells transfered and the strain combination; however, eventually most of the

remaining T cells become unresponsive to all activation stimuli, giving rise to the profound T cell suppression characteristic of GVHR (20, 21).

2.1.3. Cytokine production

During the proliferative phase, the activated T cells also produce interleukins. mRNA transcripts for IL-2 can be detected in the spleen within 24 hours of grafting (59), and high levels of IL-2 are produced throughout the first week post transplant (57, 60). Interferon-gamma (IFN-γ) is also produced at elevated levels, and can be measured in the serum of patients preceeding the clinical manifestations of acute GVHR (61). A Th1-like cytokine profile, dominated by IL-2 and IFN-γ, is established in early GVHR (62 - 64). Tumor necrosis factor-α (TNF-α) mRNA is increased approximately 4-fold in the first week after transplantation (65), suggesting a T cell source, since T cell activation is maximal at this point and macrophage have not yet been fully triggered for TNF-α production (66). Elevated levels f mRNA for IL-3 and GM-CSF are also observed (51); however, serum concentrations of these cytokines are normal or even decreased (67, 68). These seemingly contradictory findings are reconciled by the observation that splenocytes have highly upregulated IL-3 and GM-CSF receptors, in effect acting as a sink and preventing serum levels from rising (69). In the clinical setting, T cell-derived GM-CSF and IL-3 are likely to contribute to successful marrow engraftment and early recovery of

leuko- and hemato-poiesis (70). Macrophage/monocyte cytokines also play a pivotal role in GVHR (62 - 66). The level of IL-1 transcripts is elevated 100-fold in the spleen during early GVHR (65), and *in vivo* treatment with anti-IL-1 antibodies prevents GVHR in mice (65, 71). Macrophage-derived TNF-α is implicated in the pathology of tissue injury and acute death in the next phase of the GVHR (66, 72 - 77).

2.1.4. Anergy

The profound T cell unresponsiveness which follows the lymphoproliferative phase is a hallmark of GVHR (20, 21). It is the result of the interaction of many factors; suppressor cells, humoral factors, and a T cell signalling defect have all been implicated.

When splenocytes from immunosuppressed GVH-reactive mice are co-cultured with normal, syngeneic spleen cells, the normal cells also become suppressed, indicating the presence of suppressor cell(s) or production of suppressive factor(s) (20, 78, 79). Specific T suppressor cells were identified phenotypically as Thy 1^+ $CD8^+$ cells of donor origin, mediating an anti-donor suppressive effect (80). These may potentially represent the cytostatic and cytotoxic effects of donor anti-host CTLs (20). In addition, Thy 1^- $CD4^-$ $CD8^-$ non-adherent large granular lymphocytes devoid of NK activity, a profile which corresponds to

natural suppressor cells, have been isolated from GVH-reactive animals and shown to mediate a non-specific suppressive effect on both host and donor-derived T cells (81, 82). This suppressive activity may be due in part to prostaglandin production, since it is partially reversible by addition of indomethacin to the cultures (81). Macrophages also produce elevated amounts of immunosuppressive prostaglandins during the first month after GVHR induction, and no doubt this contributes to the general immune suppression (20, 83). Another potently immunosuppressive molecule elaborated by activated macrophages is nitric oxide (NO) (84). NO degradation products have been demonstrated in the sera of animals undergoing acute GVHR (85). *In vitro* inhibition of NO synthesis in GVH-reactive splenocyte cultures partially reverses the T cell unresponsiveness (84). IFN-γ, produced by the T cells themselves, is also supressive; some T cell function is restored *in vitro* by addition of anti-IFN-γ antibody to the cultures (86 - 88). Thus, a combination of specific and non-specific cell functions and humoral factors contributes to the generalised immunosuppression, at least during the first month after transplantation.

However, when T cells are isolated from experimental animals or patients undergoing GVHR, they remain unresponsive to antigenic and mitogenic stimuli, even when cultured with antigen presenting cells from normal (non-GVHR) animals (89, 90). This complete unresponsiveness was not transferable

to co-cultured cells. It is this state of cellular unresponsiveness that is also refered to as anergy; that is, the cell is viable, but unable to respond to activation stimuli. This use of the term anergy does not imply the ability to recover function; the anergy may indeed be an irreversible stage prior to death. However, the presence of these cells in the GVH-reactive animals and patients in the absence of significant thymic function suggests that at least some cells remain in an anergic, or unresponsive, state for considerable periods of time. The factors inducing the T cell anergy during GVHR are poorly understood.

The anergic GVH-reactive T cells do not proliferate, nor do they produce detectable levels of cytokines, in response to a variety of powerful activation stimuli: mitogens such as concanavalin A and phytohemaglutinin (PHA); a combination of PHA and phorbol myristic acetate (PMA, a phorbol ester capable of activating protein kinase C, PKC); anti CD3 antibodies, either alone or in combination with IL-2 or IL-4; and allogeneic cells (89 - 91). In addition, GVH-reactive cells, from both experimental animals and patients, are unable to produce a normal calcium flux in response to TCR ligation (89, 92). On the other hand, the anergic cells express normal cell surface levels of the T cell receptor for antigen (TCR) and its associated signalling complex (CD3), of the CD4 or CD8 coreceptors, and of the IL-2 receptor (89). Finally, the cells can be activated to normal levels by a combination of PMA and calcium ionophore; but as the concentration of ionophore is decreased, the cells very quickly lose

the ability to respond (89, 92).

This spectrum of dysfunction suggests that the molecular defect may lie upstream in the TCR-mediated signalling pathway. T cell activation involves signalling through two major pathways, initiated via the TCR and the IL-2r respectively (93). TCR signaling results in early tyrosine phosphorylation of the CD3 chains, particularly CD3-ζ, likely induced by receptor associated src-family tyrosine kinases $p56^{lck}$ (lck) and $p59^{fyn}$ (fyn) (93 - 95). A possible scenario involves the activation of lck, associated non-covalently with CD4 or CD8 by cysteine-coordinated zinc (96). Activation of lck is induced by dephosphorylation of its negative regulatory tyrosine residue 505 (97), potentially by the abundant membrane-associated tyrosine phosphatase CD45 (98). The activated lck is brought into proximity of the CD3 chains by the extracellular cross-linking of CD3 and CD4/8 during their interaction with the same MHC/peptide complex on the APC. Lck can phosphorylate tyrosine residues on the CD3 chains, allowing the transmission of downstream signals; this signalling may be amplified by CD3-associated fyn (99 - 101). An additional level of proximal signal amplification may be provided by the tyrosine kinase ZAP-70 (70 kD zeta-associated protein) (102). The activation and association of ZAP-70 with the TCR/CD3 complex is believed to be dependent on lck- and/or fyn-mediated phosphorylation of the three antigen recognition activation motifs (ARAMs) on CD3 ζ (as well as single motifs on

CD3 γ, δ and ζ) (102 - 104). Once activated, these kinases may mediate further downstream signals. Signalling through the TCR ultimately leads to a calcium flux via phosphatidyl inositol metabolism, and to a cascade of protein phosphorylations via PKC activation (93). Steps upstream of these events can be bypassed by treatment of the cell with ionophore to induce the calcium flux, and with PMA to activate PKC. Since GVHR-anergized T cells can be restored to normal levels of proliferation with PMA and ionomycin, the molecular lesion lies upstream, and the downstream cascade appears intact (89, 92).

The biochemical profile of the GVHR-anergized T cells distinguishes GVHR-induced anergy from other previously described anergic states. Antigen-specific unresponsiveness was demonstrated by chemically modifying cells, then transfering them into allogeneic recipients, who were subsequently specifically tolerant to the strain of the injected cells (105). It was later shown that the chemical modification destroyed cell surface molecules with "co-stimulatory" function. When T cells are activated through their TCR in the absence of costimulatory signalling, they are activated for the primary response, but then become refractory to further restimulation (106 - 108). The pair of molecules recently identified as being the principal effector of costimulation consists of CD28, expressed on the T cell, and BB1/B7, expressed on the APC (106). Other molecules such as ICAM-1 and LFA-1 have also been implicated in costimulation and anergy (109). The biochemical basis for anergy

following activation in the absence of costimulation appears to be a dysregulation in the IL-2r signalling pathway (107). Unlike GVHR-induced anergy, this type of unresponsiveness is reversible by the addition of exogenous IL-2 (89, 90, 107), suggesting that GVHR-induced T cell anergy may represent a biochemically distinct state, thus probably induced by a different molecular mechanism. A second important case of specific T cell unresponsiveness is the acquisition of peripheral tolerance to extra-thymic antigens. In transgenic models where an antigen, for example a "foreign" MHC molecule, is expressed in an organ-specific manner, phenotypically reactive but functionally inactive T cells are found in the periphery (110 - 112). This situation most likely represents a physiological instance of the *in vitro* model of activation in the absence of costimulation. The T cell encounters its specific antigen on a normal tissue cell, devoid of "professional" antigen presenting capacity; although it might undergo an initial activation, it will be anergized for subsequent encounters and thus will be unable to mount an autoimmune inflammatory response. Precisely the opposite situation occurs in GVHR; the newly infused cells will be more likely to encounter a "professional" APC, expressing all the appropriate co-stimulatory molecules, since cells injected intravenously tend to accumulate in the spleen, liver and lungs, where they will encounter dendritic cells and marophages, alveolar macrophages, and Kupffer cells, respectively. Furthermore, in a reaction induced with $CD4^+$ cells across an MHC class II barrier, most of the cells carrying the appropriate

alloantigen, MHC class II, would most likely be dedicated APCs and able to provide excellent costimulation. This reasoning provides a physiological, as well as a biochemical, rationale for a different mechanism in GVHR-induced anergy.

Immune suppression during GVHR is not limited to the T cells compartment. A profound B cell suppression develops in parallel, but is mainly attributable to the physical disappearance of peripheral B cells and bone marrow B cell progenitors, possibly as a result of direct cell killing by donor anti-host CTLs (113). What, then, allows the profoundly immunosuppressed animal to survive? It has long been known that bactericidal activity increases during GVHR (114); in retrospect this is attributable to IFN-γ-primed macrophages. The non-specific immune response mediated by macrophages and NK cells becomes activated by the heightened T cell production of IFN-γ during early GVHR, and remains hyperactive long after the initial proliferative phase is over (65, 66). This sets the stage for the pathogenesis occuring during the next phase of the GVHR.

2.2 Pathological tissue injury and acute GVHR

GVHR is characterised by extensive lesions in most organs and tissues. The

lymphoid organs and epithelial tissues are the most commonly and severely affected (113, 115 - 123).

There is no distinct temporal separation between the phases of lymphoproliferation and histopathological injury; T cell proliferation and cytokine hyperproduction are ongoing during the early stages of tissue injury. However, the lymphoproliferative phase is required to trigger the pathological consequences of GVHR, and does so by inducing three principal effects: 1) the antigenic modulation of host target structures; 2) the generation of donor anti-host effector function; and 3) the priming of non-specific effector cells of host and donor origin.

2.2.1 Antigenic modulation of host target structures

IFN-γ produced as a consequence of T cell activation upregulates the expression of adhesion molecules and MHC antigens. Endothelial leukocyte adhesion molecule 1 (ELAM-1) and vascular cell adhesion molecule 1 (VCAM-1), involved in the migration of leukocytes into tissues, are increased in clinical GVHR (124, 125). Intercellular adhesion molecule 1 (ICAM-1, CD54), a co-stimulating molecule for antigen presentation, is upregulated on keratinocytes in patients undergoing GVHR (126). MHC class I and II expression is increased on multiple lymphoid and epithelial tissues, including interstitial

dendritic cells, microglia, Kupffer cells (127), epidermal keratinocytes (128 - 134), gut (130, 134, 135) and renal tubule epithelium (136), central nervous system parenchyma and endothelium (137, 138), the secretory duct cells of the submandibular salivary gland (139), and bile duct epithelium (140, 141). These findings suggest that cytokine-induced upregulation of adhesion molecules and MHC antigens could facilitate the migration of activated, donor anti-host effector cells into the tissues, and enhance their binding to target cells such as keratinocytes (128, 133, 136, 142). Interferon-induced upregulation of MHC class II antigens on epithelial cells preceded the appearance of tissue injury, and the degree of upregulation predicted the extent of subsequent histological injury (127). These observations are consistent with initial tissue injury mediated by alloactivated T cells reacting to host MHC antigens.

2.2.2 Generation of donor anti-host effector function

In fact, phenotypic characterisation of cells infiltrating the skin and the gut during clinical and experimental GVHR reveal heterogeneous cell types, although $CD3^+$ T cells are almost always present (143 - 156). Among the T cells, the ratio of $CD4^+$ (helper) to $CD8^+$ (cytotoxic) cells varies (143 - 149), and large granular lymphocytes (150), NK cells (151 - 155), and gamma/delta T cells (156) have also been reported. In general, $CD3^+CD4^+$ cells are more

numerous early in the reaction, while $CD3^+CD8^+$ and NK cells are predominant in chronic GVHR lesions (157). A model for the formation of GVHR epithelial lesions may be formulated: donor $CD4^+$ cells, activated by their interaction with host antigen presenting cells in the spleen, migrate into the tissues by interacting with the upregulated endothelial adhesion molecules. In the tissues, recognition of the aberrantly expressed host class II antigens triggers the T cells to bind to the target cell and secrete cytokines such as IL-2, IFN-γ, and TNF-α, which may directly induce epithelial cell death (158), or may recruit $CD8^+$ CTLs and NK cells to the site. In fact, all three cell types have been described in close apposition to necrotic keratinocytes in the lesional skin of GVH-reactive mice and humans (151, 155). This scenario assumes a class II MHC difference between donor and host to allow $CD4^+$ helper cell priming and recognition of allo-MHC class II as a target antigen. However, GVHR induced across class I MHC barriers alone, or between MHC matched animals with only minor histocompatibility differences, also results in epithelial cell lesions, albeit generally less severe (29). Compelling evidence exists for the involvement of viruses in the pathology of GVHR induced across class I MHC barriers (30 - 34). In mice, concurrent infection with CMV during GVHR resulted in a marked exacerbation of the reaction characterised by a 10-fold increase in the frequency of anti-host CTLs and enhanced NK activity (32). This effect is not seen with non-viral antigens, nor with non-infectious virus (33). Depletion of $CD8^+$ cells from the inoculum used to induce GVHR, or in

vivo depletion of $CD4^+$ (but not $CD8^+$) cells from the host, prevented the virally-induced exacerbation of the reaction (34). Based on these findings, it is thought that the virus primes the host $CD4^+$ cells for lymphokine production, and that these cells can then provide help for the activation of donor anti-host $CD8^+$ (class I restricted) CTLs (30 - 34). Human statistical data and clinical case reports support this hypothesis: seropositivity for herpes simplex and varicella zoster increased the risk of acute GVHR, while immunity to CMV was associated with a higher risk of chronic GVHR (35). In one case, cutaneous GVHR lesions appeared specifically over an area of measles exanthem, suggesting a focal role for viral antigens (159). Viral infections are very common during clinical GVHR; in fact CMV infection is present in approximately 50% of BMT recipients (160). Viral infections may be acquired *de novo* from the grafted marrow or transfused blood of seropositive donors, or, more commonly, represent reactivation of latent viruses during pre-transplant conditioning immunosuppression (160).

T cells are undoubtably the cells responsible for triggering GVHR, and are probably involved in initiating the pathological injury as well. However, as the GVHR progresses a profound T cell immunosuppression develops. T cell involvement in the tissue lesions, then, may be relatively short lived, perhaps limited to their induction, but not perpetuation. It might be argued that although splenic and lymph node T cell function is severely impaired as early

as 7 to 10 days after GVHR induction, similar dysfunction of the T cells infiltrating the tissues has not been documented; it is possible that T cells segregated in the tissues and thus subjected to a vastly different microenvironment may escape the suppression. However, experiments performed with NK-deficient donors (*beige* mice) demonstrate that T cell-dependent splenomegaly occured, but persistent tissue lesions did not, in the absence of donor-derived NK cells (161 - 163). These findings suggested that NK cells mediate much of the tissue injury once the severe T cell suppression appears. Since NK cells are relatively non-specific effectors (or at least, we don't know the specific target structure to which they're responding), the site of the tissue injury may be determined by the allo-activated T cells responding to host MHC, thus accounting for the distribution of the severe lesions to epithelial cells which can be induced to upregulate MHC antigens, in particular class II, by IFN-γ.

2.2.3 Priming of non-specific effector cells of host and donor origin

Possibly the most serious consequence of the massive cytokine production during the lymphoproliferative phase of GVHR is IFN-induced non-specific NK cell activation and macrophage priming.

As discussed above, NK cells activated by T cell-derived IFN-γ may perpetuate

tissue damage triggered by T cells. The gastrointestinal (GI) tract is an important site of pathology, characterized by crypt hyperplasia, villus atrophy, and areas of necrotic epithelium. Injection of TNF-α is sufficient to induce GI pathology resembling that of GVHR, suggesting that the T cell derived TNF-α alone may be sufficient to induce some degree of damage even before cellular infiltration of the gut (158). Once IFN-γ activates NK cells, they contribute to the injury, as shown by the prevention of intestinal lesions in anti-IFN-γ-treated GVH-reactive mice (164), or in animals depleted of NK cells (165). Conversely, when NK cell activation is further boosted with poly I:C, GVHR-induced pathology is exacerbated (166). Presumably in response to the intestinal injury, bacteria are translocated from the GI tract (167, 168), resulting in the presence of lipopolysaccharide from gram negative bacteria (LPS) in the spleen, liver and serum of GVH-reactive mice (K.S. Price *et al*, manuscript in preparation). LPS is a potent trigger for the production of inflammatory cytokines by primed macrophages, and the macrophages of GVH-reactive mice have been primed by T cell derived IFN-γ (66). Thus, two lethal feedback loops are established. In the first loop, LPS from the damaged gut triggers the IFN-γ-primed macrophages to produce TNF-α and nitric oxide (NO) (169, 170), resulting in further damage to the GI tract, and in turn, more LPS entering the animal. In the second loop, the macrophages (primed by IFN-γ and triggered by LPS) produce IL-12 (171), which stimulates NK activity, resulting in further tissue damage, including gut injury (ensuring a

plentiful LPS supply) (K. Kichian *et al*, manuscript in preparation). Furthermore, the activated NK cells secrete IFN-γ, thus maintaining macrophage priming despite the absence of functional T cells, and completing the loop. In severe GVHR, induced with a large cell dose and/or across a multiple (class I and II) MHC barrier, these feedback cycles may result in death from acute GVHR, manifested as TNF-α-induced septic shock (66). In fact, TNF-α can be detected in the serum during acute GVHR (172). Treatment of acute GVHR patients with anti-TNF-α antibodies (173) significantly alleviates GI pathology; and thalidomide, a pharmacologic TNF-α antagonists, proved beneficial in the treatment of chronic GVHR (174 - 176). Attempts have also been made to interrupt the feedback cycle at the LPS stage: complete GI decontamination with antibiotics has been very successful at reducing the frequency of clinical acute GVHR and its associated mortality (177, 178). It is interesting to note that this model also explains some old observations. In 1950, death from parabiosis intoxication (in which one or both animals of the pair usually underwent a reaction with the same immunological basis as GVHR (179)) was reported to be by septic shock (180). Another long-unexplained finding was that gnotobiotic mice failed to succumbed to GVHRs which would be lethal to the same mice raised in conventional housing (181).

In a less severe GVHR, induced with a lower T cell dose and/or across only multiple minor histocompatibility differences, the same mechanisms likely lead

to the development of chronic GVHR: persistent low grade tissue injury characterized clinically by skin rash, diarrhea, elevated liver enzymes, immunosuppression, and generalised autoimmune symptoms.

2.3 Chronic GVHR and autoimmunity

Mice (and patients) who survive acute GVHR may go on to develop chronic GVHR. During chronic GVHR, the T cell immunosuppression remains profound; patients with chronic GVHR still have marked defects of T cell proliferation and cytokine expression more than four years post transplant (182 - 184). Constitutive macrophage activation persists, as shown by elevated IL-1 mRNA transcripts throughout chronic GVHR (65), affording some immune protection to the suppressed host. Paradoxically, despite the T cell suppression, systemic autoimmune symptoms develop, resulting in a syndrome similar to scleroderma or systemic lupus erythomatosis (SLE) in murine models and patients (185, 186).

By one month after GVHR induction in mice, GVHR splenocytes no longer induced marked suppression in co-cultured normal cells (20, 78). Despite this, normal peripheral T cell function is not being reconstituted by new thymic emigrants. Taken together with the tissue lesions induced by GVHR, which

includes extensive thymic injury (120 - 123), these observations suggest that we look to the thymus for an explanation of the immune disorder of chronic GVHR.

2.3.1 GVHR-induced thymic pathology

The thymus provides the microenvironment in which T cells mature and are selected. Pre-T cells produced by the bone marrow enter the thymus as $CD4^-8^-3^-$ precursors; as they mature, they acquire the expression of CD4 and CD8 molecules. These immature, cortisone-sensitive thymocytes then begin to express low levels of CD3/TCR ($CD3^{lo}$), enabling them to interact with MHC/peptide complexes and undergo selection. As they mature and are selected in the thymic microenvironment, they upregulate CD3/TCR to peripheral levels ($CD3^{hi}$), and lose expression of one of the two accessory molecules to become mature, cortisone-resistant single positive $CD4^+CD8^-$ or $CD4^-CD8^+$ cells (187). The transition from $CD3^{lo}$ to $CD3^{hi}$, an upregulation of approximately an order of magnitude, requires the thymic microenvironment and is thought to be a consequence of positive selection (187).

Thymic stromal components positively select T cells which are able to interact at low affinity with self MHC molecules, while bone-marrow derived elements mediate the deletion of potentially autoreactive cells (188 - 190). Thymic

epithelium can also induce tolerance, but by a different mechanism; the cells are anergized but not deleted (functional as opposed to physical deletion) (191). These intrathymic events are believed to be primarily responsible for the self-restriction and self-tolerance of the peripheral T cell repertoire (192). Antibody-mediated blocking of the interactions between thymic MHC molecules and the CD4 or CD8 accessory molecules on immature T cells results in defective thymic selection (193 - 195), as does the absence of these molecules in transgenic mice with deletion mutations (196 - 201). In the absence of class I MHC (in ß2-microglobulin knockout mice), $CD8^+$ T cells are not produced in the thymus (196). In contrast, in knockout mice genetically devoid of class II MHC (197, 198), or with a defect in class II MHC expression due to the absence of the invariant chain (199), some $CD4^+$ cells are produced, but they are abnormal functionally and phenotypically. Thus, disruption of the molecular interactions leading to T cell education may prevent the cells from maturing, or may allow functionally aberrant cells to survive.

During GVHR, the thymus develops lesions of the thymic stroma and exhibits changes in thymocyte subsets (120 - 123). Stromal damage includes injury of the medullary epithelial cells, which progressively decrease in numbers during the reaction; effacement of the corticomedullary junction, with the whole thymic structure becoming disordered, and taking on a more medullary appearance; and disappearance of Hassal's corpuscles (120 - 122). The changes

in the thymocyte subsets are cortisone-dependent, since they are prevented by adrenalectomy of the host animals prior to GVHR induction (123). Cortisone-mediated effects include death of the immature $CD4^+8^+$ thymocytes, providing the major contribution to thymic atrophy; in addition, the more mature $CD4^+CD8^-$ thymocytes which are cortisone resistant in normal mice fail to acquire cortisone resistance during GVHR, resulting in a reversal of the thymic ratio of $CD4^+8^-$ to $CD4^-8^+$ from 3:1 to less than 1:3 (123). The GVHR-induced thymic histological abnormalities persist well into the chronic phase of the reaction; in fact, the return of peripheral T cell function correlates with the recovery of the thymic stromal damage in our murine model (202). These findings suggest that the GVHR-induced thymic damage may prevent the newly generated T cell precursors from acquiring normal self-restriction and self-tolerance, resulting in the peripheral manifestations of autoimmunity and immunosuppression which characterize chronic GVHR. To further support this hypothesis, similar thymic damage accompanied by generalised T cell-mediated autoimmune disease has been reported in mice and humans undergoing syngeneic GVHR (sGVHR) (203 - 211). sGVHR is induced by host irradiation followed by the transplantation of autologous cells and treatment with cyclosporine A (CsA), an immunosuppressive drug which inhibits calcineurin function, thus blocking the IL-2r signalling pathway and causing multiple abnormalities in T cell function and cytokine metabolism (212). Recently, changes in thymic function accompanied by peripheral T cell induced

autoimmunity have been reported in GVH-reactive mice treated with the anti-fungal immunosuppressive drug rapamycin (213), and in irradiated autologous BMT recipients treated with the pharmacologic calcineurin inhibitor FK 506 (214). Thus GVHR-induced alterations of the thymic stroma, coupled with late effects of the acute phase cytokine dysregulation, are likely to affect T cell maturation and selection and thus their subsequent functional abnormalities in the periphery.

2.3.2 Proliferative GVHR : a mechanistically distinct model for chronic GVHR

The GVHR described so far, and used for the experiments in this thesis, results in a profound, early-onset, and long duration immune suppression. In contrast, the induction of GVHR in some strain combinations results in a very different type of response characterized by a marked B-cell proliferation, hyper-immunoglobulin production, and systemic autoimmunity (215 - 223). These two types of reaction have been called "suppressive" and "proliferative", respectively. The proliferative GVHR does not correspond to the lymphoproliferative stage of suppressive GVHR, although the nomenclature is confusing. Classically, proliferative GVHR has been studied in the DBA/2 --> C57BL/6 x DBA/2 F1 hybrid strain combination, and contrasted to induction by C57BL/6 --> C57BL/6 x DBA/2, which results in a suppressive reaction. This model is particularly appealing since the same F1 recipient develops

completely different syndromes, depending only upon which parental strain cells are transplanted. Genetic analysis has revealed that the proliferative versus suppressive outcome of the reaction is controlled by a single, non-MHC linked locus, located on chromosome 7 (224).

The proliferative reaction consists of a cognate activation of host B cells by donor $CD4^+$ T cells, which secrete high levels of IL-4 (216 - 219, 221, 223, 225). The host B cells proliferate and produce large quantities of IgG1, IgG2a, and IgE; the serum Ig levels are 10 times higher than normal for the IgG isotypes, and 100 to 200-fold increased for IgE (221). Many autoreactive antibodies are produced, including rheumatoid factor, and anti-chromatin, anti-dsDNA, anti-mitochondrial, anti-nuclear and anti-nucleolar specificities (217, 225, 226 - 231). Although an association between autoimmune antibody and $CD5^+$ B cells (B1 cells) has been suggested in other systems (232), the cellular source of the abnormal antibody in the GVHR is conventional B cells (233). The consequent pathology includes manifestations of collagen-vascular disease, syndromes resembling SLE, scleroderma, and polymyositis, and glomerulonephritis (220, 222, 229 - 231, 234 - 236). The antibody specificities and pathology seen in proliferative GVHR is similar to those of many clinical systemic autoimmune diseases, and provides a good model for scleroderma, SLE, and glomerulonephritis. The induction of proliferative GVHR and subsequent pathology can be prevented by depleting the donor inoculum of $CD4^+$ cells, or

the recipient of B cells, or by treating the recipient with monoclonal anti-IL-4 antibodies (218).

Immunosuppression is also a feature of the proliferative reaction; however, it does not involve a T cell signalling defect, but rather consists of cytokine-mediated suppression of certain specific functions (219). Production of IL-10 from both lymphoid and monocytic sources is considerably elevated; this, together with the high IL-4 secretion, causes suppression of IL-2 and IFN-γ (219). Indeed, mRNA levels of IL-2 are below normal in animals undergoing proliferative GVHR, and delayed type hypersensitivity reactivity is highly impaired (219, 237). Reports vary on the degree of suppression of IFN-γ production; it appears to be somewhat suppressed, but the amounts which are produced contribute to the hyper-IgE secretion (216, 219, 221). Hence, treatment of the recipient with anti-IFN-γ antibodies decreases the serum IgE levels, but does not alter IgG levels nor abolish pathology (221). The cytokine profile demonstrated during this reaction (high IL-4 and IL-10, low IL-2 and IFN-γ) account for the pathologic features of B cell immunoproliferation and antibody secretion together with suppression of cell mediated immunity (219).

Immunoproliferative GVHR, then, appears to be a Th2 mediated disease, in contrast to the suppressive reaction, which has features of a Th1 response. The genetically controlled response to the same (F1) antigens giving rise to a

Th1 versus Th2 response is reminiscent of Leishmania infection in mice (238). C57BL/6 (resistant) mice infected with the parasite develop a protective Th1 response, and survive, while Balb/c (sensitive) mice produce a Th2 reaction, and succumb to lethal parasitemia. Resistant mice become sensitive if they are rendered genetically incapable of IFN-γ production (IFN-γ knockout mice) (239). Interestingly, in the GVHR model, a different manipulation achieves the same effect. Recipient mice are sub-lethally irradiated and allowed to recover for 2 to 3 months. Th2 type cells recover prior to Th1 cells after irradiation; the pre-irradiation splenic ratio of 1 Th1 : 0.6 Th2 cells (assayed by *in vitro* cytokine profiles) in CBA mice decreases to 1 : 7 three months post-irradiation (240). After recovery from irradiation, these Th2 biased recipients are grafted with lymphoid cells from a strain which would normally provoke a suppressive GVHR. These mice, however, developed a proliferative disease, demonstrating that the cytokine potential of the host T cells may also affect the outcome. Since many patients are irradiated prior to transplant, this mechanism may account for some of the clinical cases of proliferative GVHR. In addition, proliferative GVHR has been reported in a series of cases of severe combined immune deficiency (SCID) patients receiving BMT to correct their congenital defect (241); the SCID *in vivo* environment may be similar to that of a mildly irradiated host in its ability to potentiate Th2 responses.

The proliferative experimental model gives rise to some ambiguity in the

GVHR nomenclature. In general, "chronic GVHR" refers to the syndrome experienced by survivors of the acute suppressive reaction, and is considered clinically as occuring 3 months or more after transplantation. However, as many of the symptoms of clinical chronic GVHR are seen in proliferative GVHR, this experimental model is also often termed "chronic GVHR". These probably represent different mechanisms of autoimmune induction; the former depends on thymic damage and is analogous to CsA-mediated autoreactivity, and the latter represents peripheral cytokine dysregulation, similar to the pathological switch to Th2 cytokines during some parasitic infections, and perhaps during AIDS (242 - 243). Both models appear to have clinical correlates in GVH-reactive patients.

3. Clinical graft-versus-host reaction: incidence, prevention and treatment.

Clinical GVHR can develop whenever Simonsen's classical conditions are met. Most frequently, this occurs during allogeneic BMT. Every effort is made to ensure optimal HLA matching, in order to minimize the antigenic differences between donor and recipient. However, only 20 to 25% of patients requiring a BMT will have a sibling with a fully matched HLA locus; other patients must resort to unrelated matched donors (244). The incidence of GVHR depends on the initial disease, the pre-transplant preparative regimen, the method of treating the bone marrow, the post-transplant GVHR prophylaxis, the patients' age, the experience of the transplant centre, and the extent of disparity between donor and host mHA (for example, female --> male transplants are associated with increased risks); however, the overall incidence of acute GVHR in matched sibling grafts is around 40 to 60%, and 50 to 75% in unrelated donor BMT (245 - 248). About half of the patients with acute GVHR will go on to develop chronic GVHR, resulting in considerable GVHR-related morbidity (245, 248). GVHR-related mortality is usually associated with the induced immune suppression, commonly resulting in lethal sepsis and pneumonias (249, 250). Thus, GVHR is the principal barrier to the extensive and successful use of BMT in treating a wide range of diseases. Nevertheless, BMT is presently the treatment of choice for most leukemias and some lymphomas (245 - 248, 251 - 253), and is the only treatment for accidental gamma

irradiation (254); in addition, it has been used to treat aplastic anemia (255), certain solid tumors (256), and genetic disorders including sickle cell anemia (257), thalassemia (258), Hurler's mucopolysaccharidosis (259), Wiscott-Aldrich disease (259), Chediak-Higashi disease (259), and more commonly, SCID (259, 260).

Situations other than BMT which also meet Simonsen's conditions arise clinically. In gut, lung and liver transplants, a significant amount of donor lymphoid tissue is present in the graft, and concomittant graft-versus-host and host-versus-graft reactions may occur (261 - 264). Blood transfusions can lead to very severe acute GVHR if the recipient is immunosuppressed, for example in neonates and patients with immune deficiencies such as AIDS; in a series of transfusion-associated GVHR, mortality was reported at 90% (265 - 267). Blood transfered from mother to fetus during gestation or delivery can lead to immunosuppression diagnosed as SCID, but actually represents a transfusion-induced GVHR (268 - 270).

Many strategies have been developed to prevent GVHR; however, the multiplicity of differing protocols in use at various transplant centers shows that no single formula is entirely effective. Two main approaches to GVHR prophylaxis are used clinically; 1) T cell depletion of the host marrow; and 2) pharmacologic immunosuppressive therapy of the recipient. Since T cells

trigger the GVHR, it was assumed that T cell depletion would prevent GVHR. Indeed, recipients of depleted marrow seldom develop even mild GVHR (271 - 275). However, T cell depletion leads to a higher frequency of graft failure (272, 273), and an increased leukemic relapse rate (274 - 278). Treatment of the recipient with GM-CSF in an attempt to facilitate engraftment and promote early bone marrow function has become routine, but does not replace the presence of donor T cells in the graft (70). Although GM-CSF therapy is effective in promoting an early rise in neutrophil counts and hence leads to earlier hospital discharge and lower costs, it does not change the overall transplant related mortality (70). An interesting situation in which engraftment may take place despite T cell depletion is in fetuses diagnosed pre-natally with genetic disorders correctable by BMT, such as SCID. In utero treatment by transfer of parental bone marrow depleted of T cells to prevent GVHR may engraft more easily in the developing fetal bone marrow microenvironment (244). In patients treated for a malignancy, T cell depletion is seldom considered since the resulting decrease in the risk of GVHR is more than counterbalanced by the increased incidence of neoplastic relapse, leading to an overall drop in disease-free survival. In fact, the graft-versus-leukemia (GVL) effect is sometimes induced deliberately to treat leukemic relapse by transfusing the BMT recipient with donor peripheral blood lymphocytes (279). Thus, it appears that modulating the GVHR may prove preferable to preventing it entirely (280).

Standard pharmacological GVHR prophylaxis and treatment includes corticosteroids, methotrexate, and CsA to downregulate T cell activity (281, 282). Recently, non-mitogenic anti-CD3 (283, 284) and anti-TCR (285) antibodies have also been used with some alleviation of symptoms. Conversely, a mitogenic anti-CD3 antibody (OKT3) caused an elevation in serum TNF-α, exacerbating the acute GVHR (286). Another approach has been to block TNF-α production or function. Pentoxyfilline has several effects on the immune system, including TNF-α inhibition (287). In one trial, it was reported to be effective at relieving intestinal and skin manifestations of GVHR (288), while in a second study it proved of no benefit, and in fact caused serious side-effects (289). Thalidomide also inhibits TNF-α, and is reportedly effective at controlling chronic GVHR (174 - 176). Unfortunately, most therapeutic strategies further immunosuppress the host, often resulting in infectious complications. Despite all the new approaches to prevention and treatment, GVHR mortality and morbidity remains a serious clinical problem in which T cell immune suppression plays a central role.

4. Objectives of the present studies.

The studies presented herein examine the mechanisms by which suppressive GVHR induces T cell dysfunction. There appear to be two distinct phases of T cell suppression; an initial T cell anergy which follows the allogeneic stimulation and proliferation of acute GVHR, and a subsequent unresponsiveness which develops after tissue injury, during chronic GVHR.

In a first series of experiments, the early T cell signalling defect was investigated. It is clear from previous work that there are several mechanisms of T cell suppression operating during acute GVHR, including the production of circulating factors which can transfer the suppression non-specifically (20, 21, 79 - 92). However, an intrinsic T cell signalling defect has also been documented (89 - 92). Thus, in Chapter 2, the molecular basis for the T cell signalling defect is investigated. A GVHR-induced decrease in the T cell signal transducing tyrosine kinases is described, providing a molecular mechanism for the T cell unresponsiveness during GVHR. Furthermore, the physiological regulation of the T cell tyrosine kinases is demonstrated to be glucocorticoid-dependent.

The acute GVHR, then, leaves the periphery devoid of functional T cells, so that reconstitution of cell-mediated immune function depends on new thymic

emigrants. In Chapter 3, the contribution of the GVHR-dysplastic thymus to the long term T cell defect is addressed. The thymocytes are examined for phenotypic abnormalities diagnostic of defective thymic function. Positive and negative selection are found to be abnormal, demonstrated by aberrant maturation and incomplete deletion of potentially autoreactive cells. Concurrently with the thymocyte abnormalities, a defect in thymic MHC class II expression is described. Thus, the GVHR-induced disruption of the thymic microenvironment provokes aberrant thymic education; this in turn may lead to repopulation of the suppressed periphery with abnormal T cells, giving rise to the immunosupressive and autoimmune manifestations of chronic GVHR.

The long term defect in the T cell compartment, then, may depend on aberrant thymic education. This in turn results from thymic injury; in fact, normal peripheral T cell function returns when thymic healing is completed (202). But what determines the extent of tissue injury during the acute phase of the reaction? The thymic lesions need not be induced by an allogeneic reaction to result in the production of autoreactive cells. During experimental sGVHR, induced by autologous BMT followed by treatment with CsA, phenotypically autoreactive cells appear in the thymus (290). However, for autoimmune symptoms to develop, these cells must be transfered to an irradiated recipient (290). Furthermore, thymic irradiation is crucial for the maintainance of the CsA-induced thymic defect; an unirradiated thymus retains its phenotypic

abnormalities only transiently after CsA treatment is stopped, preventing the accumulation of abnormal T cells (291). Thus, irradiation is central in the induction of lesions, and in potentiating the peripheral manifestations of the thymic damage.

The skin is a good model system for the study of GVHR-induced lesions. In human GVHR, epidermal damage is a characteristic manifestation of the disease (119, 124, 129, 131, 132, 145); in the P --> F1 murine model, however, such lesions are very difficult to demonstrate. In Chapter 4, the effect of local irradiation on the generation of GVHR tissue lesions is examined. Local irradiation, independent of systemic irradiation, is sufficient to markedly exacerbate the cutaneous GVHR-induced injury; by analogy, this suggests that irradiation may sensitize thymic epithelium to GVHR-induced lesions, thus amplifying the phase of pathological injury and giving rise to a long term defect in the thymic microenvironment.

References.

1. Dempster, W.J. 1953. Kidney homotransplantation. *Brit. J. Surg.* 40: 447.

2. Simonsen, M. 1953. Biological incompatibility in kidney transplantation in dogs. *Acta Path. Microbiol. Scand.* 32: 36.

3. Fowler, R. and C.D. West. 1960. Evidence against the "graft versus host" hypothesis in renal transplantation. *Trans. Bull.* 26: 133.

4. Simonsen, M. 1962. Graft versus host reactions. Their natural history, and applicability as tools of research. *Progr. Allergy* 6: 349.

5. Murphy, J.B. 1916. The effect of adult chicken organ graft on the chick embryo. *J. Exp. Med.* 24: 1.

6. Billingham, R.E., L. Brent, and P.B. Medawar. 1953. 'Actively acquired tolerance' of foreign cells. *Nature* 172: 603.

7. Billingham, R.E., and L. Brent. 1957. A simple method for inducing tolerance of skin homografts in mice. *Trans. Bull.* 4: 67.

8. Trentin, J.J. 1957. Induced tolerance and "homologous disease" in X-irradiated mice protected with homologous bone marrow. *Proc. Soc. exp. Biol., N.Y.* 96: 139.

9. Barnes, D.W.H., P.L.T. Ilbery, and J.F. Loutit. 1958. Avoidance of 'secondary disease' in radiation chimeras. *Nature* 181: 488.

10. Gorer, P.A., and E.A. Boyse. 1959. Pathological changes in F1 hybrid mice following transplantation of spleen cells from donor of the parental strains. *Immunol. (Lond.)* 2: 182.

11. Howard, J.G., and M.F.A. Woodruff. 1961. Effect of the graft-versus-host reaction on the immunological responsiveness of the mouse. *Proc. Roy. Soc. B* 154: 532.

12. Simonsen, M., J. Engelbreth-Holm, E. Jensen, and H. Poulsen. 1958. A study of the graft-versus-host reaction in transplantation to embryos, F1 hybrids, and irradiated animals. *Ann. N.Y. Acad. Sci.* 73: 834.

13. Trentin, J.J. 1958. An entity resembling homologous disease induced by transplantation of lymphoid tissue in unirradiated mice. *Fed. Proc.* 17: 461.

14. Burnet, D., and F.M. Burnet. 1961. Analysis of major histocompatibility factors in a stock of closely inbred White Leghorn fowls using a graft-versus-host reaction on the chorioallantoic membrane. *Austr. J. exp. Biol. Med.* 39: 101.

15. Martinez, C., J.M. Smith, and R.A. Good. 1961. Runt disease in adult tolerant mice induced by intravenous injection of immunologically competent cells. *Proc. Soc. exp. Biol., N.Y.* 106: 572.

16. Davies, A.J.S., and S.M.A. Doak. 1960. Fate of homologous adult spleen cells injected into newborn mice. *Nature* 187: 610.

17. Simonsen, M. 1957. The impact on the developing embryo and newborn animal of adult homologous cells. *Acta Path. Microbiol. Scand.* 40: 480.

18. Copelan, E. A. 1992. Conditioning regimens for allogeneic bone marrow transplantation. *Blood Rev.* 6: 234.

19. Bieder, A., L. Weiss, and S. Slavin. 1992. The role of recombinant cytokines and other immunomodulators on engraftment following allogeneic bone marrow transplantation in mice. *Bone Marrow Transplant.* 9: 421.

20. Lapp, W.S., T. Ghayur, M. Mendes, M. Seddik, and T.A. Seemayer. 1985. The

functional and histological basis for graft-versus-host-induced immunosuppression. *Immunol. Rev.* 88: 107.

21. Moser, M., T. Iwasaki, and G.M. Shearer. 1985. Cellular interactions in graft-versus-host-induced T cell immune deficiency. *Immunol. Rev.* 88: 135.

22. Champlin, R. E. 1990. T-cell depletion for bone marrow transplantation: effects on graft rejection, graft-versus-host disease, graft-versus-leukemia, and survival. *Cancer Treat. Res.* 50: 99.

23. Sarawar, S. R., S. M. Sparshott, P. Sutton, C. P. Yang, I. V. Hutchinson, and E. B. Bell. 1993. Rapid re-expression of CD45RC on rat CD4 T cells in vitro correlates with a change in function. *Eur. J. Immunol.* 23: 103.

24. Thiele, D. L., S. E. Bryde, and P. E. Lipsky. 1988. Lethal graft-vs-host disease induced by a class II MHC antigen only disparity is not mediated by cytotoxic T cells. *J. Immunol.* 141: 3377.

25. Tary-Lehmann, M., A.G. Rolink, P.V. Lehmann, Z.A. Nagy, and U. Hurtenbach. 1990. Induction of graft-versus-host-associated immunodeficiency by CD4+ T cell clones. *J. Immunol.* 145: 2092.

26. Lehmann, P. V., G. Schumm, D. Moon, U. Hurtenbach, F. Falcioni, S. Muller, and Z. A. Nagy. 1990. Acute lethal graft-versus-host reaction induced by major histocompatibility complex class II-reactive T helper cell clones. *J. Exp. Med.* 171: 1485.

27. Watanabe, H., M. Fujiwara, T. Mashiko, and M. Ito. 1989. T-cell clones with L3T4-positive or Lyt-2-positive phenotypes responding to mutant MHC class II antigen and inducing graft versus host reaction. *J. Invest. Dermatol.* 93: 691.

28. Moser, M., S. O. Sharrow, and G. M. Shearer. 1988. Role of L3T4+ and Lyt-2+

donor cells in graft-versus-host immune deficiency induced across a class I, class II, or whole H-2 difference. *J. Immunol.* 140: 2600.

29. Korngold, R. 1992. Lethal graft-versus-host disease in mice directed to multiple minor histocompatibility antigens: features of CD8+ and CD4+ T cell responses. *Bone Marrow Transplant.* 9: 355.

30. Cray, C. and R. B. Levy. 1989. The ability of murine cytomegalovirus and class I major histocompatibility complex-disparate parental cells to induce alterations characteristic of severe graft-versus-host reactions. *Transplantation* 48: 1057.

31. Via, C. S., J. D. Shanley, and G. M. Shearer. 1990. Synergistic effect of murine cytomegalovirus on the induction off acute graft-vs-host disease involving MHC class I differences only. Analysis of in vitro T cell function. *J. Immunol.* 145: 3283.

32. Cray, C. and R. B. Levy. 1990. Virus-associated immune responses in mice undergoing GVHR exacerbated by concurrent MCMV infection. *Transplantation* 50: 1027.

33. Cray, C. and R. B. Levy. 1990. The presence of infectious virus but not conventional antigen can exacerbate graft-versus-host reactions. *Scand. J. Immunol.* 32: 177.

34. Cray, C. and R. B. Levy. 1993. CD8+ and CD4+ T cells contribute to the exacerbation of class I MHC disparate graft-vs-host reaction by concurrent murine cytomegalovirus infection. *Clin. Immunol. Immunopathol.* 67: 84.

35. Ringden, O. 1992. Correlation of pretransplant viral serology and complications of bone marrow transplantation. *Ann. Hematol.* 64 Suppl: A143.

36. Korngold, R. 1991. Pathophysiology of graft-versus-host disease directed to minor histocompatibility antigens. *Bone Marrow Transplant.* 7 Suppl 1: 38.

37. OKunewick, J. P., D. L. Kociban, and M. J. Buffo. 1990. Comparative effects of various T cell subtypes on GVHD in a murine model for MHC-matched unrelated donor transplant. *Bone Marrow Transplant.* 5: 145.

38. Wallny, H. J. and H. G. Rammensee. 1990. Identification of classical minor histocompatibility antigen as cell-derived peptide. *Nature* 343: 275.

39. Bril, H., B. D. Molendijk-Lok, L. M. Hussaarts-Odijk, and R. Benner. 1984. Synergism of T lymphocyte subsets in the response to Mls-locus coded antigens during graft-versus-host reaction. *Cell. Immunol.* 83: 370.

40. Atkinson, K., M. Cooley, H. Farrelly, E. O'Flaherty, M. Ashby, and J. Biggs. 1987. CD4+ T cells appear capable of initiating graft-versus-host disease across non-major histocompatibility complex (MHC) barriers in man. *Bone Marrow Transplant.* 2: 79.

41. van Els, C. A., E. Zantvoort, N. Jacobs, A. Bakker, J. J. Van Rood, and E. Goulmy. 1990. Graft-versus-host disease associated T helper cell responses specific for minor histocompatibility antigens are mainly restricted by HLA-DR molecules. *Bone Marrow Transplant.* 5: 365.

42. Dellabona, P., J. Peccoud, J. Kappler, P. Marrack, C. Benoist, and D. Mathis. 1990. Superantigens interact with MHC class II molecules outside of the antigen groove. *Cell* 62: 1115.

43. Kourilsky, P. and J. M. Claverie. 1989. MHC restriction, alloreactivity, and thymic education: a common link? *Cell* 56: 327.

44. Irschick, E. U., F. Hladik, D. Niederwieser, W. Nussbaumer, E. Holler, E. Kaminski, and C. Huber. 1992. Studies on the mechanism of tolerance or graft-versus-host disease in allogeneic bone marrow recipients at the level of cytotoxic T-cell precursor frequencies. *Blood* 79: 1622.

45. Niederwieser, D., A. Grassegger, J. Aubock, M. Herold, D. Nachbaur, A. Rosenmayr, A. Gachter, W. Nussbaumer, S. Gaggl, and M. Ritter. 1993. Correlation of minor histocompatibility antigen-specific cytotoxic T lymphocytes with graft-versus-host disease status and analyses of tissue distribution of their target antigens. *Blood* 81: 2200.

46. Roosnek, E., S. Hogendijk, S. Zawadynski, D. Speiser, J. M. Tiercy, C. Helg, B. Chapuis, A. Gratwohl, J. Gmur, and R. Seger. 1993. The frequency of pretransplant donor cytotoxic T cell precursors with anti-host specificity predicts survival of patients transplanted with bone marrow from donors other than HLA-identical siblings. *Transplantation* 56: 691.

47. Theobald, M., T. Nierle, D. Bunjes, R. Arnold, and H. Heimpel. 1992. Host-specific interleukin-2-secreting donor T-cell precursors as predictors of acute graft-versus-host disease in bone marrow transplantation between HLA-identical siblings. *NEJM.* 327: 1613.

48. Theobald, M. and D. Bunjes. 1993. Pretransplant detection of human minor histocompatibility antigen-specific naive and memory interleukin-2-secreting T cells within class I major histocompatibility complex (MHC)-restricted CD8+ and class II MHC-restricted CD4+ T-cell subsets. *Blood* 82: 298.

49. Batchelor, J. R., A. P. Schwarer, Z. J. Yin, A. J. Barrett, J. M. Goldman, and R. I. Lechler. 1993. Helper T-lymphocyte precursor frequencies predict risks of graft-versus-host disease in bone marrow transplantation. *Transplant. Proc.* 25: 1237.

50. Schwarer, A. P., Y. Z. Jiang, P. A. Brookes, A. J. Barrett, J. R. Batchelor, J. M. Goldman, and R. I. Lechler. 1993. Frequency of anti-recipient alloreactive helper T-cell precursors in donor blood and graft-versus-host disease after HLA-identical sibling bone-marrow transplantation. *Lancet* 341: 203.

51. Troutt, A. B. and A. Kelso. 1992. Enumeration of lymphokine mRNA-containing

cells in vivo in a murine graft-versus-host reaction using the PCR. *Proc. Natl. Acad. Sci. USA.* 89: 5276.

52. Kelso, A. 1990. Frequency analysis of lymphokine-secreting CD4+ and CD8+ T cells activated in a graft-versus-host reaction. *J. Immunol.* 145: 2167.

53. Troutt, A. B. and A. Kelso. 1993. Lymphokine synthesis in vivo in an acute murine graft-versus-host reaction: mRNA and protein measurements in vivo and in vitro reveal marked differences between actual and potential lymphokine production levels. *Internat. Immunol.* 5: 399.

54. Troutt, A. B. and A. Kelso. 1992. Lymphokine production in vivo in a murine graft-versus-host reaction. *Transplant. Proc.* 24: 2317X.

55. Kelso, A. and A. B. Troutt. 1993. Activation of lymphokine mRNA and protein synthesis in vivo in a murine graft-vs-host reaction. *Transplant. Proc.* 25: 122.

56. Ford, W. L., B. Rolstad, S. Fossum, S. V. Hunt, M. E. Smith, and S. M. Sparshott. 1981. The stimulus to host cell proliferation in graft-versus-host reactions. *Scand. J. Immunol.* 14: 705.

57. Via, C. S. 1991. Kinetics of T cell activation in acute and chronic forms of murine graft-versus-host disease. *J. Immunol.* 146: 2603.

58. Hakim, F. T., S. O. Sharrow, S. Payne, and G. M. Shearer. 1991. Repopulation of host lymphohematopoietic systems by donor cells during graft-versus-host reaction in unirradiated adult F1 mice injected with parental lymphocytes. *J. Immunol.* 146: 2108.

59. Garlisi, C. G., K. J. Pennline, S. R. Smith, M. I. Siegel, and S. P. Umland. 1993. Cytokine gene expression in mice undergoing chronic graft-versus-host disease. *Molecular Immunol.* 30: 669.

60. Via, C. S. and F. D. Finkelman. 1993. Critical role of interleukin-2 in the development of acute graft-versus-host disease. *Internat. Immunol.* 5: 565.

61. Niederwieser, D., M. Herold, W. Woloszczuk, W. Aulitzky, B. Meister, H. Tilg, G. Gastl, R. Bowden, and C. Huber. 1990. Endogenous IFN-gamma during human bone marrow transplantation. Analysis of serum levels of interferon and interferon-dependent secondary messages. *Transplantation* 50: 620.

62. Jadus, M. R. and H. T. Wepsic. 1992. The role of cytokines in graft-versus-host reactions and disease. *Bone Marrow Transplant.* 10: 1.

63. Antin, J. H. and J. L. Ferrara. 1992. Cytokine dysregulation and acute graft-versus-host disease. *Blood* 80: 2964.

64. Ferrara, J. L. 1993. Cytokine dysregulation as a mechanism of graft versus host disease. *Current Opinions Immunol.* 5: 794.

65. Abhyankar, S., D. G. Gilliland, and J. L. Ferrara. 1993. Interleukin-1 is a critical effector molecule during cytokine dysregulation in graft versus host disease to minor histocompatibility antigens. *Transplantation* 56: 1518.

66. Nestel, F. P., K. S. Price, T. A. Seemayer, and W. S. Lapp. 1992. Macrophage priming and lipopolysaccharide-triggered release of tumorr necrosis factor alpha during graft-versus-host disease. *J. Exp. Med.* 175: 405.

67. Bruserud, O., G. Ehninger, W. Hamann, and G. Pawelec. 1993. Secretion of IL-2, IL-3, IL-4, IL-6 and GM-CSF by CD4+ and CD8+ TCR alpha beta+ T-cell clones derived early after allogeneic bone marrow transplantation. *Scand. J. Immunol.* 38: 65.

68. Hakim, F. T., D. H. Pluznik, and G. M. Shearer. 1990. Factors contributing to the decrease in concanavalin A-induced colony-stimulating factors in acute suppressive

graft-versus-host disorder. *Transplantation* 49: 781.

69. Hakim, F. T., D. H. Pluznik, and G. M. Shearer. 1990. Alterations in T cell-derived colony-stimulating factors associated with GVH-induced immune deficiency. *Transplantation* 49: 773.

70. Armitage, J. O. 1992. The use of granulocyte-macrophage colony-stimulating factor in bone marrow transplantation. *Sem. Hematol.* 29: 14.

71. Mowat, A. M., A. K. Hutton, P. Garside, and M. Steel. 1993. A role for interleukin-1 alpha in immunologically mediated intestinal pathology. *Immunology* 80: 110.

72. Piguet, P. F., G. E. Grau, B. Allet, and P. Vassalli. 1987. Tumor necrosis factor/cachectin is an effector of skin and gut lesions of the acute phase of graft-vs.-host disease. *J. Exp. Med.* 166: 1280.

73. Holler, E., H. J. Kolb, A. Moller, J. Kempeni, S. Liesenfeld, H. Pechumer, W. Lehmacher, G. Ruckdeschel, B. Gleixner, and C. Riedner. 1990. Increased serum levels of tumor necrosis factor alpha precede major complications of bone marrow transplantation. *Blood* 75: 1011.

74. Holler, E. 1991. TNF-alpha in allogeneic bone marrow transplantation. Significance for immunologic reconstitution, acute graft versus host disease and risk of complications after bone marrow transplantation. *Fortschritte der Medizin* 109: 263.

75. Girardin, E. and J. M. Dayer. 1993. Cytokines and antagonists in septic shock. *Schweizer. Medizin. Wochen.* 123: 480.

76. Garside, P. and A. M. Mowat. 1993. Natural killer cells and tumour necrosis factor-alpha-mediated enteropathy in mice. *Immunology* 78: 335.

77. Garside, P., C. Bunce, R. C. Tomlinson, B. L. Nichols, and A. M. Mowat. 1993. Analysis of enteropathy induced by tumour necrosis factor alpha. *Cytokine* 5: 24.

78. Holda, J.T., T. Maier, and H.N. Claman. 1985. Murine graft-versus-host disease across minor barriers: immunosuppressive aspects of natural suppressor cells. *Immunol. Rev.* 88: 87.

79. Tsoi, M. S., R. Storb, S. Dobbs, E. Santos, and E. D. Thomas. 1981. Specific suppressor cells and immune response to host antigens in long-term human allogeneic marrow recipients: implications for the mechanisms of graft-host tolerance and chronic graft-versus-host disease. *Transplant. Proc.* 13: 237.

80. Pickei, K., and H.K. Hoffmann. 1977. The Ly phenotype of suppressor T cells arising in mice subjected to graft-versus-host reaction. *J. Exp. Med.* 145: 1169.

81. Choi, K.L., T. Maier, J.H. Holda, and H.N. Claman. 1988. Suppression of cytotoxic T cell generation by natural suppressor cells from mice with GVHD is partially reversed by indomethacin. *Cell. Immunol.* 112: 271.

82. Moore, S. C., S. A. Theus, J. B. Barnett, and L. S. Soderberg. 1992. Cytokine regulation of bone marrow natural suppressor cell activity in the suppression of lymphocyte function. *Cell. Immunol.* 141: 398.

83. Lapp, W.S., M. Mendes, H. Kirchner, and D. Gemsa. 1980. Prostaglandin synthesis by lymphoid tissue of mice experiencing a graft-versus-host-reaction: relationship to immunosuppression. *Cell. Immunol.* 50: 271.

84. Hoffman, R. A., J. M. Langrehr, S. M. Wren, K. E. Dull, S. T. Ildstad, S. A. McCarthy, and R. L. Simmons. 1993. Characterization of the immunosuppressive effects of nitric oxide in graft vs host disease. *J. Immunol.* 151: 1508.

85. Langrehr, J. M., A. R. Muller, H. A. Bergonia, T. D. Jacob, T. K. Lee, W. H. Schraut, J. R. Lancaster,Jr., R. A. Hoffman, and R. L. Simmons. 1992. Detection of nitric oxide by electron paramagnetic resonance spectroscopy during rejection and graft-versus-host disease after small-bowel transplantation in the rat. *Surgery* 112: 395.

86. Klimpel, G. R., C. R. Annable, M. G. Cleveland, T. R. Jerrells, and J. C. Patterson. 1990. Immunosuppression and lymphoid hypoplasia associated with chronic graft versus host disease is dependent upon IFN-gamma production. *J. Immunol.* 144: 84.

87. Huchet, R., M. Bruley-Rosset, C. Mathiot, D. Grandjon, and O. Halle-Pannenko. 1993. Involvement of IFN-gamma and transforming growth factor-beta inn graft-vs-host reaction-associated immunosuppression. *J. Immunol.* 150: 2517.

88. Wall, D. A. and K. C. Sheehan. 1994. The role of tumor necrosis factor and interferon gamma in graft-versus-host disease and related immunodeficiency. *Transplantation* 57: 273.

89. Levy, R. B., M. Jones, and C. Cray. 1990. Isolated peripheral T cells from GvHR recipients exhibit defective IL-2R expression, IL-2 production, and proliferation in response to activation stimuli. *J. Immunol.* 145: 3998.

90. Tsoi, M. S., T. Mori, S. Brkic, S. Dobbs, S. Gillis, E. Santos, E. D. Thomas, and R. Storb. 1984. Ineffective cellular interaction and interleukin 2 deficiency causing T cell defects in human allogeneic marrow recipients early after grafting and in those with chronic graft-versus-host disease. *Transplant. Proc.* 16: 1470.

91. Klingemann, H. G., F. R. Kohn, and G. L. Phillips. 1991. Proliferation of peripheral lymphocytes to interleukin-2 and interleukin-4 after marrow transplantation. *Eur. Cytokine Net.* 2: 131.

92. Guillaume, T., O. Hamdan, P. Staquet, M. Sekhavat, B. Chatelain, A. Bosly, D. B. Rubinstein, Y. Humblet, C. Doyen, and B. Coiffier. 1993. Blunted rise in intracellular calcium in CD4+ T cells in response to mitogen following autologous bone marrow transplantation. *Br. J. Haematol.* 84: 131.

93. Cantrell, D. A., J. D. Graves, M. Izquierdo, S. Lucas, and J. Downward. 1992. T lymphocyte activation signals. *Ciba Found. Symp.* 164: 208.

94. Weiss, A. 1993. T cell antigen receptor signal transduction: a tale of tails and cytoplasmic protein-tyrosine kinases. *Cell.* 73: 209.

95. Klausner, R.D., and L.E. Samelson. 1991. T cell antigen receptor activation pathways: the tyrosine kinase connection. *Cell.* 64: 875.

96. Turner, J.M., M.H. Brodsky, B.A. Irving, S.D. Levin, R.M. Perlmutter, and D.R. Littman. 1990. Interaction of the unique N-terminal region of tyrosine kinase p56lck with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. *Cell.* 60: 755.

97. Amrein, K. E. and B. M. Sefton. 1988. Mutation of a site of tyrosine phosphorylation in the lymphocyte-specific tyrosine protein kinase, p56lck, reveals its oncogenic potential in fibroblasts. *Proc. Natl. Acad. Sci. USA.* 85: 4247.

98. Ledbetter, J. A., J. P. Deans, A. Aruffo, L. S. Grosmaire, S. B. Kanner, J. B. Bolen, and G. L. Schieven. 1993. CD4, CD8 and the role of CD45 in T-cell activation. *Current Opinion Immunol.* 5: 334.

99. Davidson, D., L. M. Chow, M. Fournel, and A. Veillette. 1992. Differential regulation of T cell antigen responsiveness by isoforms of the src-related tyrosine protein kinase p59fyn. *J. Exp. Med.* 175:1483.

100. da Silva, A. J., M. Yamamoto, C. H. Zalvan, and C. E. Rudd. 1992. Engagement

of the TcR/CD3 complex stimulates p59fyn(T) activity: detection of associated proteins at 72 and 120-130 kD. *Molecular Immunology.* 29:1417.

101. Cooke, M.P., K.M. Abraham, K.A. Forbush, and R.M. Perlmutter. 1991. Regulation of T cell receptor signalling by a *src* family protein-tyrosine kinase ($p59^{fyn}$). *Cell.* 65: 281.

102. Letourneur, F., and R.D. Klausner. 1991. T cell and basophil activation through the cytoplasmic tail of T cell receptor ζ family proteins. *Proc. Natl. Acad. Sci. USA.* 88: 8905.

103. Romeo, C., M. Amiot, and B. Seed. 1992. Sequence requirements for induction of cytolysis by the T cell antigen/Fc receptor ζ chain. *Cell.* 68: 889.

104. Reth, M. 1989. Antigen receptor tail clue. *Nature.* 338: 383.

105. Jenkins, M. K. and R. H. Schwartz. 1987. Antigen presentation by chemically modified splenocytes inducess antigen-specific T cell unresponsiveness in vitro and in vivo. *J. Exp. Med.* 165: 302.

106. Jenkins, M. K., P. S. Taylor, S. D. Norton, and K. B. Urdahl. 1991. CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J. Immunol.* 147: 2461.

107. Kang, S.-M., B. Beverly, A.-C. Tran, K. Brorson, R.H. Schwartz, and M.J. Lenardo. 1992. Transactivation by AP-1 is a molecular target of T cell clonal anergy. *Science* 257: 1134.

108. June, C. H., J. A. Ledbetter, P. S. Linsley, and C. B. Thompson. 1990. Role of the CD28 receptor in T-cell activation. *Immunol. Today* 11: 211.

109. Jendrisak, G., J. Gamero, T. Mohanakumar, and M. Jendrisak. 1993. Clonal

anergy induction by monoclonal antibodies to CD4, LFA-1, and ICAM-1. *Transplant. Proc.* 25: 828.

110. Burkly, L. C., D. Lo, O. Kanagawa, R. L. Brinster, and R. A. Flavell. 1989. T-cell tolerance by clonal anergy in transgenic mice with nonlymphoid expression of MHC class II I-E. *Nature* 342: 564.

111. Lo, D., L. C. Burkly, R. A. Flavell, R. D. Palmiter, and R. L. Brinster. 1989. Tolerance in transgenic mice expressing class II major histocompatibility complex on pancreatic acinar cells. *J. Exp. Med.* 170: 87.

112. Morahan, G., J. Allison, and J. F. Miller. 1989. Tolerance of class I histocompatibility antigens expressed extrathymically. *Nature* 339: 622.

113. Xenocostas, A., D. G. Osmond, and W. S. Lapp. 1991. The effect of the graft-versus-host reaction on B lymphocyte production in bone marrow of mice. Depressed genesis of early progenitors prior to mu heavy chain expression. *Transplantation* 51: 1089.

114. Howard, J.G. 1961. Increased sensitivity to bacterial endotoxin of F1 hybrid mice undergoing graft-versus-host reaction. *Nature* 190: 1122.

115. Guy-Grand, D., and P. Vassali. 1987. Gut injury in mouse graft-versus-host reaction. *Adv. Exp. Med. Biol.* 216A: 661.

116. Mowat A.McI., M.V. Felstein. 1990. Experimental studies of immunologically mediated enteropathy. V. Destructive enteropathy during an acute graft-versus-host reaction in adult BDF1 mice. *Clin. Exp. Immunol.* 79: 279.

117. Charley, M.R., J.L. Bangert, B.L. Hamilton, J.N. Gilliam, and R.D. Sontheimer. 1983. Murine Graft-versus-host skin disease: a chronologic and quantitative analysis of two histologic patterns. *J. Invest. Dermatol.* 81: 412.

118. Farmer, E. R. 1986. The histopathology of graft-versus-host disease. *Adv. Dermatol.* 1: 173.

119. Ford, W.L., and B.F. Hellsop. 1987. Allogeneic lymphocyte cytotoxicity: evidence that target cells include most, if not all, donor lymphocytes. *Transplantation* 43: 753.

120. Seddik, M., T.A. Seemayer, and W.S. Lapp. 1980. T cell functional defect associated with thymic epithelial cell injury induced by a graft-versus-host reaction. *Transplantation* 29: 61.

121. Seemayer, T.A., W.S. Lapp, and R.P. Bolande. 1977. Thymic involution in murine graft-versus-host reaction. Epithelial injury mimicking human thymic dysplasia. *Am. J. Pathol.* 88: 119.

122. Seemayer, T.A., W.S. Lapp, and R.P. Bolande. 1978. Thymic epithelial injury in graft-versus-host reactions following adrenalectomy. *Am. J. Pathol.* 93: 325.

123. Kornbluth, M., E. You-Ten, J. Desbarats, S. Gamache, A. Xenocostas, and W.S. Lapp. 1991. T cell subsets in the thymus of graft-versus-host immunosuppressed mice. Sensitivity of the $L3T4^{+}Lyt\text{-}2^{-}$ subset to cortisone. *Transplantation.* 51: 262.

124. Norton, J., J. P. Sloane, D. Delia, and M. F. Greaves. 1993. Reciprocal expression of CD34 and cell adhesion molecule ELAM-1 on vascular endothelium in acute cutaneous graft-versus-host disease. *J. Pathol.* 170: 173.

125. Norton, J., J. P. Sloane, N. al-Saffar, and D. O. Haskard. 1991. Vessel associated adhesion molecules in normal skin and acute graft-versus-host disease. *J. Clin. Pathol.* 44: 586.

126. Norton, J. and J. P. Sloane. 1991. ICAM-1 expression on epidermal keratinocytes in cutaneous graft-versus-host disease. *Transplantation* 51: 1203.

127. Parfrey, N. A., H. Ste-Croix, and G. J. Prud'homme. 1989. Evidence that nonlymphoid tissue injury in acute graft-versus-host disease is limited to epithelial cells aberrantly expressing MHC antigens. *Transplantation* 48: 655.

128. Nickoloff, B. J., D. M. Lewinsohn, and E. C. Butcher. 1987. Enhanced binding of peripheral blood mononuclear leukocytes to gamma-interferon-treated cultured keratinocytes. *Am. J. Dermatol.* 9: 413.

129. Sloane, J. P., C. J. Elliott, and R. Powles. 1988. HLA-DR expression in epidermal keratinocytes after allogeneic bone marrow transplantation. Relationship to histology, rash, marrow purging, and systemic graft-versus-host disease. *Transplantation* 46: 840.

130. Barclay, A. N. and D. W. Mason. 1982. Induction of Ia antigen in rat epidermal cells and gut epithelium by immunological stimuli. *J. Exp. Med.* 156: 1665.

131. Beschorner, W. E., E. R. Farmer, R. Saral, W. L. Stirling, and G. W. Santos. 1987. Epithelial class II antigen expression in cutaneous graft-versus-host disease. *Transplantation* 44: 237.

132. Breathnach, S. M. and S. I. Katz. 1983. Keratinocytes synthesize Ia antigen in acute cutaneous graft-vs-host disease. *J. Immunol.* 131: 2741.

133. Niederwieser, D., J. Aubock, J. Troppmair, M. Herold, G. Schuler, G. Boeck, J. Lotz, P. Fritsch, and C. Huber. 1988. IFN-mediated induction of MHC antigen expression on human keratinocytes and its influence on in vitro alloimmune responses. *J. Immunol.* 140: 2556.

134. Sviland, L., A. D. Pearson, M. A. Green, E. J. Eastham, A. J. Malcolm, S. J. Proctor, and P. J. Hamilton. 1989. Expression of MHC class I and II antigens by keratinocytes and enterocytes in acute graft-versus-host disease. Newcastle Bone Marrow Transplant Group. *Bone Marrow Transplant.* 4: 233.

135. Bland, P. W. and C. V. Whiting. 1992. Induction of MHC class II gene products in rat intestinal epithelium during graft-versus-host disease and effects on the immune function of the epithelium. *Immunology* 75: 366.

136. Halloran, P. F., J. Jephthah-Ochola, J. Urmson, and S. Farkas. 1985. Systemic immunologic stimuli increase class I and II antigen expression in mouse kidney. *J. Immunol.* 135: 1053.

137. Hickey, W. F. and H. Kimura. 1987. Graft-vs.-host disease elicits expression of class I and class II histocompatibility antigens and the presence of scattered T lymphocytes in rat central nervous system. *Proc. Natl. Acad. Sci. USA.* 84: 2082.

138. Kajiwara, K., A. Hirozane, T. Fukumoto, T. Orita, T. Nishizaki, T. Kamiryo, and H. Ito. 1991. Major histocompatibility complex expression in brain of rats with graft-versus-host disease. *J. Neuroimmunol.* 32: 191.

139. Hirozane, A., Y. Fujikura, T. Sawada, F. Shinozaki, and T. Fukumoto. 1992. Analysis of major histocompatibility complex (MHC) class I antigen in the rat salivary gland. *Archiv. Oral Biol.* 37: 147.

140. Miglio, F., M. Pignatelli, V. Mazzeo, M. Baraldini, G. F. Stefanini, G. Guardigli, G. Bandini, P. Ricci, S. Tura, and G. Gasbarrini. 1987. Expression of major histocompatibility complex class II antigens on bile duct epithelium in patients with hepatic graft-versus-host disease after bone marrow transplantation. *J. Hepatol.* 5: 182.

141. Takacs, L., B. Szende, A. Rot, and T. Diamanstein. 1985. Expression of MHC class II antigens on bile duct epithelium in experimental graft versus host disease. *Clin. Exp. Immunol.* 60: 449.

142. Nickoloff, B. J., D. M. Lewinsohn, E. C. Butcher, A. M. Krensky, and C. Clayberger. 1988. Recombinant gamma interferon increases the binding of peripheral

blood mononuclear leukocytes and a Leu-3+ T lymphocyte clone to cultured keratinocytes and to a malignant cutaneous squamous carcinoma cell line that is blocked by antibody against the LFA-1 molecule. *J. Invest. Dermatol.* 90: 17.

143. Renkonen, R. 1986. Bone marrow transplantation in the rat. V. Lymphoid cell subclasses in the target organs during acute graft-versus-host disease. *Transplantation* 41: 704.

144. Renkonen, R. and P. Hayry. 1987. Cellular infiltrates in the target organs associated with acute graft-versus-host disease. *Bone Marrow Transplant.* 2: 333.

145. Piguet, P. F., A. Janin-Mercier, P. Vassalli, and J. H. Saurat. 1987. Epidermal lesions of the GVHR: evaluation of the role of different MHC and non MHC loci and of the Ly-2+ and L3T4+ T lymphocytes. *J. Immunol.* 139: 406.

146. Sviland, L., A. M. Dickinson, P. J. Carey, A. D. Pearson, and S. J. Proctor. 1990. An in vitro predictive test for clinical graft-versus-host disease in allogeneic bone marrow transplant recipients. *Bone Marrow Transplant.* 5: 105.

147. Girolomoni, G., C. Pincelli, G. Zambruno, M. Andreani, C. Giardini, G. Lucarelli, and A. Giannetti. 1991. Immunohistochemistry of cutaneous graft-versus-host disease after allogeneic bone marrow transplantation. *J. Dermatol.* 18: 314.

148. Murphy, G. F., D. Whitaker, J. Sprent, and R. Korngold. 1991. Characterization of target injury of murine acute graft-versus-host disease directed to multiple minor histocompatibility antigens elicited by either CD4+ or CD8+ effector cells. *Am. J. Path.* 138: 983.

149. Renkonen, R. and P. Hayry. 1986. Bone marrow transplantation in the rat. II. Quantitation of cellular infiltrates in parenchymal target organs in acute graft-versus-host disease. *Scand. J. Immunol.* 23: 351.

150. Guillen, F. J., J. Ferrara, W. W. Hancock, D. Messadi, E. Fonferko, S. J. Burakoff, and G. F. Murphy. 1986. Acute cutaneous graft-versus-host disease to minor histocompatibility antigens in a murine model. Evidence that large granular lymphocytes are effector cells in the immune response. *Lab. Invest.* 55: 35.

151. Kasten-Sportes, C., M. Masset, F. Varrin, A. Devergie, and E. Gluckman. 1989. Phenotype and function of T lymphocytes infiltrating the skin during graft-versus-host disease following allogeneic bone marrow transplantation. *Transplantation* 47: 621.

152. Acevedo, A., J. Aramburu, J. Lopez, J. Fernandez-Herrera, J. M. Fernandez-Ranada, and M. Lopez-Botet. 1991. Identification of natural killer (NK) cells in lesions of human cutaneous graft-versus-host disease: expression of a novel NK-associated surface antigen (Kp43) in mononuclear infiltrates. *J. Invest. Dermatol.* 97: 659.

153. Leskinen, R., E. Taskinen, L. Volin, T. Ruutu, and P. Hayry. 1992. Immunohistology of skin and rectum biopsies in bone marrow transplant recipients. *Apmis* 100: 1115.

154. Roy, J., J. L. Platt, and D. J. Weisdorf. 1993. The immunopathology of upper gastrointestinal acute graft-versus-host disease. Lymphoid cells and endothelial adhesion molecules. *Transplantation* 55: 572.

155. Takata, M., T. Imai, and T. Hirone. 1993. Immunoelectron microscopy of acute graft versus host disease of the skin after allogeneic bone marrow transplantation. *J. Clin. Pathol.* 46: 801.

156. Norton, J., N. al-Saffar, and J. P. Sloane. 1991. An immunohistological study of gamma/delta lymphocytes in human cutaneous graft-versus-host disease. *Bone Marrow Transplant.* 7: 205.

157. Paller, A. S., A. Nelson, L. Steffen, L. Gottschalk, and H. Kaizer. 1988.

T-lymphocyte subsets in the lesional skin of allogeneic and autologous bone marrow transplant patients. *Archiv. Dermatol.* 124: 1795.

158. Garside, P., C. Bunce, R. C. Tomlinson, B. L. Nichols, and A. M. Mowat. 1993. Analysis of enteropathy induced by tumour necrosis factor alpha. *Cytokine* 5: 24.

159. Fenyk, J. R.,Jr., C. M. Smith, P. I. Warkentin, W. Krivit, R. W. Goltz, J. E. Neely, M. E. Nesbit, N. K. Ramsay, P. F. Coccia, and J. H. Kersey. 1978. Sclerodermatous graft-versus-host disease limited to an area of measles exanthem. *Lancet* 1: 472.

160. Winston, D. J., W. G. Ho, and R. E. Champlin. 1990. Cytomegalovirus infections after allogeneic bone marrow transplantation. *Rev. Infect. Diseases* 12 Suppl 7: S776.

161. Haliotis, T., J.K. Ball, D. Dexter, and J.C. Roder. 1985. Spontaneous and induced primary oncogenesis in natural killer (NK)-cell-deficient beige mutant mice. *Int. J. Cancer* 35: 505.

162. Ghayur, T., T.A. Seemayer, P.A.L. Kongshavn, J.G. Gartner, and W.S. Lapp. 1987. Graft-versus-host reactions in the beige mouse. An investigation of the role of host and donor natural killer cells in the pathogenesis of graft-versus-host disease. *Transplantation* 44: 261.

163. Halle-Panenko, O., and M. Bruley-Rosset. 1985. Decreased graft-versus-host reaction and T cell cytolytic potential of beige mice. *Transplantation* 39: 85.

164. Mowat, A. M. 1989. Antibodies to IFN-gamma prevent immunologically mediated intestinal damage in murine graft-versus-host reaction. *Immunology* 68: 18.

165. Garside, P., M. V. Felstein, E. A. Green, and A. M. Mowat. 1991. The role of interferon alpha/beta in the induction of intestinal pathology in mice. *Immunology* 74: 279.

166. Peres, A., S. Amlani, M. Kornbluth, T.A. Seemayer, and W.S. Lapp. 1989. The effects of polyinosinic:polycytidylic acid on the graft-versus-host reaction. III: Increased severity of the reaction with delayed pI:C treatment. *J. Immunol.* 48: 80.

167. Price, B. A., N. S. Cumberland, C. L. Ingham Clark, P. Malcolm, P. W. Crane, P. A. Lear, and R. F. Wood. 1992. Changes in small intestinal microflora following small bowel transplantation in the rat and bacterial translocation in rejection and graft-versus-host disease. *Transplant. Proc.* 24: 1194X.

168. Price, B. A., N. S. Cumberland, C. L. Clark, A. G. Pockley, P. A. Lear, and R. F. Wood. 1993. The effect of rejection and graft-versus-host disease on small intestinal microflora and bacterial translocation after rat small bowel transplantation. *Transplantation* 56: 1072.

169. Langrehr, J. M., N. Murase, P. M. Markus, X. Cai, P. Neuhaus, W. Schraut, R. L. Simmons, and R. A. Hoffman. 1992. Nitric oxide production in host-versus-graft and graft-versus-host reactions in the rat. *J. Clin. Invest.* 90: 679.

170. Garside, P., A. K. Hutton, A. Severn, F. Y. Liew, and A. M. Mowat. 1992. Nitric oxide mediates intestinal pathology in graft-vs.-host disease. *Eur. J. Immunol.* 22: 2141.

171. Brunda, M. J. 1994. Interleukin-12. *J. Leukocyte Biol.* 55: 280.

172. Pirenne, J., M. D'Silva, G. Degiovanni, N. Jacquet, and D. L. Dunn. 1993. Relevance of tumor necrosis factor to graft-versus-host disease after small bowel transplantation. *Transplant Internat.* 6: 258.

173. Herve, P., M. Flesch, P. Tiberghien, J. Wijdenes, E. Racadot, P. Bordigoni, E. Plouvier, J. L. Stephan, H. Bourdeau, and E. Holler. 1992. Phase I-II trial of a monoclonal anti-tumor necrosis factor alpha antibody for the treatment of refractory severe acutee graft-versus-host disease. *Blood* 79: 3362.

174. Heney, D., C. C. Bailey, and I. J. Lewis. 1990. Thalidomide in the treatment of graft-versus-host disease. *Biomed. Pharmacotherapy* 44: 199.

175. Heney, D., D. R. Norfolk, J. Wheeldon, C. C. Bailey, I. J. Lewis, and D. L. Barnard. 1991. Thalidomide treatment for chronic graft-versus-host disease. *Br. J. Haematol.* 78: 23.

176. Vogelsang, G. B., E. R. Farmer, A. D. Hess, V. Altamonte, W. E. Beschorner, D. A. Jabs, R. L. Corio, L. S. Levin, O. M. Colvin, and J. R. Wingard. 1992. Thalidomide for the treatment of chronic graft-versus-host disease. *NEJM.* 326: 1055.

177. Vossen, J. M., P. J. Heidt, H. van den Berg, E. J. Gerritsen, J. Hermans, and L. J. Dooren. 1990. Prevention of infection and graft-versus-host disease by suppression of intestinal microflora in children treated with allogeneic bone marrow transplantation. *Eur. J. Clin. Microbiol. Infect. Diseases* 9: 14.

178. Beelen, D. W., E. Haralambie, H. Brandt, G. Linzenmeier, K. D. Muller, K. Quabeck, H. G. Sayer, U. Graeven, H. K. Mahmoud, and U. W. Schaefer. 1992. Evidence that sustained growth suppression of intestinal anaerobic bacteria reduces the risk of acute graft-versus-host disease after sibling marrow transplantation. *Blood* 80: 2668.

179. Nakic, B., Z. Nakic, and V. Silobrcic. 1960. Graft versus host reaction in parabiotic disease. *Nature* 186: 322.

180. Hall, C.E., and O. Hall. 1956. On the nature of parabiosis intoxication: shock as the precipitating cause. *J. Exp. Med.* 103: 263.

181. Truitt, R. L. and M. Pollard. 1976. Allogeneic bone marrow chimerism in germ-free mice. IV. Therapy of "Hodgkin's-like" reticulum cell sarcoma in SJL mice. *Transplantation* 21: 12.

182. Witherspoon, R.P., D. Matthews, R. Storb, K. Atckinson, M. Cheever, H.J. Deeg, K. Doney, J. Kalbfleisch, D. Noel, and R. Prentice. 1984. Recovery of in vivo cellular immunity after human marrow grafting. Influence of time postgrafting and acute graft-versus-host disease. *Transplantation* 37: 145.

183. D'Andrea, A. D., E. Bayever, K. M. Haines, R. W. Wilmott, R. A. Polin, C. S. August, and S. D. Douglas. 1986. Serial evaluation of lymphocyte function in bone marrow-grafted patients. *Diagnostic Immunol.* 4: 281.

184. Tutschka, P. J. 1988. Infections and immunodeficiency in bone marrow transplantation. *Ped. Infect. Disease J.* 7: S22.

185. Jaffee, B. D. and H. N. Claman. 1983. Chronic graft-versus-host disease (GVHD) as a model for scleroderma. I. Description of model systems. *Cell. Immunol.* 77: 1.

186. Chosidow, O., M. Bagot, J. P. Vernant, J. C. Roujeau, C. Cordonnier, M. Kuentz, J. Wechsler, C. Andre, R. Touraine, and J. Revuz. 1992. Sclerodermatous chronic graft-versus-host disease. Analysis of seven cases. *J. Am. Acad. Dermatol.* 26: 49.

187. Petrie, H.T., P. Hugo, R. Scollay, and K. Shortman. 1990. Lineage relationships and developmental kinetics of immature thymocytes: CD3, CD4, and CD8 acquisition in vivo and in vitro. *J. Exp. Med.* 172: 1583.

188. Yoshikai, Y., M. Ogimoto, G. Matsuzaki and K. Nomoto. 1990. Bone marrow-derived cells are essential for intrathymic deletion of self-reactive T cells in both the host- and donor-derived thymocytes of fully allogeneic bone marrow chimeras. *J. Immunol.* 145: 505.

189. J.L. Roberts, S.O. Sharrow, and A. Singer. 1990. Clonal deletion and clonal anergy in the thymus induced by cellular elements with different radiation sensitivities. *J. Exp. Med.* 171:935.

190. Bill, J., and E. Palmer. 1989. Positive selection of $CD4^+$ T cells mediated by MHC class II-bearing stromal cells in the thymic cortex. *Nature* 341: 649.

191. Salaun, J., A. Bandeira, I. Khazaal, F. Calman, M. Coltey, A. Coutinho and N.M. LeDouarin. 1990. Thymic epithelium tolerizes for histocompatibility antigens. *Science* 247:1471.

192. Von Boemer, H., H.S. Teh and P. Kisielow. 1989. The thymus selects the useful, negiects the useless and destroys the harmful. *Immunology Today* 10:57.

193. Kruisbeek, A.M., J.J. Mond, B.J. Fowlkes, J.A. Carmen, S. Bridges, and D.L. Longo. 1985. Absence of the $Lyt\text{-}2^-L3T4^+$ lineage of T cells in mice treated neonatally with anti-I-A correlates with absence of intrathymic I-A-bearing antigen-presenting cell function. *J. Exp. Med.* 161: 1029.

194. MacDonald H.R., H. Hengartner, and T. Pedrazzini. 1988. Intrathymic deletion of self-reactive cells prevented by neonatal anti-CD4 antibody treatment. *Nature* 335:174.

195. Zuniga-Pflucker, J.C., L.A. Jones, D.L. Longo, and A.M. Kruisbeek. 1990. CD8 is required during positive selection of $CD4^-/CD8^+$ T cells. *J.Exp.Med.* 171: 427.

196. Zilstra, M., M. Bix, N.E. Simister, J.M. Loring, D.H. Raulet, and R. Jaenisch. 1990. ß2 microglobulin deficient mice lack $CD4^-8^+$ cytolytic T cells. *Nature* 344: 742.

197. Cosgrove, D., D. Gray, A. Dierich, J. Kaufman, M. Lemeur, C. Benoist, and D. Mathis. 1991. Mice lacking MHC class II molecules. *Cell* 66: 1051.

198. Grusby, M.J., R.S. Johnson, V.E. Papaioannou, and L.H. Glimcher. 1991. Depletion of $CD4^+$ T cells in major histocompatibility complex class II-deficient mice. *Science* 253: 1417.

199. Viville, S., J. Neefjes, V. Lotteau, A. Dierich, M. Lemeur, H. Ploegh, C. Benoist, and D. Mathis. 1993. Mice lacking the MHC class II-associated invariant chain. *Cell* 72: 635.

200. Rahemtulla A., W.P. Fung-Leung, M.W. Schilham, T.M. Kündig, S.R. Sambhara, A. Narendran, A. Arabian, A. Wakeham, C.J. Paige, R.M. Zinkernagel, R.G. Miller, and T.W. Mak. 1991. Normal development and function of $CD8^+$ cells but markedly decreased helper cell activity in mice lacking CD4. *Nature* 353: 180.

201. Fung-Leung W.-P., M.W. Schilhan, A. Rahemtulla, T.M. Kundig, M. Vollenweider, J. Potter, W. van Ewik, and T. Mak. 1991. CD8 is needed for development of cytotoxic T cells but not helper T cells. *Cell* 65: 443.

202. Ghayur, T., T.A. Seemayer, A. Xenocostas and W.S. Lapp. 1988. Complete sequential regeneration of graft-vs.-host induced severely dysplastic thymuses. *Am.J.Pathol.* 133:25.

203. Beschorner, W. E., C. A. Shinn, A. C. Fischer, G. W. Santos, and A. D. Hess. 1988. Cyclosporine-induced pseudo-graft-versus-host disease in the early post-cyclosporine period. *Transplantation* 46: 112S.

204. Bos, G. M., G. D. Majoor, R. G. Willighagen, and P. J. van Breda Vriesman. 1989. Chronic cyclosporine-induced autoimmune disease in the rat: a new experimental model for scleroderma. *J. Invest. Dermatol.* 93: 610.

205. Bacigalupo, A., A. Maiolino, M. T. Van Lint, D. Occhini, F. Gualandi, M. Clavio, T. Lamparelli, J. Tong, and A. M. Marmon. 1990. Cyclosporin A and chronic graft versus host disease. *Bone Marrow Transplant.* 6: 341.

206. Shinozawa, T., W. E. Beschorner, and A. D. Hess. 1990. The thymus and prolonged administration of cyclosporine. Irreversible immunopathologic changes associated with autologous pseudo-graft-versus-host disease. *Transplantation* 50: 106.

207. Carella, A. M., E. Gaozza, A. Congiu, P. Carlier, S. Nati, M. Truini, A. Castellaneta, and M. Viale. 1991. Cyclosporine-induced graft-versus-host disease after autologous bone marrow transplantation in hematological malignancies. *Ann. Hematol.* 62: 156.

208. Bryson, J. S., B. E. Caywood, and A. M. Kaplan. 1991. Relationship of cyclosporine A-mediated inhibition of clonal deletion and development of syngeneic graft-versus-host disease. *J. Immunol.* 147: 391.

209. Fischer, A. C., M. K. Laulis, L. Horwitz, and A. D. Hess. 1991. Effect of cyclosporine on T lymphocyte development. Relationship to syngeneic graft-versus-host disease. *Transplantation* 51: 252.

210. Hess, A. D., L. R. Horwitz, M. K. Laulis, and E. Fuchs. 1993. Cyclosporine-induced syngeneic graft-vs-host disease: prevention of autoaggression by treatment with monoclonal antibodies to T lymphocyte cell surface determinants and to MHC class II antigens. *Clin. Immunol. Immunopathol.* 69: 341.

211. Bucy, R. P., X. Y. Xu, J. Li, and G. Huang. 1993. Cyclosporin A-induced autoimmune disease in mice. *J. Immunol.* 151: 1039.

212. Liu, J. 1993. FK506 and cyclosporin, molecular probes for studying intracellular signal transduction. *Immunol. Today* 14: 290.

213. Blazar, B. R., P. A. Taylor, D. C. Snover, S. N. Sehgal, and D. A. Vallera. 1993. Murine recipients of fully mismatched donor marrow are protected from lethal graft-versus-host disease by the in vivo administrationn of rapamycin but develop an autoimmune-like syndrome. *J. Immunol.* 151: 5726.

214. Cooper, M. H., G. G. Hartman, T. E. Starzl, and J. J. Fung. 1991. The induction of pseudo-graft-versus-host disease followingg syngeneic bone marrow transplantation using FK 506. *Transplant. Proc.* 23: 3234.

215. Fast, L. D. 1989. DBA/2J and DBA/2Ha lymphocytes differ in their ability to induce graft-vs-host disease. *J. Immunol.* 143: 2489.

216. Claman, H. N. and H. L. Spiegelberg. 1990. Immunoglobulin dysregulation in murine graft-vs-host disease: a hyper-IgE syndrome. *Clin. Immunol. Immunopathol.* 56: 46.

217. Rozendaal, L., S. T. Pals, E. Gleichmann, and C. J. Melief. 1990. Persistence of allospecific helper T cells is required for maintaining autoantibody formation in lupus-like graft-versus-host disease. *Clin. Exp. Immunol.* 82: 527.

218. Doutrelepont, J. M., M. Moser, O. Leo, D. Abramowicz, M. L. Vanderhaegen, J. Urbain, and M. Goldman. 1991. Hyper IgE in stimulatory graft-versus-host disease: role of interleukin-4. *Clin. Exp. Immunol.* 83: 133.

219. De Wit, D., M. Van Mechelen, C. Zanin, J. M. Doutrelepont, T. Velu, C. Gerard, D. Abramowicz, J. P. Scheerlinck, P. De Baetselier, and Urbain, J. 1993. Preferential activation of Th2 cells in chronic graft-versus-host reaction. *J. Immunol.* 150: 361.

220. Ito, S., M. Ueno, S. Nishi, M. Arakawa, Y. Ikarashi, T. Saitoh, and M. Fujiwara. 1992. Histological characteristics of lupus nephritis in F1 mice withh chronic graft-versus-host reaction across MHC class II difference. *Autoimmunity* 12: 79.

221. Umland, S. P., S. Razac, D. K. Nahrebne, and B. W. Seymour. 1992. Effects of in vivo administration of interferon (IFN)-gamma, anti-IFN-gamma, or anti-interleukin-4 monoclonal antibodies in chronic autoimmune graft-versus-host disease. *Clin. Immunol. Immunopathol.* 63: 66.

222. Otani, M., I. Aoki, Y. Miyagi, K. Misugi, A. Aoki, E. Hagiwara, K. Tani, Y. Ishigatsubo, and T. Okubo. 1992. Glomerulonephritis induced by murine chronic graft-versus-host reaction. *Acta Pathol. Jap.* 42: 325.

223. Aoki, A., Y. Ishigatsubo, E. Hagiwara, A. Shirai, T. Igarashi, K. Tani, A. Maruta, M. Otani, I. Aoki, and K. Misugi. 1993. Polyclonal B cell activation and antigen-specific stimulation in mice with chronic graft-versus-host reaction. *Clin. Immunol. Immunopathol.* 66: 254.

224. Fast, L. D. 1990. Identification of a single non-H-2 gene regulating graft-versus-host disease response. *J. Immunol.* 144: 4177.

225. Morris, S. C., R. L. Cheek, P. L. Cohen, and R. A. Eisenberg. 1990. Autoantibodies in chronic graft versus host result from cognate T-B interactions. *J. Exp. Med.* 171: 503.

226. Gelpi, C., M. A. Martinez, S. Vidal, A. Alguero, C. Juarez, J. A. Hardin, and J. L. Rodriguez-Sanchez. 1990. Different strains of donor parental lymphoid cells induce different models of chronic graft-versus-host disease in murine (Balb/c xx A/J)F1 hybrid hosts. *Clin. Immunol. Immunopathol.* 56: 298.

227. Kier, P., E. Penner, S. Bakos, P. Kalhs, K. Lechner, B. Volc-Platzer, J. Wesierska-Gadek, G. Sauermann, H. Gadner, and W. Emminger-Schmidmeier. 1990. Autoantibodies in chronic GVHD: high prevalence of antinucleolar antibodies. *Bone Marrow Transplant.* 6: 93.

228. Lortan, J. E., N. C. Rochfort, M. el-Tumi, and A. Vellodi. 1992. Autoantibodies after bone marrow transplantation in children with genetic disorders: relation to chronic graft-versus-host disease. *Bone Marrow Transplant.* 9: 325.

229. Siegert, W., R. Stemerowicz, and U. Hopf. 1992. Antimitochondrial antibodies in patients with chronic graft-versus-host disease. *Bone Marrow Transplant.* 10: 221.

230. Rouquette-Gally, A. M., D. Boyeldieu, A. C. Prost, and E. Gluckman. 1988. Autoimmunity after allogeneic bone marrow transplantation. A study of 53 long-term-surviving patients. *Transplantation* 46: 238.

231. Graze, P. R. and R. P. Gale. 1979. Chronic graft versus host disease: a syndrome of disordered immunity. *Am. J. Med.* 66: 611.

232. Kasaian, M. T. and P. Casali. 1993. Autoimmunity-prone B-1 (CD5 B) cells, natural antibodies and self recognition. *Autoimmunity* 15: 315.

233. Reap, E. A., E. S. Sobel, J. C. Jennette, P. L. Cohen, and R. A. Eisenberg. 1993. Conventional B cells, not B1 cells, are the source of autoantibodies in chronic graft-versus-host disease. *J. Immunol.* 151: 7316.

234. Prussick, R., M. C. Brain, I. R. Walker, and D. N. Sauder. 1991. Polymyositis: a manifestation of chronic graft-versus-host disease. *J. Am. Acad. Dermatol.* 25: 560.

235. Pals, S. T., T. Radaszkiewicz, L. Roozendaal, and E. Gleichmann. 1985. Chronic progressive polyarthritis and other symptoms of collagen vascular disease induced by graft-vs-host reaction. *J. Immunol.* 134: 1475.

236. Aoki, I., A. Aoki, M. Otani, Y. Miyagi, K. Misugi, N. Ishii, E. Hagiwara, K. Tani, T. Okubo, and Y. Ishigatsubo. 1992. A correlation between IgG class antibody production and glomerulonephritis in the murine chronic graft-versus-host reaction. *Clin. Immunol. Immunopathol.* 63: 34.

237. Saxon, A., R. E. McIntyre, R. H. Stevens, and R. P. Gale. 1981. Lymphocyte dysfunction in chronic graft-versus-host disease. *Blood* 58: 746.

238. Heinzel, F. P., M. D. Sadick, S. S. Mutha, and R. M. Locksley. 1991. Production of interferon gamma, interleukin 2, interleukin 4, and interleukin 10 by CD4+ lymphocytes in vivo during healing and progressive murine leishmaniasis. *Proc. Natl. Acad. Sci. USA.* 88: 7011.

239. Wang, Z. E., S. L. Reiner, S. Zheng, D. K. Dalton, and R. M. Locksley. 1994. CD4+ effector cells default to the Th2 pathway in interferon gamma-deficient mice

infected with Leishmania major.. *J. Exp. Med.* 179: 1367.

240. Bass, H., T. Mosmann, and S. Strober. 1989. Evidence for mouse Th1- and Th2-like helper T cells in vivo. Selective reduction of Th1-like cells after total lymphoid irradiation. *J. Exp. Med.* 170: 1495.

241. Bacchetta, R., M. Bigler, J. L. Touraine, R. Parkman, P. A. Tovo, J. Abrams, R. de Waal Malefyt, J. E. de Vries, and M. G. Roncarolo. 1994. High levels of interleukin 10 production in vivo are associated with tolerance in SCID patients transplanted with HLA mismatched hematopoietic stem cells. *J. Exp. Med.* 179: 493.

242. Clerici, M. and G. M. Shearer. 1993. A TH1-->TH2 switch is a critical step in the etiology of HIV infection. *Immunol. Today* 14: 107.

243. Gazzinelli, R. T., M. Makino, S. K. Chattopadhyay, C. M. Snapper, A. Sher, A. W. Hugin, and H. C. Morse. 1992. CD4+ subset regulation in viral infection. Preferential activation of Th2 cells during progression of retrovirus-induced immunodeficiency in mice. *J. Immunol.* 148: 182.

244. Cowan, M. J. and M. Golbus. 1994. In utero hematopoietic stem cell transplants for inherited diseases. *Am. J. Ped. Hematol. Oncol.* 16: 35.

245. Weisdorf, D., R. Haake, B. Blazar, W. Miller, P. McGlave, N. Ramsay, J. Kersey, and A. Filipovich. 1990. Treatment of moderate/severe acute graft-versus-host disease after allogeneic bone marrow transplantation: an analysis of clinical risk features and outcome. *Blood* 75: 1024.

246. Phillips, G. L., M. J. Barnett, M. C. Brain, K. W. Chan, L. B. Huebsch, H. G. Klingemann, J. Meharchand, D. E. Reece, W. B. Rybka, and J.D. Shepherd. 1991. Allogeneic bone marrow transplantation using unrelated donors: a pilot study of the Canadian Bone Marrow Transplant Group. *Bone Marrow Transplant.* 8: 477.

247. Nash, R. A., M. S. Pepe, R. Storb, G. Longton, M. Pettinger, C. Anasetti, F. R. Appelbaum, R. A. Bowden, H. J. Deeg, and K. Doney. 1992. Acute graft-versus-host disease: analysis of risk factors after allogeneic marrow transplantation and prophylaxis with cyclosporine and methotrexate. *Blood* 80: 1838.

248. Bradley, B. A., J. M. Hows, S. M. Gore, J. L. Bidwell, T. Clay, T. R. Downie, E. Gluckman, M. R. Howard, and G. J. Laundy. 1992. Current status of unrelated-donor bone marrow transplantation. The International Marrow Unrelated Search and Transplant (IMUST) Study. *Clin. Transplants* 91.

249. Wingard, J. R. 1993. Infections in allogeneic bone marrow transplant recipients. *Sem. Oncol.* 20: 80.

250. Crawford, S. W., G. Longton, and R. Storb. 1993. Acute graft-versus-host disease and the risks for idiopathic pneumonia after marrow transplantation for severe aplastic anemia. *Bone Marrow Transplant.* 12: 225.

251. Johnsen, H. E., L. Bostrom, J. Moller, J. A. Jorgensen, L. Jensen, and O. Ringden. 1992. A study of donor alloreactivity, which may predict acute graft-versus-host disease in HLA identical bone marrow transplantations for early leukaemia. *Scand. J. Immunol.* 35: 353.

252. Ringden, O., M. M. Horowitz, R. P. Gale, J. C. Biggs, J. Gajewski, A. A. Rimm, B. Speck, J. A. Veum-Stone, T. de Witte, and M. M. Bortin. 1993. Outcome after allogeneic bone marrow transplant for leukemia in older adults. *JAMA* 270: 57.

253. Young, J. W., E. B. Papadopoulos, I. Cunningham, H. Castro-Malaspina, N. Flomenberg, M. H. Carabasi, S. C. Gulati, J. A. Brochstein, G. Heller, and P. Black. 1992. T-cell-depleted allogeneic bone marrow transplantation in adults with acute nonlymphocytic leukemia in first remission. *Blood* 79: 3380.

254. Nadezhina, N. M. 1990. Experience in organizing medical care for the victims of

the accident at the Chernobyl Atomic Electric Power Station at a specialized hospital. *Meditsinskaia Radiologiia* 35: 40.

255. Sanders, J. E., R. Storb, C. Anasetti, H. J. Deeg, K. Doney, K. M. Sullivan, R. P. Witherspoon, and J. Hansen. 1994. Marrow transplant experience fo75%% 75'ren with severe aplastic anemia. *Am. J. Ped. Hematol. Oncol.* 16: 43.

256. Spitzer, G. 1991. Autologous bone marrow transplantation in solid tumors. *Current Opinion Oncol.* 3: 238.

257. Johnson, F. L., W. C. Mentzer, K. A. Kalinyak, K. M. Sullivan, and M. R. Abboud. 1994. Bone marrow transplantation for sickle cell disease. The United States experience. *Am. J. Ped. Hematol. Oncol.* 16: 22.

258. Lucarelli, G., M. Galimberti, P. Polchi, E. Angelucci, D. Baronciani, S. M. Durazzi, C. Giardini, F. Albertini, and R. A. Clift. 1992. Bone marrow transplantation in adult thalassemia. *Blood* 80: 1603.

259. Cowan, M.J. 1991. Bone marrow transplantation for the treatment of genetic diseases. *Clin. Biochem.* 24: 375.

260. Filipovich, A. H., R. S. Shapiro, N. K. Ramsay, T. Kim, B. Blazar, J. Kersey, and P. McGlave. 1992. Unrelated donor bone marrow transplantation for correction of lethal congenital immunodeficiencies. *Blood* 80: 270.

261. Jamieson, N. V., V. Joysey, P. J. Friend, R. Marcus, S. Ramsbottom, T. Baglin, P. S. Johnston, R. Williams, and R. Y. Calne. 1991. Graft-versus-host disease in solid organ transplantation. *Transplant Internat.* 4: 67.

262. Starzl, T. E., S. Todo, A. Tzakis, M. Alessiani, A. Casavilla, K. Abu-Elmagd, and J. J. Fung. 1991. The many faces of multivisceral transplantation. *Surgery Gynecol. Obstetrics* 172: 335.

263. Roberts, J. P., N. L. Ascher, J. Lake, J. Capper, S. Purohit, M. Garovoy, R. Lynch, L. Ferrell, and T. Wright. 1991. Graft vs. host disease after liver transplantation in humans: a report of four cases. *Hepatology* 14: 274.

264. Redondo, P., A. Espana, J. I. Herrero, J. Quiroga, J. A. Cienfuegos, J. R. Azanza, and J. Prieto. 1993. Graft-versus-host disease after liver transplantation. *J. Am. Acad. Dermatol.* 29: 314.

265. Greenbaum, B. H. 1991. Transfusion-associated graft-versus-host disease: historical perspectives, incidence, and current use of irradiated blood products. *J. Clin. Oncol.* 9: 1889.

266. Shivdasani, R. A. and K. C. Anderson. 1993. Transfusion-associated graft-versus-host disease: scratching the surface. *Transfusion* 33: 696.

267. Klein, H. G. 1993. Transfusion in transplant patients: the good, the bad, and the ugly. *J. Heart Lung Transplant.* 12: S7.

268. Alain, G., C. Carrier, L. Beaumier, J. Bernard, M. Lemay, and A. Lavoie. 1993. In utero acute graft-versus-host disease in a neonate with severe combined immunodeficiency. *J. Am. Acad. Dermatol.* 29: 862.

269. Hara, T., M. Nagata, Y. Mizuno, and K. Ueda. 1990. Novel T-lymphocyte population in combined immunodeficiency with features of graft-versus-host disease. *NEJM.* 322: 336.

270. Hamilton, M. S., J. Becker, and B. L. Hamilton. 1988. Maternally induced graft-vs-host disease to minor antigens as a possible etiology of an acquired immunodeficiency syndrome in mice. *Cancer Detect. Prevent.* 12: 205.

271. Pietryga, D. 1993. Prevention and treatment of acute graft-vs.-host disease. *Am. J. Ped. Hematol. Oncol.* 15: 28.

272. Quinones, R. R. 1993. Hematopoietic engraftment and graft failure after bone marrow transplantation. *Am. J. Ped. Hematol. Oncol.* 15: 3.

273. Hows, J. M. 1991. Mechanisms of graft failure after human marrow transplantation: a review. *Immunol. Letters* 29: 77.

274. Sosman, J. A. and P. M. Sondel. 1993. The graft-vs.-leukemia effect. Implications for post-marrow transplant antileukemia treatment. *Am. J. Ped. Hematol. Oncol.* 15: 185.

275. Truitt, R. L. and A. A. Atasoylu. 1991. Contribution of CD4+ and CD8+ T cells to graft-versus-host disease and graft-versus-leukemia reactivity after transplantation of MHC-compatible bone marrow. *Bone Marrow Transplant.* 8: 51.

276. Mehta, J. 1993. Graft-versus-leukemia reactions in clinical bone marrow transplantation. *Leukemia Lymphoma* 10: 427.

277. OKunewick, J. P., D. L. Kociban, L. L. Machen, and M. J. Buffo. 1991. The role of CD4 and CD8 T cells in the graft-versus-leukemia response in Rauscher murine leukemia. *Bone Marrow Transplant.* 8: 445.

278. Schreiber, K. L. and J. Forman. 1990. Effect of graft-versus-host disease on anti-tumor immunity. *J. Immunol.* 144: 2018.

279. Kolb, H. J., J. Mittermuller, C. Clemm, E. Holler, G. Ledderose, G. Brehm, M. Heim, and W. Wilmanns. 1990. Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients. *Blood* 76: 2462.

280. Noga, S. J. and A. D. Hess. 1993. Lymphocyte depletion in bone marrow transplantation: will modulation of graft-versus-host disease prove to be superior to prevention?. *Sem. Oncol.* 20: 28.

281. Kennedy, M. S., H. J. Deeg, R. Storb, K. Doney, K. M. Sullivan, R. P. Witherspoon, F. R. Appelbaum, P. Stewart, J. Sanders, and C. D. Buckner. 1985. Treatment of acute graft-versus-host disease after allogeneic marrow transplantation. Randomized study comparing corticosteroids andd cyclosporine. *Am. J. Med.* 78: 978.

282. Schwinghammer, T. L. and E. J. Bloom. 1993. Pharmacologic prophylaxis of acute graft-versus-host disease after allogeneic marrow transplantation. *Clin. Pharm.* 12: 736.

283. Remlinger, K., P. J. Martin, J. A. Hansen, K. C. Doney, A. Smith, H. J. Deeg, K. Sullivan, R. Storb, and E. D. Thomas. 1984. Murine monoclonal anti-T cell antibodies for treatment of stercid-resistant acute graft-versus-host disease. *Human Immunology* 9: 21.

284. Anasetti, C., P.J. Martin, R. Storb, F.R. Appelbaum, P.G. Beatty, J. Davis, K. Doney, H.F. Hill, P. Stewart, and K.M. Sullivan. 1992. Treatment of acute graft-versus-host disease with a nonmitogenic anti-CD3 monoclonal antibody. *Transplantation* 54: 844.

285. Beelen, D. W., H. Grosse-Wilde, U. Ryschka, K. Quabeck, H. G. Sayer, U. Graeven, and U. W. Schaefer. 1991. Initial treatment of acute graft-versus-host disease with a murine monoclonal antibody directed to the human alpha/beta T cell receptor. *Cancer Immunol. Immunotherapy* 34: 97.

286. Gleixner, B., H. J. Kolb, E. Holler, S. Liesenfeld, C. Riedner, E. Hiller, C. Seeber, J. Kempeni, A. Moller, and H. Knabe. Treatment of aGVHD with OKT3: clinical outcome and side-effects associated with release of TNF alpha. *Bone Marrow Transplant.* 8: 93.

287. Tilg, H., B. Eibl, M. Pichl, A. Gachter, M. Herold, J. Brankova, C. Huber, and D. Niederwieser. 1993. Immune response modulation by pentoxifylline in vitro. *Transplantation* 56: 196.

288. Bianco, J. A., F. R. Appelbaum, J. Nemunaitis, J. Almgren, F. Andrews, P. Kettner, A. Shields, and J. W. Singer. 1991. Phase I-II trial of pentoxifylline for the prevention of transplant-related toxicities following bone marrow transplantation. *Blood* 78: 1205.

289. Clift, R. A., J. A. Bianco, F. R. Appelbaum, C. D. Buckner, J. W. Singer, L. Bakke, W. I. Bensinger, R. A. Bowden, G. B. McDonald, and M. Schubert. 1993. A randomized controlled trial of pentoxifylline for the prevention of regimen-related toxicities in patients undergoing allogeneic marrow transplantation. *Blood* 82: 2025.

290. Gao E.-K., D. Lo, R. Cheney, O. Kanagawa, and J. Sprent. 1988. Abnormal differentiation of thymocytes in mice treated with cyclosporin A. *Nature* 336: 176.

291. Beschorner, W.E., K.A. Kelly, A.D. Heśs, and G.W. Santos. 1987. Cyclosporine and the thymus: influence of irradiation and age on thymic immunopathology and recovery. *Cell. Immunol.* 110: 350.

CHAPTER 2.

The first phase of GVHR-induced T cell suppression: a defect in T cell signalling.

Levels of $p56^{lck}$ and $p59^{fyn}$ are reduced by a glucocorticoid-dependent mechanism in graft-versus-host reaction-induced T cell anergy.[1]

by Julie Desbarats, Kong E. You-Ten and Wayne S. Lapp

from the Department of Physiology, McGill University, Montreal, QC., Canada H3G 1Y6.

Running title: Glucocorticoid-induced reduction in $p56^{lck}$ and $p59^{fyn}$ in anergic T cells

Address correspondence to Julie Desbarats at the Department of Physiology, McGill University, 3655 Drummond St., Montreal, Quebec, Canada H3G 1Y6.

[1] This paper was submitted for publication to the *Journal of Experimental Medicine* in August 1994.

A preliminary report in abstract form was published in the Journal of Cellular Biochemistry (Keystone Symposia on Molecular and Cellular Biology), 1994, Suppl. 18D, # V551.

Acknowledgments

We thank Roussel Uclaf for the generous gift of RU 38486 and Vincent Dodelet for the Jurkat clone. We gratefully acknowledge the advice, assistance and reagents provided by Drs. André Veillette and Riaz Farookhi, and the expert technical assistance of Ailsa Lee Loy, Rosmarie Siegrist-Johnstone, and Marielle Fournel.

This work was supported by grants from the Medical Research Council of Canada and the National Cancer Institute of Canada. J. Desbarats was supported by studentship awards from the Fonds de Recherches en Santé du Québec and the Medical Research Council of Canada.

Summary

The graft-versus-host reaction (GVHR) results in profound, long-lasting immunosuppression characterized by T cell unresponsiveness to antigenic and mitogenic stimuli. In this report, the roles of the protein tyrosine kinases (PTKs) $p56^{lck}$ and $p59^{fyn}$ in GVHR-induced T cell anergy were investigated. GVHR was induced by the intravenous transfer of parental lymphoid cells into F1 hybrid recipient mice. The recipient animals were examined in the early acute and late chronic phases of the disease to determine spleen and lymph node T cell function, phenotype, and expression of the PTKs involved in T cell activation. The levels of lck and fyn declined dramatically in splenic and lymph node T cells as the reaction progressed and T cell immunosuppression developed. In contrast, thymic lck and fyn levels remained normal throughout the disease. The decrease in lck and fyn occurred independently of receptor downregulation, since the surface expression of CD4, CD8, and CD3 on peripheral T cells remained normal concurrent with the reduction in their associated PTKs. GVHR-induced reduction of lck and fyn was prevented by adrenalectomy of the GVH-reactive mice, suggesting a glucocorticoid-mediated mechanism. Indeed, treatment with exogenous glucocorticoids induced lck and fyn downregulation in the lymph node T cells of normal mice, and in cultured T cell clones. We propose that the GVHR-induced increase in endogenous glucocorticoids triggers a reduction in T cell lck and fyn, leading to severe immunosuppression, which may represent a general mechanism of

glucocorticoid-mediated immune regulation.

Introduction

The graft-versus-host reaction (GVHR) induced by injection of parental lymphoid cells into F1 hybrid recipients consists of an acute syndrome of immunosuppression, lesions of epithelial and lymphoid tissues, and cachexia often leading to death. Survivors of the acute reaction develop chronic GVHR, a multisystem disease characterized by persistent immunosuppression (1, 2). Early GVHR includes a phase of massive T cell proliferation and lymphokine production, as the semi-allogeneic parental graft cells react to the host (3). After the proliferative phase, the splenic T cells from GVH-reactive mice decrease rapidly in numbers and become profoundly unresponsive to a variety of activation stimuli (4). *In vivo*, they are unable to generate self-restricted responses to antigens, and fail to provide help to B cells for Ig production, and to cytotoxic cell precursors for CTL priming (5 - 8). *In vitro*, proliferation, expression of IL-2 receptors (IL-2R) and production of IL-2 and IL-4 in response to mitogens, anti-CD3 antibodies, and interleukins are severely impaired (4, 9, 10). These functional deficits are not the result of active suppression, and can be demonstrated in purified $CD4^+$ and $CD8^+$ lymphocytes isolated from the spleens of GVH-reactive mice (4). T cells from GVH-reactive

mice (4) and bone-marrow transplanted patients (11) cannot generate or sustain levels of intracellular calcium sufficient for activation, although they do respond to high levels of ionomycin and PMA. Taken together, these observations suggest that the defective T cell signal in GVH-induced anergy is an upstream event in the induction of activation.

An early event in the T cell receptor (TCR) signalling cascade is the rapid tyrosine phosphorylation on a variety of intracellular substrates and triggering of the phosphatidylinositol pathway (12, 13). $p59^{fyn}$ and $p56^{lck}$ are src family protein tyrosine kinases (PTKs) implicated in early signalling for T cell activation (14, 15). Alternatively spliced variants of fyn are expressed in the brain and in lymphoid tissue (16, 17); the lymphoid form is found non-covalently associated with the ε, γ, ζ and η chains of the CD3/TCR complex (18), and its activity increases transiently upon TCR stimulation (19). Overexpression of fyn in hybridomas resulted in increased antigen-induced IL-2 expression (17), and in transgenic mice produced thymocyte hyper-responsiveness to TCR stimulation (20). Conversely, in fyn-knockout mice and transgenics with kinase-deficient mutant fyn, T cell activation thresholds, especially in immature cells, were elevated (21, 22). Thus, fyn seems to modulate and amplify TCR-mediated signals. Lck is associated with CD4 and CD8 through cysteine interactions (23 - 25), and with the IL-2R (26, 27). Lck may be involved in T cell activation by the early phosphorylation of the TCR-associated signal transduction molecules, notably CD3ζ, upon cross-linking of

the TCR with CD4 or CD8 (28). Mutant cell lines having lost expression of lck resembled GVHR-anergized T cells: they were unable to signal through the TCR and failed to induce intracellular calcium mobilization, although they retained the ability to proliferate in response to PMA and ionomycin (29). In addition, in a tumor model of T cell immune suppression, fyn and lck were downregulated (30).

We therefore chose to investigate the potential dysfunction of lck and fyn in GVHR-induced T cell unresponsiveness. Our data indicate that during GVHR, the levels of these PTKs were drastically reduced in peripheral T cells despite unchanged surface levels of CD3, CD4 and CD8. Furthermore, our results suggest that the reduction in the levels of signal-transducing PTKs occured in response to the GVHR-induced elevation of endogenous glucocorticoid levels.

Materials and Methods

Animals. Mice of the inbred strains A ($H\text{-}2^a$) and C57BL/6 (B6) ($H\text{-}2^b$), and the F1 hybrid B6xA (B6AF1) were used. All mice were bred and maintained in our animal colony.

GVHR induction. GVHRs were induced as described previously (31). Briefly, pooled parental spleen and lymph nodes were made into single cell suspensions and injected i.v. into F1 hybrid recipients. Viable donor cell dose

was $5x10^7$ for A strain donors and $6x10^7$ for B6 donors.

Adrenalectomy and in vivo glucocorticoid treatment. Adrenalectomies were performed as described previously (32) 10-14 days prior to GVHR induction. Adrenalectomized mice were maintained on physiological saline in place of water. Cortisone-treated mice received 2.5 mg cortisone acetate (Merck Sharp & Dohme, Kirkland, Quebec, Canada) injected intra-peritoneally 48 hours before animals were killed.

Mitogen assays. T cell proliferative responses to concanavalin A (Con A) and phytohemaglutinin (PHA) were evaluated by [^{3}H]thymidine incorporation as described previously (33). Briefly, $5x10^5$ spleen cells were cultured for 48 hours in triplicate with or without mitogen, then for a further 16 hours with 1μCi/well of [^{3}H]thymidine. DNA was harvested with an automated cell harvester and [^{3}H]thymidine incorporation was quantitated using a beta counter (LKB Instruments, Turku, Finland).

Flow cytometry analysis. Single cell suspensions of spleen or pooled lymph nodes were prepared by gentle tamping through 50-mesh stainless steel screens. Aliquots of 10^6 cells were incubated with primary antibodies at 4°C for 20 minutes, then washed and incubated at 4°C for 20 minutes with secondary reagents if necessary. Labelled cells were analysed on a FACScan® flow cytometer (Becton Dickinson & Co., Mountain View, CA).

Reagents for flow cytometry. For single color labelling, culture supernatants from hybridomas H57-597 (anti-TCR) and 145-2C11 (anti-CD3ε)

were used as a source of primary antibodies, in conjuction with goat-anti-hamster-PE (Caltag Laboratories, San Fransisco, CA) as the second step reagent. For three-color staining, anti-CD3-FITC (Boehringer Mannheim Canada Ltd., Laval, Canada), anti-CD4-PE, and anti-CD8-biotin followed by streptavidin-CyChrome (Cedarlane Laboratories Ltd., Hornby, Ont., Canada) were used.

T cell enrichment. Single cell suspensions of spleen or lymph node cells were depleted of red blood cells by osmotic shock with distilled water, of adherent cells by incubation on tissue culture treated petri dishes (Nunclon®, Copenhagen, Denmark) for 1 hr at 37°C, then of B cells by panning for 1.5 hrs at 4°C on petri dishes (Fisher Scientific Co., Ottawa, Canada) pre-coated by overnight incubation at 4°C with 2 µg/ml anti-mouse IgG+IgM antibody (Caltag Laboratories). Typically, enriched populations were 80 - 90% $CD3^+$ T cells and < 2% B cells.

Preparation of mature thymocytes. Mature, PNA^- thymocytes were isolated by incubating single cell suspensions of thymocytes at 8 x 10^8 cells / ml in PBS with an equal volume of 1 mg / ml PNA (E-Y Laboratories, Inc., San Mateo, CA) for 10 min at room temperature. The suspensions were then layered onto PBS supplemented with 20% FCS and allowed to sediment for 30 minutes. Mature, PNA^- thymocytes were harvested from the top of the suspension.

Lck and Fyn immunoblots. Immunoblots were performed as described

previously (17, 25). Briefly, cells were washed in PBS, pelleted, and lysed in lysis buffer (1% NP40 or 2% SDS as indicated, 50mM Tris, 2mM EDTA, pH 8.0) supplemented with protease and phosphatase inhibitors (100 µg/ml each leupeptin, aprotinin, and PMSF, 50 mM sodium fluoride, and 100 µm sodium orthovanadate). Equivalent amounts of protein were loaded in each lane and resolved on 8% or 12.5% SDS-PAGE gels (for detecting PTKs or phosphotyrosine residues, respectively). Proteins were transfered to nitrocellulose membranes at 40V for 2 hours in Tris/glycine buffer, and immunoblotted with rabbit antisera generously provided by A. Veillette (anti-Lck: immunization with a peptide corresponding to amino-acids 39-64 of the murine $p56^{lck}$ sequence (34); anti-Fyn: immunization with residues 25-141 of the murine $p59^{fyn}$ sequence (17)), followed by ^{125}I-Protein A (Amersham, Arlington Heights, IL). Alternatively, membranes were immunoblotted with commercial anti-lck or anti-fyn antisera (Upstate Biotechnology Institute, Lake Placid, NY) or with anti-phosphotyrosine monoclonal antibodies (P-3300, Sigma, St. Louis, MO), then developed with alkaline-phosphatase-conjugated secondary antibodies (Promega, Madison, WI).

Cell culture and in vitro glucocorticoid treatment. Jurkat cells were seeded at 10^7 cells per well in 24-well plates and cultured for 48 hours in tissue culture medium (RPMI supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 10mM HEPES and $5x10^{-5}$M 2-ME) with or without dexamethasone (10^{-6}M or 10^{-5}M as indicated) (Steraloids, Wilton, NH) and/or

RU 38486 (3 x 10^{-6}M or 3 x 10^{-5}M as indicated) (Roussel Uclaf, Romainville, France). CTLL-2 cells were cultured as above, but all wells were supplemented with half-optimal concentrations of IL-2 (supernatants from MLA 144, an IL-2 producing clone). Splenocytes (whole spleens in single cell suspension) were cultured as above with the addition of half-optimal concentrations of the T cell mitogen Concanavalin A (1.25 mg/ml, Pharmacia, Uppsala, Sweden). In each experiment some wells were treated with ethanol (2.5% v/v) to induce non-receptor dependent cell death. Viability was determined in every culture by trypan blue dye exclusion.

Results

Lck and fyn were downregulated in spleen and lymph node T cells during GVHR. In our initial studies, we examined the tyrosine phosphorylation pattern in the anergic peripheral T cells of GVH-reactive mice. Anti-phosphotyrosine immunoblotting of splenic T cell lysates revealed a specific decrease of the bands in the 45 to 65 kD range, which became progressively more pronounced as the GVHR developed (Fig. 1). These specific disappearance of these bands suggested a decrease in lck and fyn protein levels or degree of phosphorylation. Indeed, levels of lck and fyn were reduced in T cells freshly isolated from the spleens and lymph nodes of GVH-reactive mice (Fig. 2). The intensity of the GVHR, controlled by the strain combination and cell dose used

to induce the reaction, determined when the reduction in the PTK levels first occured. A marked decrease in lck and fyn was consistently observed ten to fifteen days after disease induction, and the reduction was exacerbated progressively over the course of the acute reaction (Fig. 2 *A* and *B*). During chronic GVHR, lck and fyn remained downregulated (Fig. 2 *C*). At the time of each assay for PTK levels, GVHR-induced T cell unresponsiveness was confirmed by mitogen stimulation of splenocytes. As previously extensively documented (1, 2, 4, 33, 35, 36), splenocytes from GVH-reactive mice failed to display Con A and PHA responses above background levels (data not shown).

Membrane expression of CD3, CD4 and CD8 was not decreased concomittant with the reduction in PTKs. The cell surface expression of membrane receptors associated with lck and fyn, CD4 or CD8 and CD3/TCR respectively, was examined in spleen and lymph nodes by flow cytometry (Fig. 3). Early in acute GVHR (12 days after induction), CD3/TCR expression was slightly decreased (data not shown). However, low membrane expression of CD3/TCR levels did not correlate with reduced PTK levels, since T cells from GVH-reactive mice expressed normal levels of CD3 when their associated PTKs were significantly downregulated, 18 days and onward after GVHR induction (Fig. 3 *A*). Expression of CD4 and CD8 was not altered on day 23 after GVHR induction (Fig. 3 *B*), and remained normal throughout GVHR. Thus, decreased surface expression of CD3, CD4 and CD8 did not accompany the reduction in their

associated PTKs.

Thymocyte lck and fyn levels were not altered during GVHR. Thymic function and T cell education are abnormal during GVHR (8, 31, 32), raising the possibility that the low levels of PTKs in peripheral T cells may be a consequence of their defective expression in new thymic emigrants. GVHR-induced thymic atrophy is characterized by a loss of immature PNA^+ cortical thymocytes (8, 31, 32). Therefore to compare similar thymic subsets for PTK expression, the PNA^- thymocytes were purified and used in the preparation of protein lysates. Lck and fyn levels in mature, PNA^- thymocytes of GVH-reactive animals were not significantly different from those of normal mice (Fig. 4). Reduced PTK expression was thus probably a post-thymic event unlikely to reflect a defect in new thymic emigrants.

GVHR-induced reduction in lck and fyn was prevented by adrenalectomy in GVH-reactive mice and was replicated in normal mice by exogenous cortisone. GVH-reactive mice produce high levels of endogenous glucocorticoids, maintained throughout the course of the reaction (37, 38, and manuscript in preparation, K.E. You-Ten *et al*). We investigated the involvement of glucocorticoids in the regulation of lck and fyn by adrenalectomizing (ADX) recipient mice prior to GVHR induction. In normal mice, ADX did not alter PTK expression (Fig. 5, lane 2). In contrast, in GVH-reactive mice, ADX

prevented the downregulation of T cell lck and fyn (Fig. 5, lane 5) observed in non-ADX GVH-reactive animals (Fig. 2, and Fig. 5, lane 4). Furthermore, cortisone administered to the GVH-reactive ADX mice reversed the effect of the ADX and resulted again in highly reduced levels of the PTKs (fig. 5, lane 6). To investigate whether cortisone was acting alone to induce PTK downregulation, or conversely, was synergizing with other GVHR-dependent factors, normal (non-GVH-reactive) ADX animals were treated with cortisone. We observed that a high dose of cortisone, alone, produced a marked reduction in the lymph node T cell lck and fyn of normal ADX mice (Fig. 5, lane 3).

Glucocorticoids induced a reduction in lck and fyn in T cell clones in vitro. Reduction of T cell lck and fyn by cortisone in vivo may be mediated via other cell types. To determine whether glucocorticoids could act directly on the T cells, in vitro experiments were performed. Cells of the human Jurkat T_H clone cultered for two days with dexamethasone (a synthetic glucocorticoid) demonstrated reduced levels of lck and fyn without impaired cell viability (Fig. 6, lane 2). The reduction in lck and fyn was reversed if the glucocorticoid inhibitor RU 38486 was present in the cultures, demonstrating that the response was receptor-mediated (Fig. 6, lanes 3 and 4). Furthermore, cultures treated with RU 38486 expressed higher levels of the PTKs than untreated cultures, possibly due to its antagonistic effect on the cortisol present in the FCS used to supplement the medium (Fig. 6, lanes 4 and 5). Cells treated

with a low concentration of ethanol to induce non-receptor dependent cell death did not display comparably decreased lck and fyn levels despite increased cell death (Fig. 6, lane 6). Similar results were obtained when the same experiments were carried out with freshly harvested normal splenocytes from normal mice, or with the CTLL-2 murine T cell line. Thus it appears that glucocorticoids can act directly on T cells to downregulate lck and fyn expression.

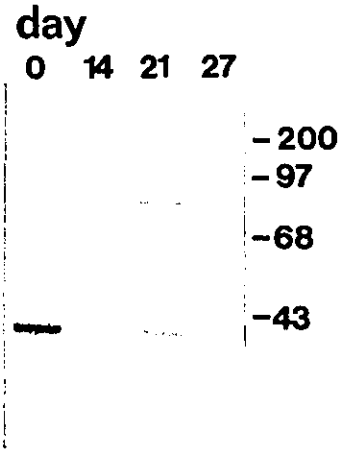


Figure 1. Anti-phosphotyrosine bands in the 45 to 65 kD range were progressively and selectively downregulated in splenic T cells during GVHR. GVHR was induced by the intravenous transfer of $5x10^7$ A strain parental lymphoid cells into B6AF1 hybrid recipients. On days 14, 21 and 27 after GVHR induction T cells (80-90% $CD3^+$ T cells and <2% $B220^+$ B cells) were enriched from the spleens. T cells were lysed 1% NP-40 buffer, and proteins were resolved on a 12.5% SDS-PAGE gel and immunoblotted with anti-phosphotyrosine antibodies.

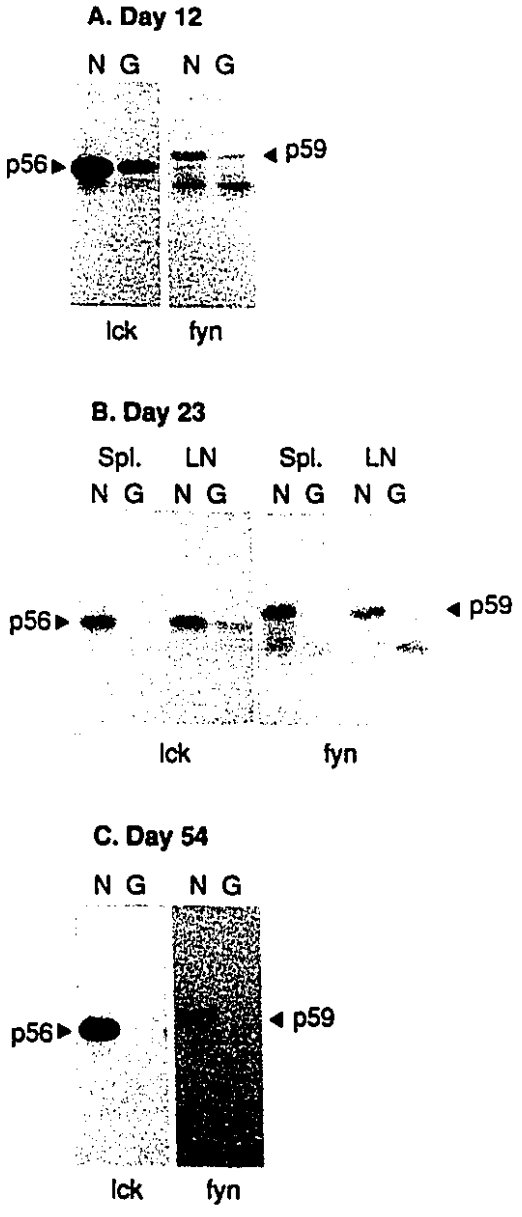
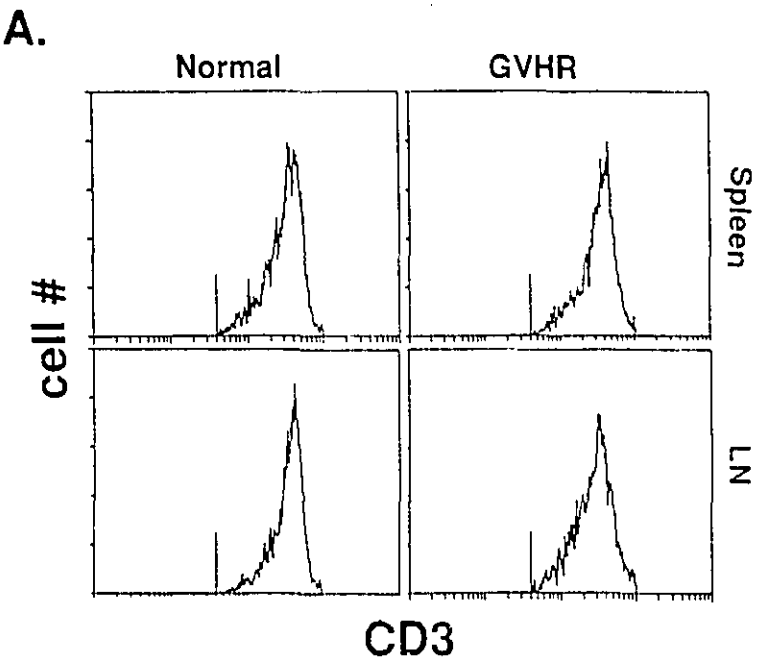


Figure 2. $p56^{lck}$ and $p59^{fyn}$ were downregulated in the T cells of GVH-reactive mice. Lck and fyn immunoblots of T cell lysates from normal (N) and GVH-reactive (G) mice were prepared from the spleen 12 days after GVHR induction (A); from the spleen (Spl.) and lymph nodes (LN) 23 days after induction (B); and from the spleen during chronic GVHR, 54 days after induction (C). GVHR was induced with $6x10^7$ C57BL/6 donor cells, resulting in a more severe reaction than that induced with 5×10^7 A strain cells (Fig. 1). Typically, changes in the levels of lck and fyn in the spleen and lymph nodes of GVH-reactive mice were comparable and followed similar kinetics.

Figure 3. Membrane receptor downregulation did not accompany reduced PTK expression. (A). Unfractionated spleen and lymph node cell suspensions were triple-labelled for CD3, CD4 and CD8. Flow cytometry histograms depict CD3 expression on a gated population consisting of both single positive $CD4^+$ and $CD8^+$ cells, showing that these subsets of T cells from GVH-reactive mice express normal levels of CD3. Staining with anti-TCR antibody (H57-597) gave identical results (not shown). (B). Splenic T cells from normal (solid lines) and GVH-reactive (dotted lines) mice express identical levels of CD3, CD4 and CD8. Labelling was performed on T cell-enriched suspensions from normal and GVH-reactive mice 23 days after GVHR induction with B6 parental donor cells. Animals examined between 18 and 54 days after disease induction showed normal cell surface receptor levels.



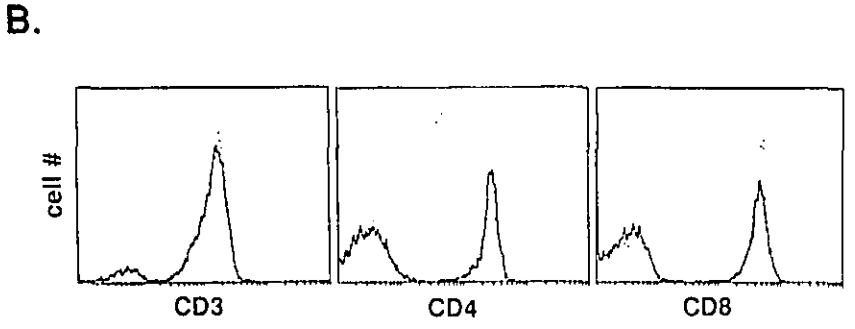


Figure 3.

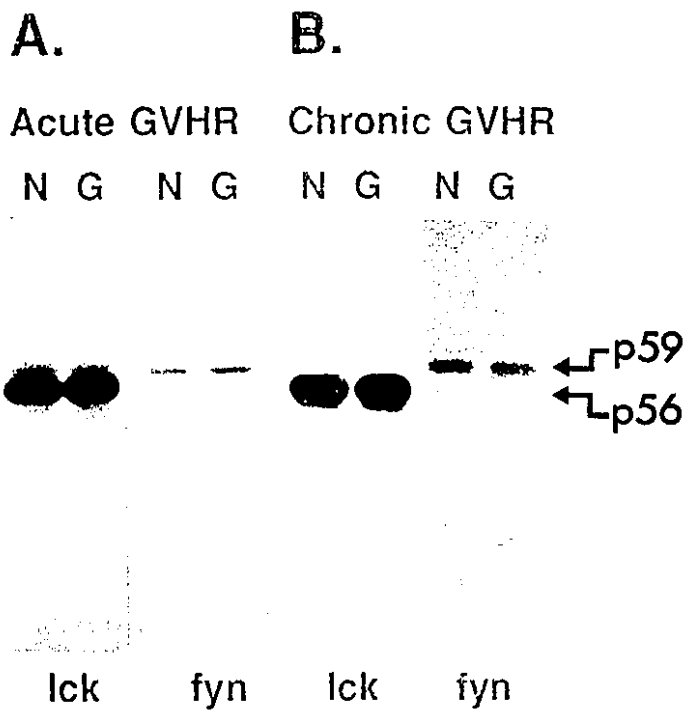


Figure 4. Thymic levels of lck and fyn are normal in GVH-reactive mice. Anti-lck and anti-fyn immunoblots of PNA^- thymocytes were performed 12 days (acute) and 54 days (chronic) after disease induction. N, normal mice; G, GVH-reactive mice induced with B6 parental donor cells.

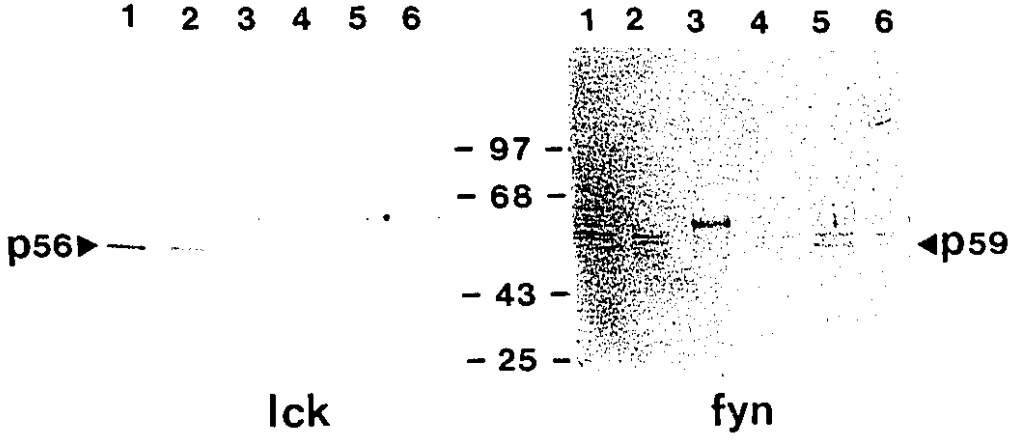


Figure 5. GVHR-induced reduction in lck and fyn was replicated in normal mice by exogenous cortisone and was prevented by adrenalectomy in GVH-reactive mice. Lymph node T cells from: lane 1, normal control mice; lane 2, adrenalectomized (ADX) mice; lane 3, ADX mice injected with cortisone; lane 4, GVH-reactive mice 10 days after disease induction; lane 5, GVH-reactive ADX mice; and lane 6, GVH-reactive ADX mice injected with cortisone. GVHR was induced with 6 x 10^7 B6 strain cells. Lysates were prepared in SDS buffer, and immunoblotted with commercial anti-lck and anti-fyn antisera.

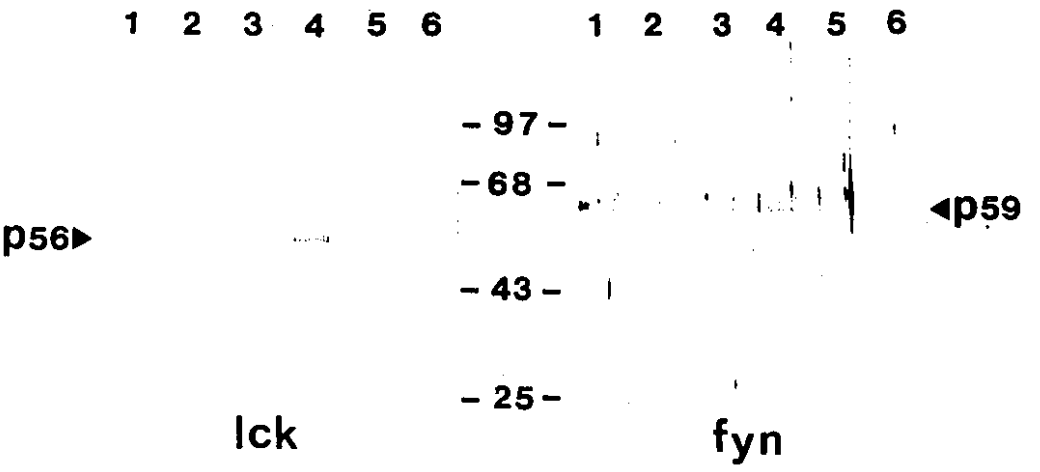


Figure 6. Lck and fyn were downregulated in dexamethasone-treated Jurkat cells and rescued by the anti-glucocorticoid RU 38486. Lane 1, Jurkat cells cultured in medium alone; lane 2, dexamethasone (10^{-6}M) added to culture; lane 3, dexamethasone (10^{-6}M) and RU 38486 (3×10^{-6} M) added to culture; lane 4, dexamethasone (10^{-6}M) and RU 38486 (3×10^{-5} M) added to culture; lane 5, RU 38486 (3×10^{-6} M) added to culture; lane 6, ethanol (2.5% final concentration) added to the medium to induce non-receptor-mediated cell death. Viability in the cultures was as follows: lane 1, 60.7%; 2, 81.6%; 3, 73.9%; 4, 66.4%; 5, 85.3%; and 6, 42.4%. Lysates were prepared in SDS buffer, and immunoblotted with commercial anti-human lck and anti-human fyn antisera.

Discussion

Our data indicate that both major PTK activities implicated in T cell activation are reduced or absent during GVHR, effectively eliminating redundancy systems for early TCR-mediated tyrosine phosphorylation. ZAP-70 is a third PTK involved in activation; however, its activation as well as its association with the TCR/CD3 complex is believed to be dependent on lck- and/or fyn-mediated phosphorylation of the three antigen recognition activation motifs (ARAMs) on CD3ζ (as well as single motifs on CD3 γ, δ and ζ) (13, 39 - 41). Thus, lack of lck and fyn may also prevent ZAP-induced phosphorylation of downstream substrates, thus crippling the cell's TCR-mediated signalling pathway. The downregulation of lck and fyn, then, provides a molecular mechanism for the profound T cell immunosuppression characteristic of GVHR. We demonstrate that the physiological mechanism regulating GVHR-induced lck and fyn downregulation was the secretion of high levels of endogenous glucocorticoids, and that this effect may be a direct consequence of T cell glucocorticoid receptor binding since it can be replicated in vitro on T cell clones, in the absence of other cell types, and can be blocked by the steroid receptor antagonist RU 38486.

Certain types of T cell unresponsiveness can be induced in the absence of glucocorticoids. A classical system for the induction of T cell anergy is by antibody-mediated CD3/TCR cross-linking in vitro, in the absence of

costimulation (42). The T cells undergo activation followed by anergy, characterised by failure to be reactivated by stimuli which normally induce strong activation. However, such anergic states are biochemically distinct from GVHR-induced T cell unresponsiveness; unlike GVHR-anergized T cells, these cells constitutively upregulate fyn expression (43) and can be rescued by exogenous IL-2 (44). In a TCR-transgenic mouse model, TCR downregulation has been reported as a mechanism for peripheral tolerance induction (45). Although we have observed transient TCR downregulation during the proliferative phase of the GVHR, the unresponsive T cells present during the later stages of the reaction express normal levels of cell surface CD3/TCR.

In contrast, GVHR-induced T cell unresponsiveness resembles superantigen-induced T cell anergy. In both cases, a stimulus capable of activating a large number of T cells triggers a sequence of massive, rapid T cell proliferation (46) followed by anergy (47 - 49) and apoptotic death of the responding cells (50). Furthermore, glucocorticoids appear to control the superantigen-induced activation and clonal deletion of peripheral T cells in vivo (51, 52). Endogenous glucocorticoid levels are elevated within two hours of treatment with bacterial superantigens; glucocorticoid antagonists largely protect the T cells from anergy and death, while supra-physiological doses of exogenous glucocorticoids augment T cell death. Our results in GVHR parallel these findings, and suggest that glucocorticoid modulation of TCR signal transduction as reported herein may in fact be a general mechanism by which

glucocorticoids regulate the immune system.

GVHR-induced T cell immunosuppression and downregulation of lck and fyn persisted long into the chronic phase of the reaction. Concurrently, endogenous glucocorticoid levels remain elevated long after the initial proliferative burst (37, 38). Immune activation triggers an elevation in circulating glucocorticoids via the direct effects of cytokines, primarily IL-1, on the hypothalamic-pituitary-adrenal axis (53). As in superantigen-induced immune activation, the initial proliferative phase probably provides sufficient IL-1 to trigger the high plasma corticosterone. However, as the reaction progresses, pathological injury to various organs, including the gut, appears (54, 55). Lipopolysaccharide (LPS) from gram negative bacteria, a potent stimulus for glucocorticoid production (56), enters the circulation via the injured gut (56), and may be responsible for the sustained high levels of endogenous glucocorticoids present throughout the GVHR. Endogenous glucocorticoid production in response to the products of gram positive (enterotoxins, bacterial superantigens) and gram negative (endotoxin, LPS) organisms may be a homeostatic regulatory mechanism to limit T cell activation and clonal expansion leading to excessive cytokine secretion, since cytokines such as tumor necrosis factor α (TNF) are the final mediators of lethal septic shock (57, 58).

The role of glucocorticoids in the modulation of T cell signalling may explain the old observation that ADX can either exacerbate or alleviate GVHR,

depending on the severity of the reaction (59 - 61). ADX mice experienced higher mortality during acute GVHR induced with high cell doses than did non-ADX controls (59, 60). Death from acute GVHR is due to cytokine-induced septic shock (3, 36), and the lack of adrenal glucocorticoids to negatively regulate cytokine production may have resulted in higher mortality. This is analogous to the acute death induced by oligoclonal T cell stimuli, such as anti-CD3 antibody or bacterial superantigen, in ADX mice or mice treated with glucocorticoid antagonists (51). Paradoxically, in GVHR induced with lower cell doses, ADX mice experienced a less severe reaction and recovered more fully and earlier than non-ADX animals (61). These mice did not undergo glucocorticoid-induced PTK downregulation, and thus, despite transient T cell immunosuppression probably mediated by prostaglandins (1) and interferon-γ (62), escaped the profound, long term immunosuppression which precludes a rapid recovery. Again, an analogy can be drawn with superantigen-treated mice, in which the reactive T cell clones were rescued from peripheral deletion by pre-treatment with an anti-glucocorticoid (51).

Mature thymocytes, unlike peripheral T cells, maintained normal levels of lck and fyn throughout the GVHR, although the thymocytes had undoubtably been exposed to the high levels of circulating glucocorticoids since the thymuses of GVH-reactive mice undergo steroid-dependent involution (8, 31, 32). This suggested that T cells responding to antigen may be preferentially sensitive to the glucocorticoid-mediated reduction in PTKs. The

T cells present in the thymus were mainly of host origin and therefore not alloactivated during GVHR.

Glucocorticoids are involved in the regulation of many T cell-mediated disease states. Abnormalities of glucocorticoid metabolism are involved in autoimmune disease in several animal models, such as thyroiditis in the OS chicken and experimental autoimmune encephalitis (EAE) in the Lewis rat (63). In fact, EAE may be triggered in normally resistant rat strains if the animals are ADX prior to disease induction (63). In this case, the glucocorticoid-mediated decrease of T cell immune function is protective. Conversely, the profound, long-lasting T cell anergy contributes significantly to the morbidity and mortality of clinical GVHR (1, 2). Furthermore, T signalling abnormalities resembling those of GVHR have been observed in other immunosuppressive states such as AIDS (64); and similar PTK defects have been reported in tumor-induced T cell suppression (30). Our findings suggest a mechanism by which glucocorticoids may directly modulate T cell activation and function, and thus may contribute to our understanding of the T cell pathophysiology in these complex clinical problems.

References

1. Lapp, W.S., T. Ghayur, M. Mendes, M. Seddik, and T.A. Seemayer. 1985. The functional and histological basis for graft-versus-host-induced immunosuppression. *Immunol. Rev.* 88: 107.

2. Moser, M., T. Iwasaki, and G.M. Shearer. 1985. Cellular interactions in graft-versus-host-induced T cell immune deficiency. *Immunol. Rev.* 88: 135.

3. Ferrara, J.L. 1993. Cytokine dysregulation as a mechanism of graft versus host disease. *Current Opinions Immunol.* 5: 794.

4. Levy, R.B., M. Jones, and C. Cray. 1990. Isolated peripheral T cells from GvHR recipients exhibit defective IL-2R expression, IL-2 production, and proliferation in response to activation stimuli. *J. Immunol.* 145: 3998.

5. Moser, M., T. Mizuochi, S.O. Sharrow, A. Singer, and G.M. Shearer. 1987. Graft-vs-host reaction limited to a class II MHC difference results in a selective deficiency in $L3T4^+$ but not in $Lyt2^+$ T helper cell function. *J. Immunol.* 138: 1355.

6. Moser, M., S.O. Sharrow, and G.M. Shearer. 1988. Role of $L3T4^+$ and $Lyt2^+$ donor cells in graft-versus-host immune deficiency induced across a class I, class II, or whole H-2 difference. *J. Immunol.* 140: 2600.

7. Moller, G. 1971. Suppressive effect of graft-versus-host reaction on immune response to heterologous red cells. *Immunology.* 20: 597.

8. Seddik M., T.A. Seemayer, and W.S. Lapp. 1984. The graft-versus-host reaction and immune function. I. T-helper cell immuno-deficiency associated with graft-versus-host induced thymic epithelial cell damage. *Transplantation.* 37: 281.

9. Mendes, M.L., H. Rode, A. Peres, P.A.L. Konshavn, and W.S. Lapp. 1985.

Interleukin-1 and interleukin-2 defects associated with murine graft-versus-host-induced immunodeficiency. *Transplantation.* 39: 418.

10. Joseph, L.J., T. Iwasaki, T.R. Malek, and G.M. Shearer. 1985. Interleukin 2 receptor dysfunction in mice undergoing a graft-vs-host reaction. *J. Immunol.* 135: 1846.

11. Soiffer, R.J., L. Bosserman, C. Murray, K. Cochran, J. Daley, and J. Ritz. 1990. Reconstitution of T cell function after CD6-depleted allogeneic bone marrow transplantation. *Blood.* 75: 2076.

12. Rudd, C.E., O. Janssen, K.V.S. Prasad, M. Raab, A. da Silva, J.C. Telfer, and M. Yamamoto. 1993. *src*-related protein tyrosine kinases and their surface receptors. *Biochim. Biophys. Acta.* 1155: 239.

13. Weiss, A. 1993. T cell antigen receptor signal transduction: a tale of tails and cytoplasmic protein-tyrosine kinases. *Cell.* 73: 209.

14. Klausner, R.D., and L.E. Samelson. 1991. T cell antigen receptor activation pathways: the tyrosine kinase connection. *Cell.* 64: 875.

15. Veillette, A. and D. Davidson. 1992. Src-related protein tyrosine kinases and T-cell receptor signalling. *Trends Genet.* 8:61.

16. Cooke, M.P., and R.M. Perlmutter. 1989. Expression of a novel form of the *fyn* proto-oncogene in hematopoietic cells. *New Biol.* 1:66.

17. Davidson, D., L. M. Chow, M. Fournel, and A. Veillette. 1992. Differential regulation of T cell antigen responsiveness by isoforms of the src-related tyrosine protein kinase p59fyn. *J. Exp. Med.* 175:1483.

18. Timson Gauen, L. K., A. N. Kong, L. E. Samelson, and A. S. Shaw. 1992. p59fyn

tyrosine kinase associates with multiple T-cell receptor subunits through its unique amino-terminal domain. *Mol. Cell. Biol.* 12:5438.

19. da Silva, A. J., M. Yamamoto, C. H. Zalvan, and C. E. Rudd. 1992. Engagement of the TcR/CD3 complex stimulates p59fyn(T) activity: detection of associated proteins at 72 and 120-130 kD. *Molecular Immunology.* 29:1417.

20. Cooke, M.P., K.M. Abraham, K.A. Forbush, and R.M. Perlmutter. 1991. Regulation of T cell receptor signalling by a *src* family protein-tyrosine kinase ($p59^{fyn}$). *Cell.* 65: 281.

21. Appleby, M.W., J.A. Gross, M.P. Cooke, S.D. Levin, X. Qian, and R.M. Perlmutter. 1992. Defective T cell receptor signaling in mice lacking the thymic isoform of p59fyn. *Cell.* 70: 751.

22. Stein, P.L., H.M. Lee, S. Rich, and P. Soriano. 1992. pp59fyn mutant mice display differential signaling in thymocytes and peripheral T cells. *Cell.* 70: 741.

23. Turner, J.M., M.H. Brodsky, B.A. Irving, S.D. Levin, R.M. Perlmutter, and D.R. Littman. 1990. Interaction of the unique N-terminal region of tyrosine kinase p56lck with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. *Cell.* 60: 755.

24. Hoeveler, A. and B. Malissen. 1993. The cysteine residues in the cytoplasmic tail of CD8 alpha are required for its coreceptor function. *Molecular Immunology.* 30: 755.

25. Veillette, A., M.A. Bookman, E.M. Horak, L.E. Samelson, and J.B. Bolen. 1989. Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase p56lck. *Nature.* 338: 257.

26. Hatakeyama, M., T. Kono, N. Kobayashi, A. Kawahara, S.D. Levin, R.M. Perlmutter, and T. Taniguchi. 1991. Interaction of the IL-2 receptor with the

src-family kinase p56lck: identification of novel intermolecular association. *Science.* 252: 1523.

27. Minami, Y., T. Kono, K. Yamada, N. Kobayashi, A. Kawahara, R.M. Perlmutter, and T. Taniguchi. 1993. Association of p56lck with IL-2 receptor beta chain is critical for the IL-2-induced activation of p56lck. *EMBO J.* 12: 759.

28. Veillette, A., N. Abraham, L. Caron, and D. Davidson. 1991. The lymphocyte-specific tyrosine protein kinase p56lck. *Sem. Immunol.* 3: 143.

29. Straus, D.B., and A. Weiss. 1992. Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. *Cell.* 70: 585.

30. Mizoguchi, H., J.J. O'Shea, D.L. Longo, C.M. Loeffler, D.W. McVicar, and A.C. Ochoa. 1992. Alterations in signal transduction molecules in T lymphocytes from tumor-bearing mice. *Science.* 258: 1795.

31. Desbarats, J., and W.S. Lapp. 1993. Thymic selection and thymic major histocompatibility complex class II expression are abnormal in mice undergoing graft-versus-host reactions. *J. Exp. Med.* 178: 805.

32. Kornbluth, M., E. You-Ten, J. Desbarats, S. Gamache, A. Xenocostas, and W.S. Lapp. 1991. T cell subsets in the thymus of graft-versus-host immunosuppressed mice. Sensitivity of the L3T4$^+$Lyt-2$^-$ subset to cortisone. *Transplantation.* 51: 262.

33. Seddik, M., T.A. Seemayer, and W.S. Lapp. 1986. The graft-versus-host reaction and immune function. III. Functional pre-T cells in the bone marrow of graft-versus-host-reactive mice displaying T cell immunodefociency. *Transplantation.* 41: 238.

34. Veillette, A. and M. Fournel. 1990. The CD4 associated tyrosine protein kinase p56lck is positively regulated through its site of autophosphorylation. *Oncogene.*

5:1455.

35. Seddik, M., T.A. Seemayer, and W.S. Lapp. 1979. In vitro study of the effects of the GVH reaction on T cell immunocompetence. In: Kaplan, J.G., ed., The molecular basis of immune cell function. Elsevier (Amsterdam), 549.

36. Nestel, F.P., K.S. Price, T.A. Seemayer, and W.S. Lapp. 1992. Macrophage priming and LPS-triggered release of TNF-alpha during graft-versus-host disease. *J. Exp. Med.* 175: 405.

37. Khairallah, M., C. Spach, F. Maitre, and R. Motta. 1988. Endocrine involvement in minor (non-H-2) graft versus host reaction in mice: dissociated effect on corticosterone and aldosterone plasma levels. *Endocrinology.* 123: 1949.

38. Hoot, G.P., J.R. Head, and W.S.T. Griffin. 1983. Increased free plasma corticosterone and adrenal hyperactivity associated with graft-versus-host disease. *Transplantation* 35: 478.

39. Reth, M. 1989. Antigen receptor tail clue. *Nature.* 338: 383.

40. Letourneur, F., and R.D. Klausner. 1991. T cell and basophil activation through the cytoplasmic tail of T cell receptor ζ family proteins. *Proc. Natl. Acad. Sci. USA.* 88: 8905.

41. Romeo, C., M. Amiot, and B. Seed. 1992. Sequence requirements for induction of cytolysis by the T cell antigen/Fc receptor ζ chain. *Cell.* 68: 889.

42. Gajewski, T.F., D. Qian, P. Fields, and F.W. Fitch. 1994. Anergic T lymphocyte clones have altered inositol phosphate, calcium, and tyrosine kinase signaling pathways. *Proc. Natl. Acad. Sci.* 91: 38.

43. Quill, H., M.P. Riley, E.A. Cho, J.E. Casnellie, J.C. Reed, and J. Torigoe. 1992.

Anergic Th1 cells express altered levels of the protein tyrosine kinases p56lck and p59fyn. *J. Immunol.* 149: 2887.

44. Kang, S.-M., B. Beverly, A.-C. Tran, K. Brorson, R.H. Schwartz, and M.J. Lenardo. 1992. Transactivation by AP-1 is a molecular target of T cell aclonal anergy. *Science* 257: 1134.

45. Schönrich, G., U. Kalinke, F. Momburg, M. Malissen, A.-M. Schmitt-Verhulst, B. Malissen, G.J. Hämmerling, and B. Arnold. 1991. Down-regulation of T cell receptors on self-reactive T cells as a novel mechanism for extrathymic tolerance induction. *Cell.* 65: 293.

46. White, J., A. Herman, A.M. Pullen, R. Kubo, J.W. Kappler, and P. Marrack. 1989. The Vß-specific superantigen Staphylococcal Enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. *Cell.* 56: 27.

47. Rammensee, H.-G., R. Kroschewski, and B. Frangoulis. 1989. Clonal anergy induced in mature Vß6$^+$ T lymphocytes on immunizing Mls-1^b mice with Mls-1^a expressing cells. *Nature.* 339: 541.

48. Kawabe, Y., and A. Ochi. 1990. Selective anergy of Vß8$^+$, CD4$^+$ T cells in Staphylococcal Enterotoxin B-primed mice. *J. Exp. Med.* 172: 1065.

49. Rellahan, B.L., L.A. Jones, A.M. Kruisbeek, A.M. Fry, and L.A. Mathis. 1990. In vivo induction of anergy in peripheral Vß8$^+$ T cells by Staphylococcal Enterotoxin B. *J. Exp. Med.* 172: 1091.

50. Webb, S., C. Morris, and J. Sprent. 1990. Extrathymic tolerance of mature T cells: clonal elimination as a consequence of immunity. *Cell.* 63: 1249.

51. Gonzalo, J.A., A. Gonzalez-Garcia, C. Martinez-A., and G. Kroemer. 1993. Glucocorticoid-mediated control of the activation and clonal deletion of peripheral T

cells in vivo. *J. Exp. Med.* 177: 1239.

52. Lussow, A.R., T. Crompton, O. Karapetian, and H.R. MacDonald. 1993. Peripheral clonal deletion of superantigen-reactive T cells is enhanced by cortisone. *Eur. J. Immunol.* 23: 578.

53. H.O. Besedovsky, A. del Rey, E. Sorkin, and C.A. Dinarello. 1986. Immunoregulatory feedback between interleukin 1 and glucocorticoid hormones. *Science* 233: 652.

54. Guy-Grand, D., and P. Vassali. 1987. Gut injury in mouse graft-versus-host reaction. *Adv. Exp. Med. Biol.* 216A: 661.

55. Mowat A.McI., M.V. Felstein. 1990. Experimental studies of immunologically mediated enteropathy. V. Destructive enteropathy during an acute graft-versus-host reaction in adult BDF1 mice. *Clin. Exp. Immunol.* 79: 279.

56. Rivier, C., R. Chizzonite, and W. Vale. 1989. In the mouse, the activation of the hypothalamic-pituitary-adrenal axis by a lipopolysaccharide (endotoxin) is mediated through interleukin-1. *Endocrinology* 125: 2800.

57. Tracey, K.J. and A. Cerami. 1993. Tumor necrosis factor: an updated review of its biology. *Crit. Care Med.* 21: S415.

58. Spooner, C.E., N.P. Markowitz, and L.D. Saravolatz. 1992. The role of tumor necrosis factor in sepsis. *Clin. Immunol. Immunopathol.* 62: 511.

59. Heim, L.R., C. Martinez, and R.A. Good. 1967. Cause of homologous disease. *Nature* 214: 26.

60. Heim, L.R., R.A. Good, and C. Martinez. 1966. Influence of adrenalectomy on homologous disease. *Proc. Soc. Exp. Biol. Med.* 122: 107.

61. Kaplan, H.S., and B.H. Rosston. 1959. Studies on a wasting disease induced in F1 hybrid mice injected with parental strain lymphoid cells. *Stanford Med. Bull.* 17: 77.

62. Liu, Y., and C.A. Janeway. 1990. Interferon γ plays a critical role in induced cell death of effector T cell: a possible third mechanism of self-tolerance. *J. Exp. Med.* 172: 1735.

63. Wick, G., S. Schwarz, and G. Kroemer. 1993. Immunoendocrine communication via the hypothalamo-pituitary-adrenal axis in autoimmune diseases. *Endocrine Rev.* 14: 539.

64. Hofman, B., J. Moller, E. Langhoff, K. Damgard Jakobsen, N. Odum, E. Dickmeiss, L.P. Ryder, O. Thastrup, O. Scharff, B. Foder, P. Platz, C. Petersen, L. Mathiesen, T. Hartvig-Jensen, P. Skinhoj, and A. Svejgaard. 1989. Stimulation of AIDS lymphocytes with calcium ionophore (A23187) and phorbol ester (PMA): studies of cytoplasmic free Ca, IL-2 receptor expression, IL-2 production, and proliferation. *Cell. Immunol.* 119: 14.

CHAPTER 3.

The second phase of GVHR-induced T cell suppression:

a defect in thymic education.

The acute GVHR results in death of most peripheral T cells, and profound unresponsiveness of the remainder. The T cell anergy is accompanied by a glucocorticoid-induced downregulation of lck and fyn, the tyrosine kinases which transduce TCR signals. However, the mature (PNA$^-$, cortisone resistant) thymocytes appear to have normal PTK levels, despite their exposure to glucocorticoids. In the absence of proliferative signals such as those experienced by the allo-stimulated peripheral T cells, the thymocytes may be protected from glucocorticoid-induced PTK downregulation. Why, then, are they unable to reconstitute the periphery, even, in the clinical setting, years after the acute reaction? Peripheral T cell function begins to reappear when the thymic histopathology resolves, suggesting that GVHR-induced thymic lesions may result in abnormal T cell maturation and selection. To investigate this hypothesis, the thymocyte phenotype and and the thymic microenvironment were examined during GVHR.

Thymic Selection and Thymic Major Histocompatibility Complex Class II Expression Are Abnormal in Mice Undergoing Graft-versus-Host Reactions[1]

by Julie Desbarats and Wayne S. Lapp.

From the Department of Physiology, McGill University, Montreal, Quebec, Canada, H3G 1Y6

Running title: Altered Thymic Selection in Graft-vs.-Host Reactive Mice

Address correspondence to Julie Desbarats at the Department of Physiology, McGill University, 3655 Drummond St., Montreal, Quebec, Canada H3G 1Y6.

[1] This paper was published in the *Journal of Experimental Medicine* 178: 805, 1993.

Acknowledgments

We thank Ailsa Lee Loy and Rosmarie Siegrist-Johnstone for their excellent technical assistance, and Dr. Francis Glorieux and Rose Travers, Genetics Unit, Shriner's Hospital for Crippled Children, Montreal, for the use of and assistance with their fluorescence microscope.

This work was supported by grants from the Medical Research Council of Canada and the National Cancer Institute of Canada. J.Desbarats was supported by studentship awards from the Fonds de Recherches en Santé du Québec and the Medical Research Council of Canada.

Summary

The graft-versus-host reaction (GVHR) results in damage to the epithelial and lymphoid compartments of the thymus and thus in abnormal maturation and function of thymocytes in mice undergoing GVHR. In this report, the effects of GVHR on thymic T cell receptor (TCR) expression and usage have been investigated. GVHR was induced in unirradiated F1 hybrid mice by the intravenous transfer of parental lymphoid cells. Expression of the CD3/TCR complex on thymocyte subsets defined by CD4 and CD8 was studied by three color flow cytometry. The level of CD3/TCR was decreased on $CD4^+CD8^-$, but not $CD4^-CD8^+$, mature thymocytes. The lack of upregulation of CD3/TCR on CD4 single positive thymocytes, but not on their $CD8^+$ counterparts, suggested an abnormality of class II MHC expression in the thymuses of mice undergoing GVHR. Immunofluorescence staining of thymic frozen sections revealed that MHC class II expression was dramatically decreased in GVH-reactive mice. GVHR-induced changes in positive and negative selection were evaluated by determining the incidence of specific Vß TCR segment usage in the thymus. In normal mice, thymocyte usage of any given Vß segment was highly consistent between individuals of the same strain and age; however, a marked divergence in the incidence of TCR $V\beta6^{hi}$ and $V\beta8^{hi}$ cells between GVH-reactive littermate mice was observed, suggesting that thymic positive selection had become disregulated in these animals. Furthermore, negative selection was

defective; the incidence of phenotypically self-reactive Vß6^{hi} T cells was significantly greater in the thymuses of GVH-reactive mice bearing the endogenous superantigen Mls-1^{a} than in untreated controls. Thus, mice undergoing GVHR showed defective TCR upregulation on CD4^{+}CD8^{-} thymocytes, and changes in TCR usage reflecting aberrant thymic selection, in conjunction with decreased expression of MHC class II. Most abnormalities of TCR expression and usage on CD4^{+} thymocytes observed in GVH-reactive mice were analogous to those of class II knockout mice.

Introduction

The graft-versus-host reaction (GVHR) induced by injection of parental lymhoid cells into unirradiated F1 hybrid recipients consists of an acute syndrome of immunosuppression, epithelial cell lesions, and cachexia often leading to death. Survivors of the acute reaction develop chronic GVHR, a multisystem disease characterized by autoimmune features and persistent immunosuppression (1-3). These manifestations of chronic GVHR represent T cell functional abnormalities possibly resulting from defective education within the GVHR-dysplatic thymus (4,5). Indeed, the thymus sustains severe histopathological damage during acute GVHR, including injury to medullary

epithelial cells, effacement of the corticomedullary junction, progressive disappearance of Hassal's corpuscles (6), and cortisone-dependent effects such as death of the immature $CD4^+8^+$ thymocytes (4). In addition, the immune activation which occurs early in GVHR results in the release of cytokines such as interferon (7), which primes macrophages (8) and modulates MHC antigens in peripheral tissues, and may similarly affect the thymus. Thus, the thymic microenvironment is markedly altered by GVHR; furthermore, histological abnormalities persist well into the chronic phase of the reaction (9).

In normal animals, the thymus provides the microenvironment for T cell maturation and selection. Pre-T cells enter the thymus as $CD4^-8^-3^-$ precursors; as they mature, they become double positive $CD4^+8^+$ immature, cortisone-sensitive thymocytes and acquire low levels of CD3/TCR ($CD3^{lo}$); they then lose expression of one of the two accessory molecules and become mature, cortisone-resistant single positive $CD4^+8^-$ or $CD4^-8^+$ T cells with peripheral levels of CD3/TCR expression ($CD3^{hi}$). The transition from $CD3^{lo}$ to $CD3^{hi}$, an upregulation of approximately an order of magnitude, requires the thymic microenvironment and is thought to be a consequence of positive selection (10).

Thymic stromal components positively select T cells able to interact at low affinity with self MHC molecules, while bone-marrow derived elements mediate the deletion of potentially autoreactive cells (11-14). These intrathymic events are believed to be primarily responsible for the self-restriction and self-tolerance of the peripheral T cell repertoire (15). Antibody-

mediated blocking of the interactions between thymic MHC molecules and the CD4 or CD8 accessory molecules on immature T cells has resulted in defective thymic selection (16-18), as has the absence of these molecules in transgenic mice with deletion mutations (19-24). Thus GVHR-induced alterations of the thymic stroma, whether by direct injury or by cytokine-triggered modulation of cell surface molecules, are likely to affect T cell maturation and selection.

To investigate this hypothesis, the level of CD3/TCR expression and the incidence of representative positively or negatively selected TCRs were studied during acute and chronic GVHR. The effects of GVHR on positive selection were monitered by following the upregulation of CD3 expression on thymocytes and by changes in the incidences of specific TCR Vß segment usage in the thymus. Prior to selection, the incidence of each Vß segment is germline-controlled; however, the final incidence of each Vß among mature thymocytes is determined by thymic selection processes (27). Thus in unmanipulated age- and sex-matched mice, the incidence of any given TCR Vß segment among mature (CD3/TCR^{hi}) thymocytes is highly consistent and results from positive selection pressures. We therefore chose to use deviations in normal incidences prior to antigen-driven expansion in the periphery as an indicator of abnormal positive selection. Negative selection results in the intrathymic deletion of potentially autoreactive clones; in mice bearing the endogenous retrovirus encoding the Mls-1^a antigen, T cells bearing Mls-1^a reactive Vß segments such as Vß6 are deleted (26,27). Thus, we induced GVHR in a strain combination

in which both the donor and recipient were Mls-1^{a+}, and monitored the incidence of normally deleted V$\beta 6^{hi}$ T cells in the thymuses of GVH-reactive mice in order to detect defective negative selection. Our results demonstrate aberrant positive and negative selection concomitant with decreased thymic MHC class II expression during GVHR. Our findings demonstrated remarkable similarities between the $CD4^+$ thymocytes in GVH-reactive mice and class II knockout mice; this suggested that the abnormal level and distribution of thymic class II during GVHR could explain the impaired thymic maturation and defective peripheral function of the class II restricted T cells observed in GVH-reactive mice.

Materials and methods

Animals. Mice of the inbred strains CBA/J (H-2^k, Mls-1^a), A (H-2^a, Mls-1^b), and C57BL/6 (B6) (H-2^b, Mls-1^b), and the F1 hybrids B6xCBA/J, AxCBA/Ca (H-$2^{a/k}$, Mls-$1^{b/b}$), and B6xA (B6AF1) were used. All mice were bred and maintained in our animal colony.

GVHR induction. GVHRs were induced as described previously (4). Briefly, pooled parental spleen and lymph nodes were made into single cell suspensions

and injected i.v. into unirradiated F1 hybrid recipients. Cell dose varied from $2x10^7$ to $6x10^7$ viable donor lymphoid cells depending on the strain combination and the required severity of the GVHR.

Direct plaque-forming cell (PFC) response to sheep red blood cells (SRBC). Mice were immunized i.v. with $5x10^8$ SRBC in 0.3 ml saline. Four days later the direct splenic PFC response was assessed by the method of Cunningham and Szenberg (29) as modified in this laboratory (30).

Mitogen assays. T cell proliferative responses to concanavalin A (Con A) and B cell responses to lipopolysaccharide (LPS) were evaluated by ^{3}H-thymidine incorporation as described previously (31). Briefly, $5x10^5$ spleen cells were cultured for 48 hours in triplicate with or without mitogen, then for a further 16 hours with 1μCi/well ^{3}H-thymidine. DNA was harvested with an automated cell harvester and ^{3}H-thymidine incorporation was quantitated on a beta counter (LKB Instruments, Turku, Finland). Data are expressed as: net cpm = cpm of mitogen stimulated culture - cpm of unstimulated culture. The counts in unstimulated cultures (background) did not exceed 6000 cpm for normal mice, and 2500 cpm for GVH-reactive mice.

Flow cytometry analysis. Single cell suspensions of thymocytes were prepared by gentle tamping through 50-mesh stainless steel screens. Suspensions were

washed, made up to $2x10^7$ cells/ml in RPMI 1640 supplemented with 10% FCS, and incubated for 1 hour at 37°C. Aliquots of thymocytes (10^6 cells per sample) were incubated with primary antibodies at 4°C for 20 minutes, then washed and incubated at 4°C for 20 minutes with secondary reagents if necessary. Labelled thymocytes were resuspended in 1 ml RPMI and analysed by flow cytometry. For each sample, 12000 to 25000 events were acquired on a FACScan flow cytometer (Becton Dickinson & Co., Mountain View, CA) with light scatter gating to exclude erythrocytes and debris, and propidium iodide exclusion of dead cells whenever possible (for 1 and 2 color analyses). Data were analysed with Consort 30 or FACScan Research Software. TCR and CD3 labelling produced two positive peaks, of "hi" and "lo" intensity fluorescence, corresponding to immature and mature thymocytes (10,32,33). Analysis gates were set to assess the TCR^{hi} or $CD3^{hi}$ populations.

Reagents for flow cytometry. Culture supernatants from hybridomas KJ16-133 (anti-Vß8.1+8.2 antibody, kindly provided by Dr. P. Marrack (34)) and 44-22-1 (anti-Vß6, a gift from Dr. G. Prudhomme, originally obtained from Dr. H. Hengartner (35)) were diluted 1:2 in PBS and used as a source of primary antibodies in conjuction with goat-anti-rat-FITC (Kirkegaard and Perry Laboratories, Gaithersburg, MD) as the second step reagent. Anti-CD3-FITC (Boehringer Mannheim Canada Ltd., Laval, Canada) was used as single step reagent, either alone or for three color analysis with anti-CD4-phycoerythrin,

and anti-CD8-biotin followed by streptavidin-allophycocyanin (Becton Dickinson & Co.). Reagents obtained commercially were employed at the recommended dilutions.

Immunofluorescence labelling of frozen sections. Thymuses were removed, rinsed in HBSS, and immediately embedded in Tissue-Tek O.C.T. compound (Miles Inc. Diagnostics Division, Elkhart, IN) on dry ice. The tissue blocks were cut into 12 µm sections on an American Optical Histostat microtome at -20°C and the sections were mounted on gelatin-coated slides. The sections were incubated with normal goat serum (Cedarlane Laboratories, Hornby, Ont., Canada) for 1 hour at room temperature to block non-specific binding, washed, then incubated overnight at 4°C with hybridoma P7/7 supernatant (anti MHC class II specificity, kindly provided by Dr. T. Owens, McGill University). The slides were washed, incubated for one hour with goat-anti-rat-FITC (Kirkegaard and Perry Laboratories, Gaithersburg, MD), washed again, and mounted in Gelvatol (glycerol and polyvinyl alcohol in Tris-HCl buffer). Background staining was assessed by substituting PBS alone for P7/7 supernatant during the overnight incubation.

Results

CD3 expression is altered in the thymus during GVHR. CD3 expression was examined in the thymuses of mice 25 days after induction of GVHR. B6AF1 mice injected with $5x10^7$ B6 parental lymphoid cells developed an intense, lethal reaction as shown by complete supression of splenic mitogen responses (Fig. 1 legend) and 70% GVHR-induced mortality (the remaining 30% GVH-reactive mice were sacrificed on day 25). Thymocytes were analysed by flow cytometry for expression of CD3 on subsets defined by the CD4 and CD8 accessory molecules (Fig. 1). The distribution and levels of CD3 on the most immature subset, the $CD4^-8^-$ thymocytes, were unchanged in mice undergoing GVHR. In untreated mice, the majority of $CD4^+8^+$ thymocytes expressed low levels of CD3 after an incubation at 37°C; this cortisone-sensitive $CD3^{lo}$ $CD4^+8^+$ subset was largely ablated by the high levels of circulating glucocorticoids that we and others have observed during severe GVHR (36; and in preparation, K.E. You-Ten and W.S. Lapp). A small subpopulation of the $CD4^+8^+$ thymocytes, representing the most mature cells of this subset, expressed high levels of CD3; only this small subpopulation of $CD3^{hi}$, $CD4^+8^+$ cortisone resistant cells persisted during GVHR. In normal mice, the most mature thymic subsets lose expression of one of the two accessory molecules and expressed peripheral levels of CD3. However, $CD4^-8^+$ GVH-reactive thymocytes showed higher than normal mean levels of CD3, while the $CD4^+8^-$ thymocytes

had lower levels of CD3 expression, suggesting a selective failure of CD3 upregulation on $CD4^+8^-$ thymocytes in mice undergoing GVHR (Fig. 1).

Thymic TCR usage is altered during severe GVHR. Potential alterations in positive selection were addressed by analysing the incidence of TCRs which are normally positively selected, as inferred by their high proportional representation among mature thymocytes and peripheral T cells. In this study, only mature thymocytes were considered in order to examine the post-selection repertoire while avoiding bias due to peripheral clonal expansion in response to antigenic stimulation.

In the B6AF1 mice described above, the incidence of $V\beta8^{hi}$ thymocytes was analysed by flow cytometry (Fig. 2). Severe GVHR induces high glucocorticoid production, resulting in the disappearance of the cortisone-sensitive $CD4^+8^+$ thymocytes and consequent thymic involution and increased incidence of mature thymocytes (4, 36, and in preparation , K.E. You-Ten and W.S. Lapp); to correct for this effect, results were expressed in terms of the ratio of $V\beta8^{hi}$ thymocytes to total mature thymocytes, as determined by $CD3^{hi}$ expression. The incidence of $V\beta8^{hi}$ thymocytes varied by an order of magnitude among GVH-reactive mice, whereas it was highly consistent among the untreated, age- and sex-matched control mice. Results indicate that the range of Vß8 incidence in B6AF1 GVH-reactive mice spanned from 3% to more than 25% of the mature thymocytes, a ten-fold variability. Both increases and

decreases in Vß8^{hi} incidence with respect to normal were observed in GVH-reactive animals, suggesting that the normally tightly regulated thymic selection processes were affected stochastically by the GVHR. The high incidence of Vß8 cells was unlikely donor infiltration of mature cells into the host thymus since studies using a DNA probe to detect donor cells in similar reactions never revealed high chimerism this early in GVHR (Wisse, You-Ten, and Lapp, in preparation).

Altered incidences of Vß usage were also detected in the reciprocal GVHR, induced by injecting $2x10^7$ A strain cells into B6AF1 recipients to produce a severe lethal reaction (Fig. 3). All GVH-reactive mice were completely immunosuppressed, confirming GVHR induction, at the time of assay (Fig. 4). As above, results were expressed as a ratio of Vß6^{hi} or Vß8^{hi} to CD3^{hi} thymocytes to normalize specific receptor usage; the increased variability in incidence among the mice undergoing GVHR was again remarkable when compared to the consistency of usage in the untreated control mice. The incidences of mature thymocytes expressing Vß6 and Vß8 were examined in the same mice; although both increased in variability within the GVH-reactive group, these changes were not coordinated within a given thymus: an increase in the incidence of Vß6^{hi} did not correlate consistently with a concomittant increase or decrease in Vß8^{hi} incidence, again suggesting a stochastic variation in receptor usage during GVHR.

Thymic TCR usage is altered during non-lethal GVHR. We chose to study a non-lethal GVHR model to determine if our findings were reproducible in a less severe GVHR. AxCBA/Ca F1 hybrid recipients of $5x10^7$ A strain lymphoid cells developped a chronic GVHR with complete immunosupression (Table I) but no mortality. Results were expressed directly as percentages of total thymocytes rather than as ratios of mature cells since the relatively mild GVHR did not result in cortisone-dependent thymic atrophy. As early as 14 days after GVHR induction, changes were detected in the incidence of $V\beta6^{hi}$ and $V\beta8^{hi}$ thymocytes (Table II). Again, the variability in thymic TCR incidences was increased during GVHR, as demonstrated by the high standard deviations, the large ranges, and the significantly greater mean dispersion, of specific Vß incidences in the GVHR groups.

Negative selection is defective in CBA/JxB6 ($Mls^{a/b}$) mice undergoing GVHR. A potential defect in negative selection in CBA/JxB6 mice undergoing GVHR was detected by examining their thymuses for the presence of T cells bearing phenotypically autoreactive receptors, which would normally be deleted. Both CBA/J (donor) and CBA/JxB6 F1 (recipient) mice were Mls-1^{a+} and thus delete T-cells expressing Vß6, 7, 8.1 or 9, resulting in a very low incidence (<0.2% of thymocytes) of these cells in the thymuses of normal young adult mice (26,27,37-39). Mls-1^a strain donors were employed so that the graft used to induce GVHR would not contain $V\beta6^+$ cells; thus the presence of cells

expressing Vß6 at high levels (Vß6^{hi}) in the recipient thymus could not be attributed to donor cell infiltration. In addition, in this system any donor-derived cells able to mediate negative selection would also delete Vß6^{+} thymocytes in the event of eventual stromal chimerism. Finally, this model was chosen to avoid irradiation, which can cause GVHR-independent thymic damage, thus complicating interpretation of the results (40).

As shown in Fig. 5 and table III, the incidence of potentially self-reactive thymocytes expressing Vß6 at high levels was significantly increased by 21 days after induction of GVHR. In most GVH-reactive mice, a distinct Vß6^{hi} population was clearly visible by flow cytometry three weeks after induction of GVHR (Fig. 5). The increased incidence of Vß6^{hi} thymocytes was maintained during the acute phase of the reaction, and was observed to undergo a further increase during the chronic phase, 55 days after induction of GVHR (Table III). As in the AxCBA/Ca F1 recipients, the GVHR induced by $5x10^7$ cells in this strain combination produced complete immunosuppression (Table I) but was not lethal and relatively mild, resulting in minimal cortisone-dependent thymic involution which resolved after the acute phase. Thus the increased incidence of Vß6^{hi} cells did not represent merely a decrease in the percentage of immature thymocytes. Furthermore, the increased incidence of potentially autoreactive cells was mirrored by the absolute number of Vß6^{hi} thymocytes, which was as much as 6-fold greater in chronic GVH-reactive animals than in normal age-matched control mice.

Thymic MHC class II expression is decreased during GVHR. Class II expression was examined in the thymuses of AxCBA/Ca mice injected with $5x10^7$ lymphoid cells from A strain parental donors, and B6AF1 mice injected with $2x10^7$ A strain cells or $6x10^7$ B6 strain cells. At these cell doses, the animals were completely immunosuppressed at the time of class II labelling, between 13 to 15 days after GVHR induction. In every strain combination examined, all of the GVH-reactive mice showed greatly diminished class II staining (Fig. 6). In normal mice, there was a clear histologic demarcation between the thymic cortex and medulla, with a corresponding clear-cut difference in class II expression, with the medullary staining appearing more intense (Fig. 6A). During severe GVHR, the cortex atrophies due to the cortisone-mediated disappearance of the $CD4^+8^+$ thymocytes, and the cortico-medullary junction disappears (1). At low magnification, class II staining in the thymic medulla was dramatically decreased, and appeared no brighter than normal cortical staining; at higher power, small areas of normal brightness could be seen interspersed with areas devoid of any detectable label (Fig. 6B). Preliminary time course studies indicated that one week after GVH induction, no change in class II expression was apparent; by two weeks after cell transfer, the GVH-reative thymus was largely devoid of class II; and after three weeks, some sparse but bright class II staining appeared at the periphery of the thymus. This labelling probably corresponded to class II expressed on macrophages infiltrating the thymus at this stage of GVHR.

Thus, both the distribution and intensity of class II expresion was altered in the GVH-reactive thymus.

Table 1. *Immunosupression of GVH-reactive mice assayed by their PFC response to SRBC.*

Strain Combination	Mean PFC / 10^6 Splenocytes ± standard deviation			
		Day after GVHR induction		
	Normal F1	14	21	28
A -> AxCBA/Ca	1323 ± 195	0 ± 0	2 ± 3	0 ± 0
CBA/J -> CBA/JxB6	1158 ± 228	0 ± 0	5 ± 4	0 ± 0

Table 2. *Variability of the incidence of TCR Vß usage by mature thymocytes in GVH-reactive AxCBA/Ca mice.*[*]

Group	Vß6^{hi} Mean ± SD (Range)	Vß6^{hi} Mean dispersion[‡]	Vß8^{hi} Mean ± SD (Range)	Vß8^{hi} Mean dispersion[‡]
Untreated mice	1.58 ± 0.11 (1.40 - 1.60)	0.09	2.51 ± 0.44 (2.09 - 3.10)	0.35
Day 14 GVH-reactive mice	2.59[‖] ± 0.88 (1.58 - 3.65)	0.66[**]	1.56[§] ± 1.32 (0.05 - 2.51)	1.01[**]
Day 21 GVH-reactive mice	2.62[**] ± 0.64 (2.14 - 3.56)	0.47[**]	4.22[**] ± 0.72 (3.55 - 5.24)	0.51[§]
Day 28 GVH-reactive mice	3.20[**] ± 0.90 (2.30 - 4.10)	0.60[‖]	5.80[**] ± 1.42 (4.70 - 5.30)	1.07[**]
GVH-reactive mice[‡‡]	2.77[**] ± 0.77 (1.58 - 4.10)	0.64[**]	3.90[§] ± 2.02 (0.05 - 7.40)	1.47[¶]

[*] The incidence of Vß6^{hi} and Vß8^{hi} cells among total thymocytes is shown for untreated and GVH-reactive AxCBA/Ca F1 hybrid mice. GVHR was induced by the injection of $5x10^7$ A strain lymphoid cells, and confirmed by suppressed PFC responses (Table I).

[‡] Mean dispersion (or mean deviation), $\Sigma|x-\bar{x}|/N$, is a measure of the variability of the data.

[§]$p<0.1$, [‖]$p<0.01$, [¶]$p<0.025$, and [**]$p<0.005$ compared with untreated mice by the Student's T test.

[‡‡] Statistics are shown for all the GVH-reactive animals considered together.

Table 3. *Defective negative selection in GVH-reactive B6xCBA/J (Mls-1^{a+}) F1 hybrid mice.*[*]

Group	%Vß6^{hi} Mean ± S.D.	Range
Untreated mice	0.09 ± 0.05	0.03 - 0.17
Day 14 GVH-reactive mice	0.04 ± 0.01	0.03 - 0.04
Day 21 GVH-reactive mice	0.30[‡]± 0.10	0.20 - 0.40
Day 28 GVH-reactive mice	0.31[‡]± 0.08	0.21 - 0.41
Day 55 GVH-reactive mice	0.43[‡]± 0.14	0.29 - 0.61

[*] The incidence of Vß6^{hi} cells among total thymocytes is shown for untreated and GVH-reactive B6xCBA/J (Mls-1^{a+}) F1 hybrid mice. A representative FACS profile of Vß6 expression in the thymus of a day 21 GVH-reactive mouse is shown in figure 5. GVHR was induced by the injection of $5x10^7$ CBA/J (Mls-1^{a+}) lymphoid cells and confirmed by suppressed PFC responses (Table I).

[‡] $p<0.001$ compared with untreated mice by the Student's T test.

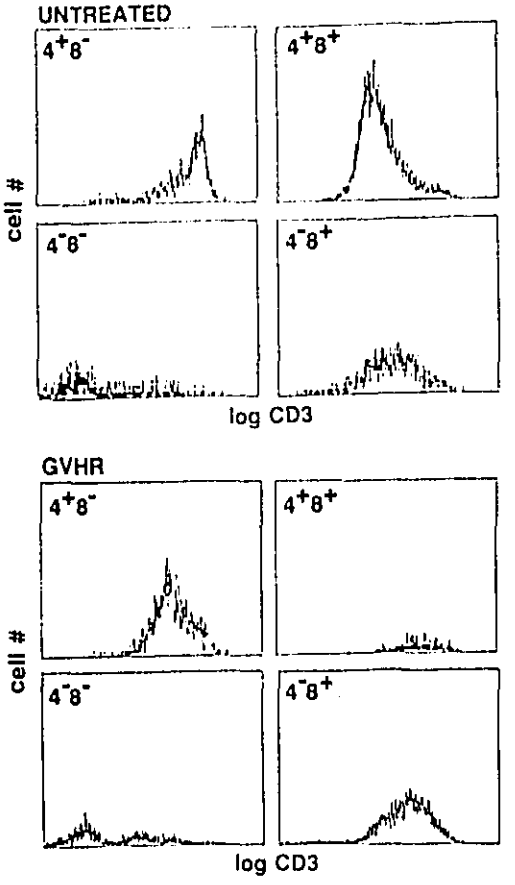


Figure 1. CD3 expression on thymocytes is affected by GVHR. Thymocytes were labelled with anti-CD4, anti-CD8 and anti-CD3 antibodies and analysed by three-color flow cytometry. The histograms represent log-CD3 fluorescence of gated CD4/CD8 populations of thymocytes from untreated and GVH-reactive B6AF1 mice 25 days after transfer of $5x10^7$ B6 lymphoid cells. GVHR was confirmed by immunosuppression assessed by mitogen responses to ConA and LPS. Net responses to ConA and LPS stimulation, respectively, were 70 578 ± 11 403 cpm and 52 980 ± 7 702 cpm in normal mice, and 592 ± 2 684 cpm and -7 488 ± 486 cpm in GVH-reactive mice.

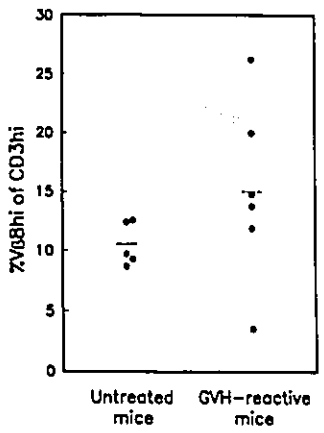


Figure 2. The incidence of TCR Vß8 usage by mature thymocytes becomes inconsistent during severe GVHR. The incidence of Vß8^{hi} cells as a percentage of total CD3^{hi} thymocytes was assessed by flow cytometry and is shown for individual B6AF1 animals. GVH-reactive mice were those described in Fig. 1.

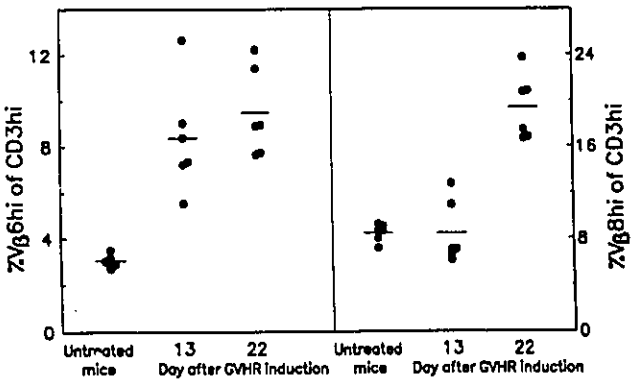


Figure 3. The incidence of TCR Vß usage by mature thymocytes becomes inconsistent early in GVHR. The incidence of Vß6^{hi} and Vß8^{hi} cells as a percentage of total CD3^{hi} thymocytes is shown for individual B6AF1 hybrid mice 13 and 22 days after the induction of GVHR by the injection of $2x10^7$ A strain lymphoid cells. GVHR was confirmed by suppressed mitogen responses (Fig. 4).

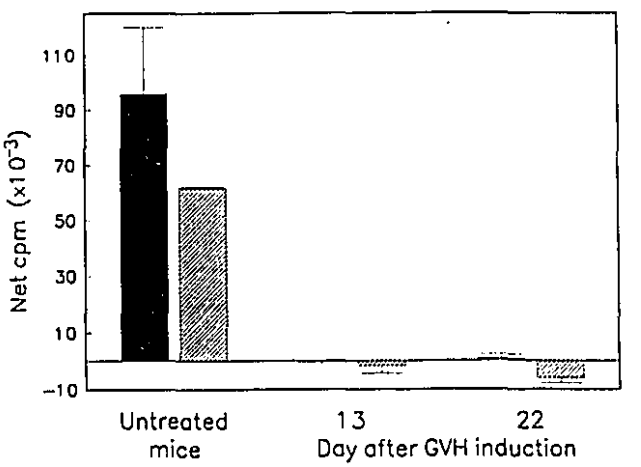


Figure 4. GVH-reactive mice are immunosuppressed. Net responses of splenocytes to the T cell mitogen ConA (solid bars) and the B-cell mitogen LPS (stippled bars) are shown. GVH-reactive mice were those described in Fig. 3.

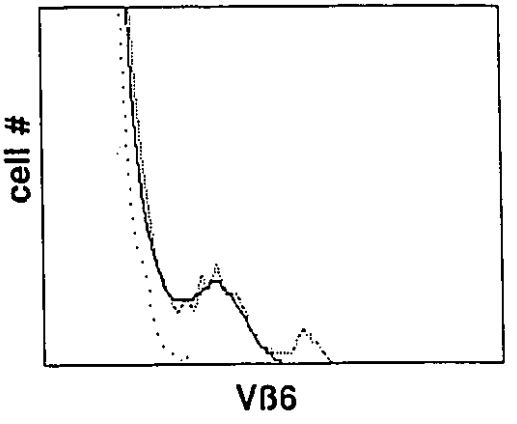


Figure 5. Negative selection is defective during GVHR. Vß6^{hi} expression in Mls-1^{a+} CBA/JxB6 F1 mice was determined by flow cytometry in untreated (___) and GVH-reactive (.....) mice 21 days after GVHR induction by the transfer of $5x10^7$ CBA/J (Mls-1^{a+}) lymphoid cells. Background staining (. . . .) represents labelling with the second antibody alone. The peaks represent TCR Vß6$^-$, Vß6^{lo}, and Vß6^{hi} expression displayed on a logarithmic scale.

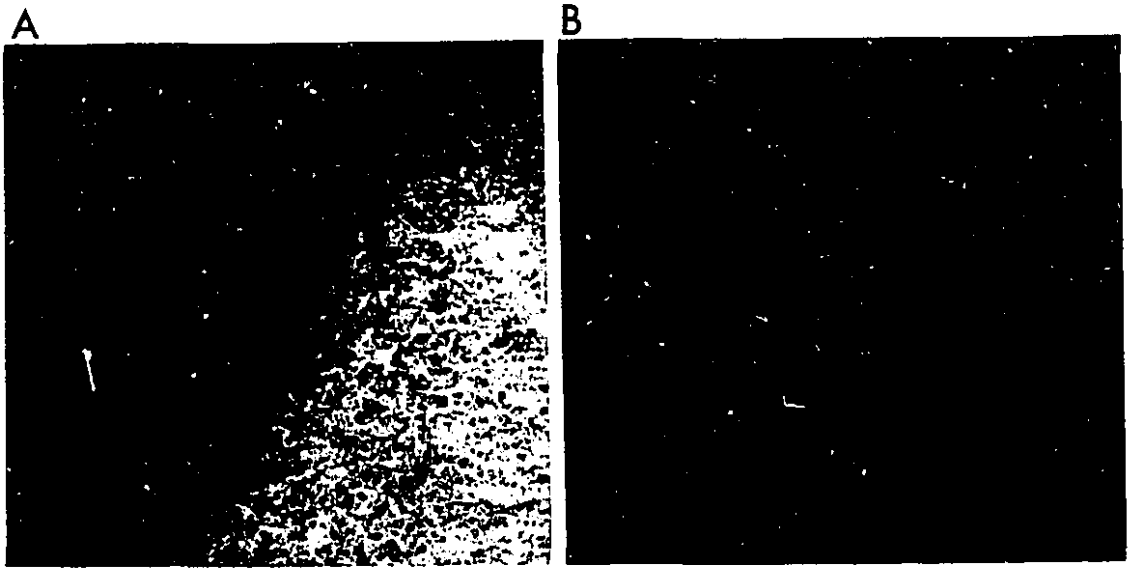


Figure 6. Immunohistochemical staining for thymic class II MHC was dramatically decreased in GVH-reactive mice. B6AF1 mice were studied 13 days after the induction of GVHR by the injection of $6x10^7$ B6 lymphoid cells. At high magnification (250x), a well-defined cortico-medullary junction was seen in normal mice, with intense class II expression in the medulla and dimmer staining in the cortex (A). In GVH-reactive mice, no thymic cortico-medullary junction was detectable; throughout the thymus of GVH-reactive mice, faint class II staining was observed interspersed with areas entirely devoid of class II (B).

Discussion

In this report, the effect of GVHR-induced thymic injury on positive and negative selection was examined. We used non-irradiated F1 hybrid recipients of parental lymphoid cells to dissect out the consequences of GVHR on thymic maturation and education of T cells. T cell maturation and positive selection appeared dramatically altered in GVH-reactive mice, as indicated by lack of upregulation of CD3 on $CD4^+8^-$ thymocytes and variability of up to an order of magnitude in the incidence of specific Vß segments on mature thymocytes. Concomitantly, the appearance of phenotypically self-reactive T cells in the GVH-damaged thymuses demonstrated impaired thymic deletion processes. The defects in selection may have resulted from the profound changes in the level and pattern of thymic MHC class II expression observed during GVHR.

We report here that thymic class II MHC expression is dramatically decreased during GVHR. Class II-bearing tissues including lymphoid organs and epithelia such as gut, lung, liver, salivary glands, and skin, are targets of GVHR-induced lesions (1, 41-46). We have previously reported that medullary epithelial cells are injured during GVHR (6); the loss of the intense staining for class II in the thymic medulla may be due to the destruction of class II-bearing stroma. This seems especially likely since viable thymic epithelial cells would be expected to upregulate their class II expression in response to the interferon-γ production induced early during GVHR (7,47). However, the

relative contributions of epithelial cell damage versus class II MHC downregulation have not yet been examined. The net result of the decreased cell surface class II expression appeared to be a defect in class II-mediated selection events.

It is interesting that most of the abnormalities of $CD4^+$ cell maturation and function reported in class II knockout mice were also observed in GVH-reactive animals. GVH-reactive animals (4, and reported herein) and class II knockout mice (21) both demonstrated decreased expression of CD3 on class II-restricted, $CD4^+8^-$ thymocytes; cortisone sensitivity of $CD4^+8^-$ thymocytes; abnormal variability in thymocyte Vß incidence between mice; paucity of $CD4^+$ cells in the thymus and the periphery; and peripheral immunosuppression. Thus, the class II dependent acquisition of functions and phenotype by $CD4^+$ thymocytes appears to be as flawed during GVHR as in the complete absence of class II expression. It seems, then, that the class II molecules remaining in the thymus during GVHR were expressed on cell types inefficient at mediating selection; or that the class II antigens were themselves altered and unable to deliver complete or correct selection signals. Reduced affinity of the TCR/MHC interaction has been shown to result in inefficient positive selection and functionally defective self-restriction in the periphery in mice transgenic for allelic variants of class II (48). During GVHR, the affinity of the class II/TCR interaction may be affected by the presentation of different, GVHR-induced peptides; acute phase proteins and endogenous viral antigens induced by the

high cytokine expression during GVHR (49) likely give rise to peptides not usually presented within the thymus, potentially displacing endogenous peptides which normally play a role in positive selection (50).

In transgenic mice with class II expression restricted to specific regions of the thymus (51; ΔX and ΔY mice), positive selection was defective in mice without cortical class II despite its normal medullary expression (52). We have observed defective positive selection despite low levels of class II expression; this supports the hypothesis that the class II antigens remaining in the GVHR-injured thymus are unable to deliver normal selection signals. In fact, we have previously reported that the cortico-medullary junction, normally clearly demarcated histologically, is lost during GVHR (1); this may be in part a reflection of loss of certain characteristic cortical cell able to mediate positive selection.

Our findings greatly resemble those reported in cyclosporine A (CsA)-treated mice. CsA treatment in conjunction with thymic irradiation also triggers GVHR-like thymic histopathology, disappearance of medullary class II expression (53), and the appearance of autoreactive (Mls-reactive) T cells (54). CsA treatment in human recipients of autologous bone marrow transplants has resulted in a syndrome dubbed "syngeneic GVHR", consisting of a GVHR-like syndrome with autoimmune features (55-57). Our present results suggest that the primary etiology of the T cell selection defect in allogeneic (as well as syngeneic) GVHR may be the abnormally reduced

expression of class II MHC in the thymus.

Defective positive selection has also been described in TCR transgenic mice lacking the appropriate MHC haplotype (58). In these mice, there is an abnormal accumulation of $CD3^{hi}$ $CD4^-8^-$ cells; we have not observed an analogous phenomenon during GVHR. Thus, the changes seen in GVHR more closely resemble those which occur in the absence of class II expression than in the absence of an appropriately restricted TCR/MHC class I interaction.

Negative selection of TCR $V\beta6^+$ cells in response to $Mls\text{-}1^a$ was found to be defective in GVHR. The incidence of $V\beta6^{hi}$ thymocytes in GVH-reactive mice, however, did not reach the frequency observed in non-deleting strains; this may reflect a leakiness in the negative selection process rather than a complete failure of deletion. The latter hypothesis conforms to a view of thymic selection in which developing T cells associate with a group of stromal cells and receive most of their selection signals in a spacially restricted microenvironment: those maturing on undamaged stroma are normally tolerized and restricted, while thymocytes interacting with injured epithelia or infiltrating APCs may receive aberrant signals. This hypothesis correlates with the previously described focal nature of GVHR-induced histopathological thymic lesions (59,60), and the patchy pattern of class II loss and stochastic disruption in Vß incidences reported here.

Defective positive and negative selection observed in mice undergoing GVHR could provide a basis for the symptoms characteristic of clinical chronic

GVH disease. As in murine allogeneic GVHR, human GVHR results in thymic dysplasia (60,61), and therefore may also induce the production of aberrantly self-restricted and self-tolerized T cells. We report here that the incidence of specific Vß segments was dramatically altered during GVHR; these disproportional decreases or increases may result in a limited repertoire, and reflect defective thymic class II-mediated signalling. In fact, selective defects in $CD4^+$ T cell self-restricted responses and IL-2 production have been detected in the periphery of GVH-reactive mice (5,62,63). In turn, these functional defects contribute to the immunosuppression and susceptibility to bacterial and viral pathogens that complicate GVHR. The autoimmunity which occurs clinically in spite of the profound immunosuppression may be triggered by the self-reactive T-cells generated during GVHR. Although the phenotypically self-reactive cells arising during the GVHR were not examined for functional autoreactivy in this study, by analogy with CsA induced autoreactivity, T cells educated in the GVHR-dysplastic thymus may express autoimmune activity in irradiated hosts (54).

References

1. Lapp, W.S., T. Ghayur, M. Mendes, M. Seddik, and T.A. Seemayer. 1985. The functional and histological basis for graft-versus-host-induced immunosuppression. *Immunol. Rev.* 88: 107.

2. Via, C.S. and G.M. Shearer. 1988. Murine graft-versus-host disease as a model for the development of autoimmunity. Relevance of cytotoxic T lymphocytes. *Ann. N. Y. Acad. Sci.* 532:44.

3. Morris, S.C., R.L. Cheek, P.L. Cohen, and R.A. Eisenberg. 1990. Autoantibodies in chronic graft versus host result from cognate T-B interactions. *J. Exp. Med.* 171: 503.

4. Kornbluth, M., E. You-Ten, J. Desbarats, S. Gamache, A. Xenocostas, and W.S. Lapp. 1991. T cell subsets in the thymus of graft-versus-host immunosuppressed mice. Sensitivity of the $L3T4^{+}Lyt\text{-}2^{-}$ subset to cortisone. *Transplantation* 51: 262.

5. Fukuzawa, M., C.S. Via and G.M. Shearer. 1988. Defective thymic education of $L3T4^{+}$ T helper cell function in graft-vs-host mice. *J. Immunol.* 141:430.

6. Seddik, M., T.A. Seemayer, and W.S. Lapp. 1980. T cell functional defect associated with thymic epithelial cell injury induced by a graft-versus-host reaction. *Transplantation* 29: 61.

7. Leist, T.P., R. Heuchel, and R.M. Zinkernagel. 1988. Increased bactericidal macrophages activity induced by immunological stimuli is dependent on interferon-γ. Interference of anti-IFN-γ but not anti-IFN-α/ß with modulation of macrophage activity caused by lymphocytic choriomeningitis virus infection or systemic graft-*vs.*-host reactions. *Eur. J. Immunol.* 18: 1295.

8. Nestel, F.P., K.S. Price, T.A. Seemayer, and W.S. Lapp. 1992. Macrophage priming and LPS-triggered release of TNF-alpha during graft-versus-host disease. *J.Exp.Med.*

175: 405.

9. Ghayur, T., T.A. Seemayer, A. Xenocostas and W.S. Lapp. 1988. Complete sequential regeneration of graft-vs.-host induced severely dysplastic thymuses. *Am.J.Pathol.* 133:25.

10. Petrie, H.T., P. Hugo, R. Scollay, and K. Shortman. 1990. Lineage relationships and developmental kinetics of immature thymocytes: CD3, CD4, and CD8 acquisition in vivo and in vitro. *J. Exp. Med.* 172: 1583.

11. Yoshikai, Y., M. Ogimoto, G. Matsuzaki and K. Nomoto. 1990. Bone marrow-derived cells are essential for intrathymic deletion of self-reactive T cells in both the host- and donor-derived thymocytes of fully allogeneic bone marrow chimeras. *J. Immunol.* 145:505.

12. Matsuzaki, G., Y. Yoshikai, M. Ogimoto, K. Kishihara and K. Nomoto. 1990. Clonal deletion of self-reactive T cells at the early stage of T cell development in thymus of radiation bone marrow chimeras. *J. Immunol.* 145:46.

13. J.L. Roberts, S.O. Sharrow, and A. Singer. 1990. Clonal deletion and clonal anergy in the thymus induced by cellular elements with different radiation sensitivities. *J. Exp. Med.* 171:935.

14. Salaun, J., A. Bandeira, I. Khazaal, F. Calman, M. Coltey, A. Coutinho and N.M. LeDouarin. 1990. Thymic epithelium tolerizes for histocompatibility antigens. *Science* 247:1471.

15. Von Boemer, H., H.S. Teh and P. Kisielow. 1989. The thymus selects the useful, neglects the useless and destroys the harmful. *Immunol. Today* 10:57.

16. Kruisbeek, A.M., J.J. Mond, B.J. Fowlkes, J.A. Carmen, S. Bridges, and D.L.

Longo. 1985. Absence of the Lyt-2$^-$L3T4$^+$ lineage of T cells in mice treated neonatally with anti-I-A correlates with absence of intrathymic I-A-bearing antigen-presenting cell function. *J. Exp. Med.* 161: 1029.

17. MacDonald H.R., H. Hengartner, and T. Pedrazzini. 1988. Intrathymic deletion of self-reactive cells prevented by neonatal anti-CD4 antibody treatment. *Nature* 335:174.

18. Zuniga-Pflucker, J.C., L.A. Jones, D.L. Longo, and A.M. Kruisbeek. 1990. CD8 is required during positive selection of CD4$^-$/CD8$^+$ T cells. *J.Exp.Med.* 171: 427.

19. Zilstra, M., M. Bix, N.E. Simister, J.M. Loring, D.H. Raulet, and R. Jaenisch. 1990. ß2 microglobulin deficient mice lack CD4$^-$8$^+$ cytolytic T cells. *Nature* 344: 742.

20. Koller, B.H., P. Marrack, J.W. Kappler, and O. Smithies. 1990. Normal development of mice deficient in ß2M, MHC class I proteins, and CD8$^+$ T cells. *Science* 248: 1227.

21. Cosgrove, D., D. Gray, A. Dierich, J. Kaufman, M. Lemeur, C. Benoist, and D. Mathis. 1991. Mice lacking MHC class II molecules. *Cell* 66: 1051.

22. Grusby, M.J., R.S. Johnson, V.E. Papaioannou, and L.H. Glimcher. 1991. Depletion of CD4$^+$ T cells in major histocompatibility complex class II-deficient mice. *Science* 253: 1417.

23. Rahemtulla A., W.P. Fung-Leung, M.W. Schilham, T.M. Kündig, S.R. Sambhara, A. Narendran, A. Arabian, A. Wakeham, C.J. Paige, R.M. Zinkernagel, R.G. Miller, and T.W. Mak. 1991. Normal development and function of CD8$^+$ cells but markedly decreased helper cell activity in mice lacking CD4. *Nature* 353: 180.

24. Fung-Leung W.-P., M.W. Schilhan, A. Rahemtulla, T.M. Kundig, M. Vollenweider, J. Potter, W. van Ewik, and T. Mak. 1991. CD8 is needed for development of cytotoxic T cells but not helper T cells. *Cell* 65: 443.

25. Vandekerckhove, B.A.E., R. Baccala, D. Jones, D.H. Kono, A.N. Theofilopoulos, and M.-G. Roncarolo. 1992. Thymic selection of the human T cell receptor Vß repertoire in SCID-hu mice. *J.Exp.Med.* 176: 1619.

26. Kanagawa O., E. Palmer, and J. Bill. 1989. The T cell receptor V beta 6 domain imparts reactivity to the Mls-1^a antigen. *Cell Immunol.* 119: 412.

27. MacDonald H.R., R. Schneider, R.K. Lees, R.C. Howe, H. Acha Orbea, H. Festenstein, R.M. Zinkernagel, and H. Hengartner. 1988. T-cell receptor V beta activity and tolerance to Mls^a-encoded antigens. *Nature* 332: 40.

28. Palathumpat, V.C., M.M. Vandeputte, and M. Waer. 1990. Effects of thymus irradiation on the immune competence of T cells after total-lymphoid irradiation. *Transplantation* 50: 95.

29. Cunningham, A.J., and A. Szenberg. 1968. Further improvements in the plaque technique for detecting single antibody-forming cells. *Immunology* 14: 599.

30. Parthenais, E., and W.S. Lapp. 1978. Evidence for two types of nonspecific suppressor cells activated by the graft-versus-host reaction in mice. *Scand. J. Immunol.* 7: 215.

31. Seddik M., T.A. Seemayer, and W.S. Lapp. 1984. The graft-versus-host reaction and immune function. I. T-helper cell immuno-deficiency associated with graft-versus-host induced thymic epithelial cell damage. *Transplantation* 37: 281.

32. Kappler, J.W., N. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell* 49: 273.

33. Murphy, K.M., A.B. Heimberger, and D.Y. Loh. Induction by antigen of intrathymic apoptosis of CD4$^+$CD8$^+$TCR^{lo} thymocytes in vivo. *Science* 250: 1720.

34. Haskins, K., C. Hannum, J. White, N. Roehm, R. Kubo, J. Kappler, and P. Marrack. 1984. The antigen-specific, major histocompatibility complex restricted receptor on T cells. IV. An antibody to a receptor allotype. *J.Exp.Med.* 160:452.

35. Payne, J., B.T. Huber, N.A. Cannon, R. Scheider, M.W. Schilham, H. Acha-Orbea, H.R. MacDonald, and H. Hengartner. 1988. Two monoclonal rat antibodies with specificity for the ß-chain variable region Vß6 of the murine T-cell receptor. *Proc. Natl. Acad. Sci. USA.* 85: 7695.

36. Khairallah, M., C. Spach, F. Maitre, and R. Motta. 1988. Endocrine involvement in minor (non-H-2) graft versus host reaction in mice: dissociated effect on corticosterone and aldosterone plasma levels. *Endocrinology* 123: 1949.

37. Okada, C.Y., B. Holzmann, C. Guidos, E. Palmer, and I.L. Weissman. 1990. Characterization of a rat monoclonal antibody specific for a determinant encoded by the Vß7$^+$ gene segment. Depletion of Vß7$^+$ T cells in mice with Mls-1^a haplotype. *J. Immunol.* 144: 3473.

38. Kappler, J.W., U. Staerz, J. White, and P.C. Marrack. 1988. Self-tolerance eliminates T cells specific for Mls-modified products of the major histocompatibility complex. *Nature* 332: 35.

39. Happ, M.P., D.L. Woodland, and E. Palmer. 1989. A third T-cell receptor ß-chain variable region gene encodes reactivity to Mls-1^a gene products. *Proc. Natl. Acad. Sci. USA.* 86: 6293.

40. Fukushi, N., H. Arase, B. Wang, K. Ogasawara, T. Gotohda, R.A. Good, and K. Onoé. 1990. Thymus: a direct target tissue in graft-versus-host reaction after allogeneic bone marrow transplantation that results in abrogation of induction of self-tolerance. *Proc. Natl. Acad. Sci. USA.* 87: 6301.

41. Piguet, P.-F., A. Janin-Mercier, P. Vassalli, and J.H. Saurat. 1987. Epidermal

lesions of the GVHR: evaluation of the role of different MHC and non MHC loci and of the Ly-2$^+$ and L3T4$^+$ T lymphocytes. *J. Immunol.* 139: 406.

42. Charley, M.R., J.L. Bangert, B.L. Hamilton, J.N. Gilliam, and R.D. Sontheimer. 1983. Murine Graft-versus-host skin disease: a chronologic and quantitative analysis of two histologic patterns. *J. Invest. Dermatol.* 81: 412.

43. Guy-Grand, D., and P. Vassali. 1987. Gut injury in mouse graft-versus-host reaction. *Adv. Exp. Med. Biol.* 216A: 661.

44. Mowat A.McI., M.V. Felstein. 1990. Experimental studies of immunologically mediated enteropathy. V. Destructive enteropathy during an acute graft-versus-host reaction in adult BDF1 mice. *Clin. Exp. Immunol.* 79: 279.

45. Guy-Grand, D. and P. Vassalli. 1987. Gut injury in mouse graft-versus-host reaction. Adv. Exp. Med. Biol. 216A: 661.

46. Xenocostas, A., D.G. Osmond, and W.S. Lapp. 1991. The effect of the graft-versus-host reaction on B lymphocyte production in bone marrow of mice. Depressed genesis of early progenitors prior to μ heavy chain expression. *Transplantation* 51: 1089.

47. Galy, A.H.M., and H. Spits. 1991. IL-1, IL-4, and IFN-γ differentially regulate cytokine production and cell surface molecule expression in cultured human thymic epithelial cells. *J Immunol.* 147: 3823.

48. Berg, L.J., G.D. Frank, and M.M. Davis. 1990. The effects of MHC gene dosage and allelic variation on T cell receptor selection. *Cell* 60: 1043.

49. Gratama, J.W., W.E. Fibbe, A.M. Naipal, J. Slats, T. Stijnen, J. D'Amaro, R.L. Bolhuis, T.H. The and J. Janssen. 1986. Cytomegalovirus immunity and T lymphocytes in bone marrow donors and acute graft-versus-host disease. *Bone Marrow Transplant.* 1:141.

50. Nikolic-Zugic, J., and M.J. Bevan. 1990. Role of self-peptides in positively selecting the T-cell repertoire. *Nature* 344: 65.

51. van Ewijk, W., Y. Ron, J. Monaco, J. Kappler, P. Marrack, M. LeMeur, P. Gerlinger, B. Durand, C. Benoist, and D. Mathis. 1988. Compartmentalization of MHC class II gene expression in transgenic mice. *Cell* 53:357.

52. Bill, J., and E. Palmer. 1989. Positive selection of $CD4^+$ T cells mediated by MHC class II-bearing stromal cells in the thymic cortex. *Nature* 341: 649.

53. Beschorner, W.E., K.A. Kelly, A.D. Hess, and G.W. Santos. 1987. Cyclosporine and the thymus: influence of irradiation and age on thymic immunopathology and recovery. *Cell. Immunol.* 110: 350.

54. Gao E.-K., D. Lo, R. Cheney, O. Kanagawa, and J. Sprent. 1988. Abnormal differentiation of thymocytes in mice treated with cyclosporin A. *Nature* 336: 176.

55. Beschorner, W.E., C.A. Shinn, A.C. Fischer, G.W. Santos and A.D.Hess. 1988. Cyclosporine-induced pseudo-graft-versus-host disease in the early post-cyclosporine period. *Transplantation* 46:112S.

56. Einsele, H., G. Ehninger, E.M. Schneider, G.F. Kruger, A. Vallbracht, R. Dopfer, H. Schmidt, H.D. Waller and C.A. Muller. 1988. High frequency of graft-versus-host-like syndromes following syngeneic bone marrow transplantation. *Transplantation* 45:579.

57. Fischer, A.C., M.K. Laulis, L. Horwitz, W.E. Beschorner and A. Hess. 1989. Host resistance to cyclosporine induced syngeneic graft-versus-host disease. Requirement for two distinct lymphocyte subsets. *J.Immunol.* 143:827.

58. von Boehmer, H., J. Kirberg, and B. Rocha. 1991. An unusual lineage of α/ß T cells that contains autoreactive cells. *J. Exp. Med.* 174: 1001.

59. Seemayer, T.A., W.S. Lapp, and R.P. Bolande. 1978. Thymic epithelial injury in graft-versus-host reaction following adrenalectomy. *Am. J. Pathol.* 93: 325.

60. Seemayer, T.A., W.S. Lapp, and R.P. Bolande. 1977. Thymic involution in murine graft-versus-host reaction. *Am. J. Pathol.* 88: 119.

61. Seemayer, T.A., W.S. Lapp, and R.P. Bolande. 1977. Thymic involution in murine graft-versus-host reaction: epithelial injury mimicking human thymic dysplasia. *Am. J. Pathol.* 88:119.

62. Levy, R.B., M. Jones, and C. Cray. 1990. Isolated peripheral T cells from GvHR recipients exhibit defective IL-2R expression, IL-2 production, and proliferation in response to activation stimuli. *J. Immunol.* 145: 3998.

63. Mendes, M.L., H. Rode, A. Peres, P.A.L. Konshavn, and W.S. Lapp. 1985. Interleukin-1 and interleukin-2 defects associated with murine graft-versus-host-induced immunodeficiency. *Transplantation* 39: 418.

CHAPTER 4.

A model for GVHR-induced epithelial pathology:

the effects of irradiation.

The long term defect in the T cell compartment may depend on aberrant thymic education, giving rise to peripheral T cells which have not been adequately self-restricted or self-tolerized. This in turn results from thymic epithelial injury and aberrant class II MHC expression. But what determines the extent of tissue injury during the acute phase of the reaction? CsA-induced sGVHR results in a similar syndrome of thymic injury, phenotypically abnormal thymocytes, and peripheral autoimmunity. sGVHR is also accompanied by a decrease in thymic MHC class II; however, expression returns to normal unless the thymus is irradiated. Thus, irradiation allows long term maintainance of abnormal thymic histopathology. To explore the role of irradiation on the induction and severity of GVHR-induced epithelial histopathology, the skin is used as a model system. In the mouse, skin provides an ideal model because it is ordinarily highly resistant to GVHR-induced lesions, and thus provides a clear indication of heightened susceptibility to injury. Furthermore, this model permits skin to be removed, irradiated, and grafted to syngeneic or allogeneic recipients, allowing the effect of local irradiation to the target organ to be distinguished from the effects of systemic irradiation.

Irradiation of the skin and systemic graft-versus-host disease synergize to produce cutaneous lesions.[1]

Julie Desbarats[*], Thomas A. Seemayer[†], and Wayne S. Lapp[*]

From the Department of Physiology[*], McGill University, Montreal QC. Canada H3G 1Y6 and [†] the Department of Pathology and Microbiology, University of Nebraska Medical Centre, Omaha, NE. 68198-3135

Running head: Irradiation exacerbates cutaneous GVHD

This work was supported by the MRC of Canada (grant no. MT 3526) and the NCI of Canada. J. Desbarats was supported by studentships from the Fonds de Recherches en Santé du Québec and the MRC of Canada.

Address correspondence to J. Desbarats, Rm. 1108, McIntyre Medical Sciences Building, Department of Physiology, McGill University, 3655 Drummond St., Montreal, QC., Canada H3G 1Y6

[1] This paper was published in the *American Journal of Pathology*, 144: 883, 1994.

In this report, the relationship between irradiation and graft-versus-host disease (GVHD)-induced cutaneous injury was investigated. Unirradiated F1 hybrid mice were grafted with irradiated skin and then injected with parental strain lymphoid cells to induce GVHD. Although low grade dermal lymphoid infiltrates were observed in unirradiated skin grafts of some GVH-reactive mice, and irradiated grafts of normal animals showed occasional fibrosis, only the irradiated grafts of GVH-reactive mice developed lesions consisting of vacuolar degeneration of the epidermal junction and necrotic keratinocytes accompanied by pronounced epidermal infiltrates, characteristic of clinical cutaneous GVHD. The results suggest that cutaneous irradiation exerts a permissive effect on lesion formation in the skin of mice undergoing GVHD. Furthermore, systemic irradiation, known to exacerbate the severity of GVHD, is not required. Cutaneous lesions may be triggered by radiation injury of keratinocytes, upregulation of adhesion molecules on irradiated endothelium, destruction of protective radiosensitive intraepithelial lymphocytes, and radiation-induced priming of intradermal macrophages.

Introduction

Bone marrow transplantation (BMT) is used extensively in the treatment of hematological malignancies to reconstitute patients after anti-neoplastic radiochemotherapy. Systemic graft-versus-host disease (GVHD) frequently complicates BMT and produces significant morbidity and mortality.[1] GVHD occurs when allogeneic T cells in the donor inoculum become activated in the immunoincompetent host, resulting in massive T cell proliferation and cytokine release.[1, 2] A profound state of immunosuppression and extensive epithelial cell injury ensue, the latter probably resulting from the combined effects of activated allospecific cytotoxic T lymphocytes and cytokine-primed macrophages and natural killer cells.[3] The skin is a major target in clinical and experimental GVHD, as are the liver, lung, gastro-intestinal tract and lymphoid tissues.[1,4] Cutaneous GVHD develops in most cases of human GVHD.[5] In murine models of GVHD, cutaneous lesions have been reported in irradiated recipients injected with allogeneic lymphoid cells; in contrast, cutaneous lesions fail to develop in unirradiated GVH-reactive mice in which disease has been induced by semi-allogeneic parental lymphoid cells.[6] The preferential appearance of cutaneous lesions in irradiated human and murine recipients suggests that irradiation might affect the skin itself, making it more susceptible to GVHD-induced damage. Clinical case reports have described the development of cutaneous lesions selectively in irradiation fields, suggesting

that the irradiated skin is itself more susceptible to GVHD-induced injury.[7,8] Alternatively, the nature of systemic GVHD may be altered in irradiated recipients. GVHD in irradiated recipients is both quantitatively and qualitatively different: the reaction is more severe in irradiated recipients, and requires a smaller T cell dose for disease induction;[4] and, the pattern and magnitude of immunoglobulin production and cytokine release differ in irradiated and unirradiated GVH-reactive mice.[9]

To determine whether local irradiation is sufficient to allow the formation of cutaneous lesions during systemic GVHD, isolated skin was irradiated or left unirradiated as a control and grafted onto unirradiated AxCBA F1 hybrid mice which were then injected with A strain parental lymphoid cells to induce GVHD. Syngeneic AxCBA F1 skin was grafted onto F1 recipients to examine the interaction between local cutaneous irradiation and systemic GVHD in the absence of total body irradiation; parental A or CBA strain skin (irradiated or not) was grafted onto AxCBA F1 recipients to study the allospecificity of cutaneous lesion formation in recipients of A strain lymphoid cells.

Materials and Methods

Animals

Mice of the inbred strains CBA/Ca (CBA, H-2^k) and Strong A (A, H-2^a [$K^k/I^k/D^d$]), and F1 hybrid AxCBA (H-$2^{a/k}$) mice were used. All mice were bred

and maintained in our animal colony.

Skin grafts

Skin grafts from AxCBA, A, or CBA strain donor mice were grafted onto AxCBA F1 recipients as described previously.[10, 11] Briefly, circular grafts (0.5-1 cm diameter) were cut from shaved, ether anaesthetized donors. The grafts were irradiated at 139 rads/min from a cesium source (Atomic Energy of Canada Limited, Ottawa, Canada) to the appropriate dosage (500-1500 rads, as indicated). Control grafts were unirradiated. Recipients were ether anaesthetized and the grafts were placed onto graft beds prepared by shaving the area and removing a circular area of skin size-matched to the graft, then secured with tape. Mice received either a single graft, or paired grafts, one irradiated and one unirradiated.

GVHD Induction

Fourteen days after skin grafting, GVHD was induced by the i.v. injection of $5x10^7$ viable lymphoid cells from A strain parental donors into unirradiated AxCBA F1 hybrid skin graft recipients. The donor inoculum consisted of pooled spleen and lymph node tissue made into single cell suspension by gentle tamping through 50-mesh stainless steel screens and washed in Hanks' balanced salt solution.

Direct plaque-forming cell (PFC) response to sheep red blood cells (SRBC)

Sixteen days after GVHD induction, the PFC response to SRBC was determined to verify immunosuppression, diagnostic of systemic GVHD. Mice were immunized i.v. with $5x10^8$ SRBC in 0.3 ml saline. Four days later the direct splenic PFC response was assessed by the method of Cunningham and Szenberg[12] as modified in this laboratory.[13]

Preparation and scoring of skin biopsies.

Twenty days after GVHD induction (34d after skin grafting), mice were sacrificed and their skin grafts were biopsied by removing a strip of tissue from the centre of the graft. The biopsies were fixed in 10% buffered formalin, dehydrated in graded alcohols and xylols, and embedded in paraffin. Four 5µm serial sections from each sample were cut and stained with hematoxylin and eosin. The samples were randomized and coded for evaluation. Grading of cutaneous GVHD was performed by examination of the whole area of all four sections for each sample, according to the following scheme: grade 1, two or more necrotic keratinocytes; grade 2, several foci of necrotic keratinocytes with lymphoid infiltration of epidermis; grade 3, many necrotic keratinocytes with scattered intraepithelial lymphoid infiltrates and vacuolar degeneration of the epidermal-dermal junction; and grade 4, extensive areas of necrotic keratinocytes, marked vacuolar degeneration of the epidermal-dermal junction

and intense intraepithalial lymphoid infiltrates.

Results

GVH-reactive mice were immunosuppressed

Systemic GVHD, induced by the injection of 5×10^7 A strain parental lymphoid cells into AxCBA recipients, was confirmed by immunosuppression (Table 1). All mice injected with parental cells had a suppressed PFC response to SRBC diagnostic of GVHD.

Local irradiation increased the incidence and severity of cutaneous GVHD in syngeneic skin grafts

Cutaneous GVHD is seldom seen in unirradiated GVH-reactive mice, and then only low grade lesions are observed.[6] Correspondingly, in the GVH-reactive recipients of syngeneic, unirradiated skin grafts, graft biopsies revealed normal findings (Figure 1a) with no evidence of cutaneous GVHD in 3 of 5 animals, and only low grade cutaneous changes in the remaining two mice (Figure 2). In contrast, all irradiated grafts in GVH-reactive mice showed some degree of cutaneous GVHD (Figures 1b,c and 2), with the mean grade ranging from 2.3 to 2.5 depending on the experiment and the radiation dose (Figure 2 and Table 2). Of these, 85% displayed cutaneous GVHD more severe than grade 1. In the experiments employing paired skin grafts, one unirradiated and one

irradiated graft on the same mouse, the irradiated graft invariably displayed a more severe grade of cutaneous GVHD. The incidence and severity of cutaneous GVHD was similar throughout the dosage range of radiation tested (500 - 1500 rads), although grade 4 cutaneous GVHD was observed only with the maximal radiation dose. Thus, despite profound immunosuppression in all systemically GVH-reactive animals, only irradiated skin was seen to develop a high incidence of significant cutaneous GVHD.

Local irradiation in the absence of systemic GVHD did not produce changes mimicking cutaneous GVHD

Although it has been reported that radiation dermatitis can imitate the histological changes of acute GVHD,[14] we have not observed cutaneous GVHD-like injury in irradiated syngeneic skin grafts in the absence of systemic GVHD (Figure 2 and Table 2). The histopathology observed in irradiated skin (in the absence of systemic GVHD) was limited to frequent fibrotic changes (incidences up to 67% in some experiments at the highest radiation dosage, 1500 rads), and occasional mild lymphoid infiltrates (Figure 1d).

Cutaneous GVHD in irradiated skin is allo-specific

In addition to syngeneic skin, parental A or CBA skin was grafted onto AxCBA recipients to determine whether the radiation-induced cutaneous GVHD was allo-specific (Table 2). Systemic GVHD was induced with A strain lymphoid

cells, and therefore should produce cutaneous lesions in the CBA, but not A, skin grafts in an allo-specific reaction. As expected, results for CBA strain skin grafts were similar to those for AxCBA syngeneic grafts, with rare low-grade cutaneous GVHD in the unirradiated grafts and more severe lesions (mean grade 2.3) in irradiated grafts. The severity of the cutaneous GVHD was not significantly different between CBA and AxCBA grafts, but the incidence of serious GVHD was higher in CBA skin, with all grafts showing greater than grade 1 GVHD (Table 2). In contrast, none of the irradiated A strain grafts displayed significant cutaneous GVHD, demonstrating allospecificity in the reaction. Unirradiated A strain grafts, however, occasionally demonstrated mild cutaneous GVHD-like changes in GVH-reactive mice (Table 2).

Table 1. Systemic GVHD confirmed by depressed PFC responses to SRBC.

Mice	Mean PFCs/10^6 splenocytes ± SD		
	Exp. 1	Exp. 2	Exp. 3
Untreated	2869 ± 605	1716 ± 524	1222 ± 122
Systemic GVH-reactive	1 ± 4	2 ± 4	0 ± 0

Table 2. Severity and Incidence of Cutaneous GVHD in Grafted Skin.

	Mean grade ± SD of Cutaneous GVHD (%Incidence*)					
Strain of skin graft donor	AxCBA (syngeneic)		CBA		A	
Skin graft Irradiation†	-	+	-	+	-	+
Normal mice	0.0 ± 0.0 (0%)	0.0 ± 0.0 (0%)	0.0 ± 0±0 (0%)	0.0 ± 0.0 (0%)	0.3 ± 0.6 (0%)	0.2 ± 0.3 (0%)
Systemic GVH-reactive mice¶	0.6 ± 0.9 (20%)	2.4 ± 0.9 (85%)	0.5 ± 0.7 (0%)	2.3 ± 0.4 (100%)	1.4 ± 0.5 (50%)	0.5 ± 0.6 (0%)

* Incidence of skin grafts showing more than grade I cutaneous GVHD.

† Irradiated skin grafts received 1500 rads.

¶ Systemic GVHD was induced 20d before assay by the injection of $50x10^6$ A strain parental lymphoid cells.

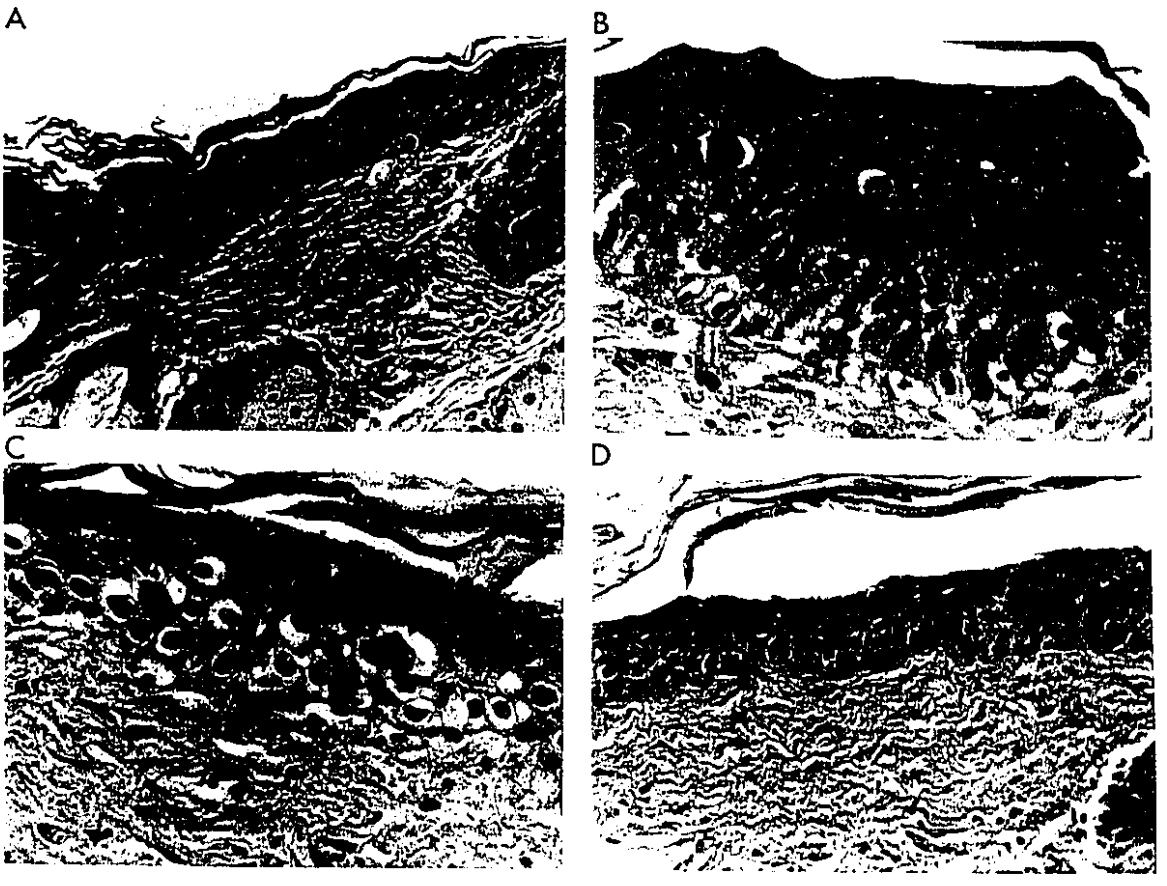


Figure 1. Histologic sections from AxCBA syngeneic skin grafts. Unirradiated or irradiated (1500 rads) skin grafts were transplanted onto syngeneic AxCBA recipients. GVHD was induced 14d after skin grafting. Skin graft sites were biopsied 20d after GVHD induction.

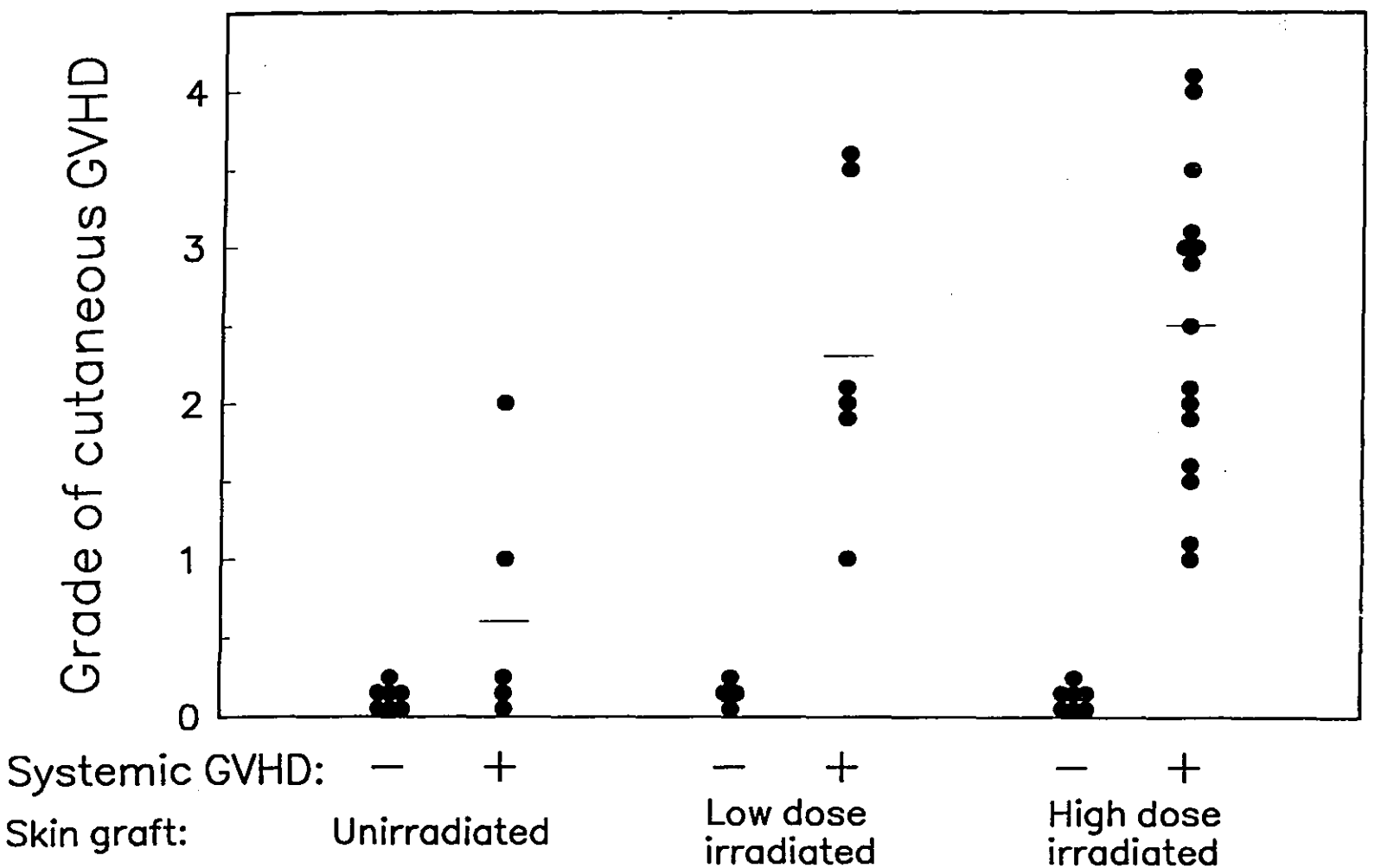
A. Unirradiated graft, untreated (non-GVH) group reveals normal histology (hematoxylin-eosin, x200).

B. Irradiated graft, GVH-reactive group reveals pronounced vacuolar degeneration of the epidermal-dermal junction, intraepithelial lymphoid infiltrates and two necrotic keratinocytes (arrows) (hematoxylin-eosin, x400).

C. Irradiated graft, GVH-reactive group reveals intense intraepidermal lymphoid infiltrate and necrotic keratinocytes (arrows) (hematoxylin-eosin, x400).

D. Irradiated graft, untreated (non-GVH) group reveals normal epidermis, and dermal fibrosis (hematoxylin-eosin, x200).

Figure 2. The effect of local irradiation on cutaneous GVHD. AxCBA skin grafts were unirradiated, irradiated with 500 rads (low dose), or 1000 - 1500 rads (high dose), then grafted onto unirradiated syngeneic AxCBA recipients. Two weeks later, systemic GVHD was induced in some of the AxCBA skin graft recipients (+) by the injection of 5×10^7 A strain parental lymphoid cells. The remaining skin grafted mice served as untransplanted (-) controls. The grade of cutaneous GVHD of each skin graft and the mean grade of each GVH-reactive group is shown.


Grade of cutaneous GVHD
0
1
2
3
4
Systemic GVHD: – + – + – +
Skin graft: Unirradiated
Low dose irradiated
High dose irradiated

Discussion

Our findings demonstrate that local irradiation of the skin and systemic GVHD synergize to produce cutaneous GVHD lesions in the mouse. Although total body irradiation may contribute to cutaneous GVHD in clinical and experimental models by exacerbating the reaction and altering the pattern of cytokine release,[9] the present results indicate that local cutaneous irradiation alone is necessary and sufficient for a high incidence of moderate to severe cutaneous GVHD. The more severe reaction in irradiated syngeneic AxCBA and parental CBA grafts as compared to A strain skin support a role for allospecificity in the formation of the cutaneous GVHD lesions, although we have not measured specific CTL activity in the mouse strain used here. The only significant GVHD-like lesions observed in A strain skin occurred in unirradiated grafts, and were probably the result of passenger leukocytes present in the grafted skin activated against the recipient by the circulating cytokines produced during systemic GVHD.

The mechanism of injury in radiation-exacerbated cutaneous GVHD is thus unlikely to be entirely mediated by non-specific effects such as epidermal radiation damage and systemic cytokine production. A potential mechanism consistent with our findings involves a two step process. In the non-specific phase, macrophages present in the skin are activated by gamma-irradiation, which has been shown to alter their morphology,[15] increase their ability to

secrete IL-1,[16] and prime them for antibody-dependent cytotoxicity[17] and responsiveness to bacterial lipopolysaccaride (LPS).[18] Macrophage priming by gamma-irradiation persists without decreasing for at least 8 days in vitro,[17] and for up to one month *in vitro* as determine by increased cytokine secretion.[19] A second macrophage-priming stimulus is provided by the high levels of interferon-gamma (IFN-γ) produced during the massive T cell proliferation early in GVHD.[3,20] Synergy between gamma-irradiation and IFN-γ priming may provide a trigger for local, low-level TNF-α production, which in turn upregulates ICAM-1 on endothelium and keratinocytes.[21] The second, allospecific phase is then initiated; host-reactive, activated T cell migration into the skin and attachment to keratinocytes facilitated by local ICAM-1 upregulation; in fact, increased ICAM-1 expression has been reported in conjunction with upregulated HLA-DR (also IFN-γ inducible) in cutaneous GVHD lesions.[22] $CD8^+$ T cells, possibly attracted by GVH-induced increases in MIP-1α[23], as well as $CD4^+$ T cells and $CD14^+$ macrophages, are all found in GVH-reactive skin;[24,25] thus, the alloreactive CTLs can then directly mediate keratinocyte death while local T cell production of IFN-γ[26] can amplify the cascade of macrophage TNF-α release. TNF-α is present at necropsy in human GVHD cutaneous lesions,[27] and can contribute directly to epidermal injury as evidenced by the presence of GVHD-like damage in the skin of TNF-α transgenic mice.[28] Although TNF-beta, a T cell product, has been reported to be decreased in the spleens of GVH-reactive mice[23], it has not been shown to

be involved in the formation of cutaneous lesions. It has been reported that local transfer of T cells into murine skin can partially overcome the requirement for irradiation in the formation of cutaneous lesions;[6] in this model, the facilitating role of ICAM-1 is obviated. However, the severity of the cutaneous GVHD fails to attain that seen in irradiated skin, suggesting that radiation effects such as keratinocyte damage and local macrophage activation contribute to the process. Finally, irradiation disregulates cutaneous immunity by destroying Langerhans cells, which normally carry antigens to the regional lymph nodes,[29] and dendritic epidermal cells, which may regulate αß T cell responses[30, 31] and mediate local suppression.[32] Thus, the radiation-induced depletion of these regulatory and suppressive cell types may prevent the normal translocation of potentially harmful immune responses to the lymph nodes, instead allowing them to proceed in the epidermis where the keratinocytes are harmed directly or by bystander effects.

Clinically, cutaneous lesions have been used in the diagnosis and grading of GVHD, and attempts have been made to correlate the severity of cutaneous GVHD with prognosis.[33] The present findings suggest that radiation treatment, as well as potential differences in radiosensitivity between individuals,[34, 35] should be taken into account when considering the grade of cutaneous GVHD for diagnostic or prognostic purposes.

Acknowledgments

We thank Ailsa Lee Loy and Rosmarie Siegrist-Johnstone for their expert technical assistance.

References

1. Lapp WS, Ghayur T, Mendes M, Seddik M, Seemayer TA: The functional and histological basis for graft-versus-host-induced immunosuppression. Immunol Rev 1985, 88: 107-133

2. Pietryga D: Prevention and treatment of acute graft-vs.-host disease. Am J Pediat Hematol-Oncol 1993, 15:28-48

3. Nestel FP, Price KS, Seemayer TA, Lapp WS: Macrophage priming and lipopolysaccharide-triggered release of tumor necrosis factor α during graft-versus-host disease. J Exp Med 1992, 175:405-413

4. Korngold R: Biology of graft-vs.-host disease. Am J Pediat Hematol-Oncol 1993, 15:18-27

5. Darmstadt GL, Donnenberg AD, Vogelsang GB, Farmer ER, Horn TD: Clinical, laboratory, and histopathologic indicators of the development of progressive acute graft-versus-host disease. J Invest Dermatol 1992, 99:397-402

6. Kawai K, Matsumoto Y, Watanabe H, Ito M, Fujiwara M: Induction of cutaneous

graft-versus-host disease by local injection of unprimed T cells. Clin Exp Immunol 1991, 84:359-366

7. Zwaan FE, Jansen J, Noordijk EM: Graft-versus-host disease limited to area of irradiated skin. Lancet 1980, 1:1081-1082

8. Socie G, Gluckman E, Cosset JM, Devergie A, Girinski T, Esperou H, Dutreix J: Unusual localization of cutaneous chronic graft-versus-host disease in the radiation fields in four cases. Bone Marrow Transplant 1989, 4:133-135

9. Claman HN, Spiegelberg HL: Immunoglobulin dysregulation in murine graft-vs-host disease: a hyper-IgE syndrome. Clin Immunol Immunopathol 1990, 56:46-53

10. Bliss JQ: The development of a congenic pair from the A and CBA strains of mice. Transplantation 1965, 3:730-736

11. Lapp WS, Bliss JQ: Significance of allelic dosage and graft size on tolerance induction by H-1-incompatible skin grafts. Transplantation 1970, 10:389-395

12. Cunningham AJ, Szenberg A: Further improvements in the plaque technique for detecting single antibody-forming cells. Immunology 1968, 14:599-600

13. Parthenais E, Lapp WS: Evidence for two types of nonspecific suppressor cells activated by the graft-versus-host reaction in mice. Scand J Immunol 1978, 7:215-220

14. LeBoit PE: Subacute radiation dermatitis: a histologic imitator of acute cutaneous graft-versus-host disease. J Am Acad Dermatol 1989, 20:236-241

15. Seed TM, Niiro GK: Alpha-particle irradiation-induced change in bronchopulmonary macrophage morphology, in vitro. Scanning Microsc 1991, 5:565-71

16. Wasserman J, Petrini B, Wolk G, Vedin I, Glas U, Blomgren H, Ekre HP, Strannegard O: Cytokine release from mononuclear cells in patients irradiated for breast cancer. Anticancer Res 1991, 11:461-464

17. Duerst R, Werberig K: Cells of the J774 macrophage cell line are primed for antibody-dependent cell-mediated cytotoxicity following exposure to gamma-irradiation. Cell Immunol 1991, 136:361-372

18. Elzer PH, Rowe GE, Enright FM, Winter AJ: Effects of gamma radiation and azathioprine on Brucella abortus infection in BALB/c mice. Am J Vet Res 1991, 52:838-844

19. Rubin P, Finkelstein J, Shapiro D: Molecular biology mechanisms in the radiation induction of pulmonary injury syndromes: interrelationship between the alveolar macrophage and the septal fibroblast. Int J Radiat Oncol, Biol, Phys 1992, 24:93-101

20. Leist TP, Heuchel R, Zinkernagel RM. Increased bactericidal macrophage activity induced by immunological stimuli is dependent on interferon-γ. Interference of anti-IFN-γ but not anti-IFN-αß with modulation of macrophage activity caused by lymphocytic choriomeningitis virus infection or systemic graft-*vs.*-host reactions. Eur J Immunol 1988, 18: 1295-1298

21. Krutmann J, Kock A, Schauer E, Parlow F, Moller A, Kapp A, Forster E, Schopf E, Luger TA: Tumor necrosis factor-ß and ultraviolet radiation are potent regulators of human keratinocyte ICAM-1 expression. J Invest Dermatol 1990, 95:127-131

22. Norton J, Sloane JP: ICAM-1 expression on epidermal keratinocytes in cutaneous graft-versus-host disease. Transplantation 1991, 51:1203-1206

23. Allen RD, Staley TA, Sidman CL: Differential cytokine expression in acute and chronic murine graft-versus-host-disease. Eur J Immunol 1993, 23:333-337

24. Girolomoni G, Pincelli C, Zambruno G, Andreani M, Giardini C, Lucarelli G, Giannetti A: Immunohistochemistry of cutaneous graft-versus-host disease after allogeneic bone marrow transplantation. J Dermatol 1991, 18:314-323

25. Kasten-Sportes C, Masset M, Varrin F, Devergie A, Gluckman E: Phenotype and function of T lymphocytes infiltrating the skin during graft-versus-host disease following allogeneic bone marrow transplantation. Transplantation 1989, 47:621-624

26. Dickinson AM, Sviland L, Dunn J, Carey P, Proctor SJ: Demonstration of direct involvement of cytokines in graft-versus-host reactions using an in vitro human skin explant model. Bone Marrow Transplant 1991, 7:209-216

27. Rowbottom AW, Norton J, Riches PG, Hobbs JR, Powles RL, Sloane JP: Cytokine gene expression in skin and lymphoid organs in graft versus host disease. J Clin Pathol 1993, 46:341-345

28. Cheng J, Turksen K, Yu QC, Schreiber H, Teng M, Fuchs E: Cachexia and graft-vs.-host-disease-type skin changes in keratin promoter-driven TNF-α transgenic mice. Genes Dev 1992, 6:1444-1456

29. Zambruno G, Girolomoni G, Manca V, Andreani M, Galimberti M, Lucarelli G, Giannetti A: Epidermal Langerhans cells after allogeneic bone marrow transplantation: depletion by chemotherapy conditioning regimen alone. J Cutaneous Pathol 1992, 19:187-192

30. Ferrick DA, Sydora B, Wallace V, Gemmell-Hori L, Kronenberg M, Mak TW: Self-reactive γδ T lymphocytes: implications for T-cell ontogeny and reactivity. Immunol Rev 1991, 120:51-69

31. Ptak W, Askenase PW: γδ T cells assist αβ T cells in adoptive transfer of contact sensitivity. J Immunol 1992, 149:3503-3508

32. Welsh EA, Kripke ML: Murine Thy-1$^+$ dendritic epidermal cells induce immunologic tolerance in vivo. J Immunol 1990, 144:883-891

33. Sviland L, Pearson AD, Green MA, Baker BD, Eastham EJ, Reid MM, Hamilton PJ, Proctor SJ, Malcolm AJ: Immunopathology of early graft-versus-host disease – a prospective study of skin, rectum, and peripheral blood in allogeneic and autologous bone marrow transplant recipients. Transplantation 1991, 52:1029-1036

34. Geara FB, Peters LJ, Ang KK, Wike JL, Sivon SS, Guttenberger R, Callender DL, Malaise EP, Brock WA: Intrinsic radiosensitivity of normal human fibroblasts and lymphocytes after high- and low-dose-rate irradiation. Cancer Res 1992, 52:6348-6352

35. Tucker SL, Turesson I, Thames HD: Evidence for individual differences in the radiosensitivity of human skin. Eur J Cancer 1992, 28A:1783-1791

CHAPTER 5.

Conclusions and Discussion

1. Summary of results.

The results presented in this thesis address some of the mechanisms and processes by which GVHR induces T cell functional abnormalities.

The studies herein demonstrate that the early T cell signalling defect of GVHR is associated with a decrease in the levels of $p56^{lck}$ and $p59^{fyn}$, protein tyrosine kinases implicated in the TCR signal transduction pathway. This decrease is specific, since the levels of other T cell phosphoproteins do not drop during the course of the reaction. GVHR-induced hyperproduction of endogenous glucocorticoids appears to provoke the decrease in T cell lck and fyn levels, since mice adrenalectomized prior to GVHR induction are protected from this reduction, and normal (non GVH-reactive) mice injected with cortisone experience a similar drop in T cell lck and fyn. The glucocorticoid-mediated reduction in T cell PTKs can be demonstrated *in vitro* in T cell clones, in the absence of other cell types, and can be prevented by addition of the anti-glucocorticoid RU 38484 to the cultures. Thus, the glucocorticoids appear to induce lck and fyn downregulation directly by binding T cell glucocorticoid receptors.

Further studies indicate that the GVHR leads to a decrease in class II MHC expression in the thymus; concurrently, abnormal positive and negative T cell

selection are observed. The decrease in thymic class II may result from GVHR-induced injury and eventual disappearance of most thymic epithelial cells, and from a modulation of surface class II expression, which has been reported to occur in response to glucocorticoids (1). The thymocyte abnormalities accompanying this pathological decrease in thymic MHC class II mimick those reported in class II and invariant chain knockout mice (2, 3), suggesting that the GVHR-induced downregulation in thymic MHC class II might be directly responsible for the altered thymocyte phenotype. These alterations include reduced numbers of $CD4^+$ cells in the thymus and periphery, reduced expression of TCR/CD3 on $CD4^+CD8^-$ thymocytes, abnormal and inconsistent TCR Vß-segment usage, and the appearance of phenotypically autoreactive thymocytes.

Finally, results show that local irradiation increases the frequency and severity of GVHR-induced epithelial lesions. Since the irradiation was limited to the target tissue in these experiments, the exacerbation of tissue injury could not be attributed to the elimination of circulating radiosensitive host suppressor cells able to minimize donor-induced histopathology, nor to any other systemic radiation-induced changes in the host. However, the injury resulting from the interaction of local irradiation and systemic GVHR was observed specifically in tissue allogeneic to the donor, whether it was syngeneic or semi-allogeneic to the host, suggesting the involvement of donor T cells in the induction of the

lesions.

2. GVHR induced T cell suppression: a synthesis of results.

Based on the present results, a model may be constructed to demonstrate how the GVHR-induced immunoproliferation and subsequent formation of histopathological lesions contribute to T cell suppression and dysfunction, and ultimately lead to the third phase of the reaction, chronic GVHR.

Immunoproliferation

When the donor T cells are injected into the semi-allogeneic recipient, they are confronted by an array of foreign MHC antigens (as well as shared MHC haplotypes carrying peptides derived from allogeneic proteins) which immediately stimulates them to proliferate and produce cytokines. Host antigen presenting cells (APCs) interact with donor T cells, stimulating cytokine production, in particular IL-1, which increases up to 100-fold early in GVHR (4). The increased secretion of IL-1 activates the hypothalamic-pituitary-adrenal (HPA) axis, resulting in elevated serum cortisol levels (5, 6). Concurrently, the donor T cells undergo massive proliferation and differentiate into effector cells, including CTLs; and secrete cytokines such as IFN-γ, which

upregulate adhesion molecules and MHC antigens on lymphoid, epithelial and endothelial cells, and prime macrophages and NK cells.

Induction of pathology

In the spleen, (to which the intravenously injected cells have direct access), the expanded population of activated donor CTLs may directly kill the host lymphocytes, resulting in the profound B- and T-lymphopenia characteristic of GVHR (7, 8). Other donor T cells may migrate from the circulatory system into the tissues, and initiate epithelial lesions through CTL effector function or via the secretion of cytotoxic cytokines such as TNF-α (9). If the host has been irradiated, which is usually the case in clinical BMT (10), the T cell-induced epithelial lesions, which also involve the GI tract and the thymus, are likely to be more numerous and more severe. The GI injury results in the entry of bacterial LPS, which triggers primed macrophages to produce TNF-α, thus exacerbating the tissue damage (11). Furthermore, the IL-1 secreted by the continuously LPS-activated macrophages results in sustained, abnormally high serum glucocorticoid levels (5, 6).

The T cell signalling defect

After the initial proliferative burst, most of the T cells in the peripheral lymphoid organs are donor cells which have expanded in response to host alloantigens, since many of the host T cells will have been eliminated by donor anti-host CTL activity, and the host-unresponsive donor cells will not have expanded, and thus will constitute only a minority of the remaining T cells (8). Then, about 10 days after induction of the reaction, these cells begin to become unresponsive or to die. Activation-induced cell death is a well documented phenomenon, which has been reported after *in vivo* superantigen stimulation and *in vitro* activation by TCR/CD3 cross linking (12 - 14). Thus, the elimination of donor T cells following the immunoproliferative phase of the GVHR may represent activation-induced cell death, perhaps exacerbated by interferon production (15). Other T cells survive; the reasons for the survival of some cells and the death of others, when both have been activated, is poorly understood. It may depend on several factors, including whether the cell is memory or naive, the microenvironment in which it was activated, and the strength of the activating stimulus (16).

The surviving T cells become anergic. They are profoundly unresponsive as a consequence of glucocorticoid-induced downregulation of lck and fyn, the tyrosine kinases required for signal transduction through the TCR. In adrenalectomized animals, a transient T cell immunosuppression is observed; however, it can be attributed to the production of IFN-γ from the T cells

themselves, and to macrophage-derived prostaglandins and NO, all of which have been shown to mediate immunosuppression (17 - 19). The recovery of immune function in adrenalectomized mice occurs early (20, and K.E. You-Ten *et al*, manuscript in preparation), unlike non-ADX animals, which remain profoundly immunosuppressed for months or years (21 - 23). Thus, in animals with intact HPA axes, a T cell signalling defect consisting of an interruption of the TCR signalling cascade at the level of tyrosine phosphorylation is induced by glucocorticoids, resulting in peripheral T cell unresponsiveness.

Late immunological abnormalities

During engraftment following clinical BMT, donor-derived T cell precursors from the host bone marrow begin to recolonize the thymus. The thymus, however, has undergone considerable damage as a consequence of GVHR, including destruction of much of the epithelial stroma (24, 25). By analogy with cutaneous epithelial injury, if the patient has received total body irradiation (TBI) prior to transplantation, the GVHR-induced thymic epithelial lesions may be more severe. The T cell precursor developing in the GVHR-dysplastic thymus mature in an abnormal microenvironment, characterized by a deficiency in MHC class II expression. As in class II MHC and invariant chain knockout mice, the lack of class II MHC results in phenotypic abnormalities in the developing thymocytes (2, 3). Since thymocytes pass

through a $CD4^+8^+$ double positive stage in the course of their maturation into single positive $CD4^+$ or $CD8^+$ T cells, the absence of class II MHC may result in functional abnormalities in both subsets. The CD4-class II MHC interaction in $CD4^+8^+$ thymocytes is involved in the *in vivo* repression of CD3 expression (26, 27). In the absence of class II MHC inhibition, CD3 may be upregulated early. This would allow the thymocytes to receive signals through CD3 at an earlier developmental stage than normal (28), which may lead to later functional abnormalities in both CD4 and CD8 subsets. As in class II knockout mice, however, the class II deficiency during GVHR appears to affect $CD4^+$ thymocytes more profoundly than $CD8^+$ cells. We have previously shown that the loss of $CD4^+$ thymocytes in GVH-reactive mice is a consequence of the inability of this subset to acquire cortisone resistance during GVHR (29); this functional defect may be a consequence of their failure to interact with, and be positively selected by, class II MHC molecules. The present findings also show that $CD4^+8^-$ thymocytes express abnormally low levels of CD3, indicating impaired positive selection; and some mature thymocytes express "forbidden", phenotypically autoreactive TCR-Vß segments, suggesting defective negative selection. These abnormal T cells then reconstitute the T cell depleted periphery. Thus, the chronic immune abnormalities of late GVHR may result from the inability of the GVHR-dysplastic thymus to reconstitute the periphery with normal T cells, resulting in dysfunctional cell-mediated immunity even once the production of non-specific immunosuppressive factors by macrophages

and by the endocrine system has ceased.

In suppressive P --> F1 GVHR, overt peripheral self-reactivity is not generally observed. However, allo-specific T-cell induced lesions are exacerbated by irradiation; and CsA-induced autoimmunity is observed only after irradiation (30). By analogy, autoimmune disease following thymic damage in this GVHR model may manifest itself only after irradiation of the host. In fact, a recent report demonstrates that the phenotypically autoreactive thymocytes expand when transferred into irradiated hosts (31). Furthermore, most patients undergoing chronic GVHR characterised by autoimmune-like syndromes have been irradiated (10). It would be of interest to determine if the T cells generated in the thymuses of class II knockout mice, whose phenotype is similar to that of GVH-reactive thymocytes, would be capable of inducing autoimmunity when transferred into an irradiated host.

3. Clinical implications.

GVHR is a significant clinical problem, leading to considerable morbidity and mortality. The model generated by the present results suggests a re-evaluation of the current treatment strategies.

Standard preventative therapy includes CsA and prednisone (or other glucocorticoids) (32). CsA is effective in preventing GVHR; however, it can in itself induce a GVHR-like syndrome in the post-CsA period, resulting in long term morbidity (30, 33). The present results suggest that shielding the thymus during TBI may reduce the severity of thymic epithelial lesions, leading to faster healing and thus fewer long-term T cell abnormalities. This could be combined with pulmonary shielding, already used clinically to decrease the incidence of pneumonia after BMT (34). Prednisone treatment may contribute to the early T cell signalling defect; however, it seems preferable than CsA since it is not known to produce long-term immunological abnormalities. Both of these immunosuppressive therapies, however, merely hold the reaction in abeyance, without actually eliminating the alloreactive donor cells; when the drugs are tapered or discontinued, even months after induction of the reaction, an "acute" GVHR may appear. Another currently used approach involves blocking the pathological effects of TNF-α with antibodies, thalidomide, or pentoxyfylline (35 - 38). This constitutes a downstream interruption in the cycle of tissue damage, and is perhaps less effective than a more upstream block; clinical results have been mixed (37, 38).

An effective preventative or therapeutic scheme must result in the prevention of tissue injury and immunosuppression, without loss of engraftment and GVL activity. The early cytokine burst seems to be the trigger for all subsequent

pathology; IL-1 induces glucocorticoid production and hence the T cell signalling defect, and IFN-γ primes macrophages for eventual TNF-α secretion and activates NK cells. Thus, blocking IL-1 and IFN-γ may limit the extent of tissue injury and T cell suppression; and since the cascade would be interrupted upstream of LPS translocation and macrophage TNF-α secretion, death from septic shock would be precluded. Blocking both IL-1 and IFN-γ production would provide flexible control over the extent of T cell activation, while still preventing downstream pathology; this has two main advantages. First, since pathology downstream of IFN-γ secretion could be prevented, the reaction could be permitted to run a controlled course, resulting in short-lived, cytokine-mediated pathology similar to that encountered during exogenous IL-2 administration. Most of the alloreactive cells may then succumb to activation-induced cell death, precluding the need for long term pharmacologic immunosuppressive therapy. Second, the IL-1 antagonist would be expected to effectively block long-range interaction such as activation of the HPA axis, while being less efficient at preventing proximate interactions such as signals between the T cell and the APC. T cell function, then, would be preserved, allowing the GVL effects to protect the recipient from leukemic relapse, and the production of cytokines such as IL-3 and GM-CSF to promote engraftment. The activation of the HPA axis may be safely blocked by antagonising IL-1, since the potentially dangerous secretion of IFN-γ, normally downregulated by glucocorticoids, would be controlled by treatment with an IFN-γ blocker. This

dual therapy gives control over two reciprocal mediators of the feedback cycle: a macrophage product important in T cell activation; and a T cell cytokine involved in macrophage priming. Hence, it provides a redundant means to break a positive feedback loop. Furthermore, soluble cytokine receptors may be used as antagonists, in order to prevent sensitization of the host to foreign proteins such as antibodies derived from other species, allowing several cycles of therapy in refractory cases (39).

This type of strategy attempts to control and modulate an essentially beneficial cytokine response, instead of using overwhelming pharmacologic means which create a destructive, long term disruption of immune function.

4. Insight into immune function.

Negative feedback regulation of the immune system by the HPA axis is well documented. The immune system activates the HPA axis via IL-1 secretion, resulting in increased serum glucocorticoid levels (5, 6); in turn, the glucocorticoids downregulate the production of cytokines and inflammatory mediators by monocytes and lymphocytes (40). The studies presented here suggest that glucocorticoids also act directly on T cells, regulating their level of activation through the TCR by downregulating its associated PTKs. Glucocorticoids have been reported to prevent signalling through the IL-2

receptor pathway, also by a mechanism involving reduced tyrosine phosphorylation (41); since lck normally associates with the IL-2 receptor (42) as well as with CD4, the glucocorticoid-mediated downregulation of lck and fyn reported herein may also explain that observation. The feedback loop is completed by the glucocorticoid-induced anti-inflammatory effects resulting in a decrease of IL-1 production, ending the HPA stimulation, and restoring immunological homeostasis.

This physiological downregulation of immune activation, and of T cell function in particular, by glucocorticoids, may be an important factor in the maintainance of immunological homeostasis. In patients with adrenal hypoactivity (hypocortisolism), IL-2 production in response to mitogen stimulation *in vitro* is constitutively increased; conversely, in Cushing's disease (adrenal hyperactivity), IL-2 production is decreased (43). Further evidence for a homeostatic role for glucocorticoids in T cell signalling is provided by strains of animals or groups of patients in which the HPA-T cell feedback cycle is interrupted at various points, resulting in autoimmune disease. There are several examples of abnormally low responsivenss of the hypothalamus or pituitary to IL-1, associated with autoimmune pathology. Obese strain (OS) chickens, which develop spontaneous autoimmune thyroiditis, have an aberrant, decreased HPA response to IL-1 (44, 45). A similar defect has been reported in Fisher rats, which are susceptible to the induction of a variety of

autoimmune diseases including erosive polyarthritis and experimental autoimmune encephalitis (EAE) (45). Lupus prone (NZB x NZW F1) mice may have a defect in brain IL-1 receptor expression, again resulting in an impaired HPA response to immune activation (45). In other cases, the feedback cycle is abnormal at the level of adrenal responsiveness: UCD-200 chickens, which develop a spontaneous scleroderma-like disorder, require twice the normal levels of pituitary adrenocorticotropin (ACTH) to trigger production of glucocorticoids by the adrenals (44). Furthermore, the feedback cycle may be interrupted artificially at this level, by removing the adrenals; rat strains normally resistant to the induction of EAE become susceptible after adrenalectomy (45). Finally, the T cells themselves may be hyporesponsive to glucocorticoids. This appears to be the case in autoimmune rheumatoid arthritis, in which patients' T cells express fewer than normal glucocorticoid receptors (46). The observation of overt, T cell mediated autoimmunity in situations where this feedback loop is disrupted suggests that the modulation of T cell tyrosine kinase signal transduction by glucocorticoids may play a role in the induction or maintainance of peripheral tolerance.

Apart from their role in constitutive regulation of T cell function, glucocorticoids are critical in the response to massive immune activation. By downregulating Th1 type cytokines including IFN-γ and TNF-α, glucocorticoids can prevent lethal septic shock induced by bacterial products such as

endotoxins and enterotoxins. Furthermore, the results presented here suggest that glucocorticoids also directly induce T cell unresponsiveness to proliferative signals; when a large proportion of the T cell repertoire is stimulated to proliferate and secrete interleukins, such as during GVHR or *in vivo* exposure to bacterial superantigens (enterotoxins), glucocorticoids moderate the extent of T cell activation. In the absence of this negative regulation, for example in mice treated with the anti-glucocorticoid RU 38486 or in adrenalectomized mice, the massive T cell activation results in high mortality (47, 48). Anergizing the T cells by interrupting their signal transduction cascade allows the cells to remain present, but unresponsive; thus, they do not represent an immediate threat to survival during immune hyperactivation, and yet can be preserved and potentially regain activity after the glucocorticoid levels return to baseline, ensuring a memory response to the antigen which triggered the response. In fact, glucocorticoids have been reported to antagonize activation-induced cell death (49, 50), perhaps facilitating the preservation of a memory response. In situations where glucocorticoid levels are excessively high, more of the activated cells may be eliminated, as exemplified by dose-responsive cell death of activated cells after superantigen stimulation (47). In extreme cases, this might result in an ablation of the memory response (i.e. the induction of peripheral "tolerance" to superantigens after clonal elimination of the responding cells during high dose stimulation), and more generally, may provide an explanation for "high-zone" tolerance.

Thus, in general it appears that the glucocorticoid response triggered by immune activation, resulting in subsequent downregulation of T cell activity, is beneficial. However, a problem may arise when the HPA axis is triggered from outside the immune system, or as a consequence of chronic, long term immune activation, resulting in inappropriate T cell unresponsiveness. Clinical anergy, or unresponsiveness to delayed type hypersensitivity (DTH) testing, is a well-documented phenomenon during mycobacterial infections such as tuberculosis (TB) (51, 52). Interestingly, high serum cortisol and abnormalities of the diurnal cortisol cycle, similar to those seen in GVH-reactive animals, have been reported in TB patients (53, 54). Similarly, in severely malnourished patients suffering from a variety of disorders including anorexia nervosa (55), marasmus (56), and cancer (57), T cell immunosuppression and elevated cortisol levels are also seen. These observations suggest that the glucocorticoid-dependent downregulation of lck and fyn, resulting in T cell unresponsiveness, may be a widespread clinical phenomenon contributing to the morbidity of many disparate conditions. In fact, downregulation of lck and fyn has been reported in an experimental model of tumor-induced immunosuppression (58). In these situations, anti-glucocorticoid therapy may be beneficial in restoring immune function. TB patients, for example, may mount a more effective anti-mycobacterial response if their T cell unresponsiveness was reversed. However, therapy with systemic glucocorticoid antagonists such as RU 38486 would also block the beneficial

effects of glucocorticoids on non-immune tissues; an alternative approach would be treatment with dehydroepiandosterone (DHEA), a steroid for which T cells have specific receptors, and which efficiently antagonizes glucocorticoid effects (59).

In fact, the endogenous ratio of glucocorticoids to DHEA has been implicated in the regulation of Th1 versus Th2 responses (59). A predominance of glucocorticoids tends to downregulate Th1 type responses. Exogenous DHEA, then, may restore the balance in animals or patients subjected to chronic stress, and reestablish DTH responsiveness. Perhaps, then, a genetically determined relative susceptibility of T cells to the effect of glucocorticoids may account for the striking strain-dependent dichotomy of Th1 versus Th2 type responses in murine leishmania (60), and in suppressive versus proliferative GVHR (61). It is interesting (though likely merely coincidental) that the locus which determines the proliferative versus suppressive outcome of GVHR (62), and a locus regulating the gene transcriptional response to glucocorticoids (63), are each located at unknown points on murine chromosome 7. Furthermore, there is clinical evidence suggesting genetic differences in T cell glucocorticoid responsiveness; in steroid-resistant asthma, patients' T cells express abnormally low levels of glucocorticoid receptors when they are cultured in medium supplemented with IL-2 and IL-4 (64). This phenotype may represent an adaptive response of the immune system to stress in certain genotypes,

permitting, for example, anti-parasitic Th2-like responses despite the suppression of potentially life-threatening Th-1 type responses.

Thus, results obtained in studies of GVHR-induced T cell immunosuppression have revealed general mechanisms in the regulation of T cell signalling and T cell education. These findings have lead to a unifying model of GVHR-induced T cell abnormalities, and of course, give rise to new questions, in particular concerning the regulation of T cell function by glucocorticoids.

5. References

1. Celada, A., S. McKercher, and R. A. Maki. 1993. Repression of major histocompatibility complex IA expression by glucocorticoids: the glucocorticoid receptor inhibits the DNA binding of the X box DNA binding protein. *J. Exp. Med.* 177: 691.

2. Cosgrove, D., D. Gray, A. Dierich, J. Kaufman, M. Lemeur, C. Benoist, and D. Mathis. 1991. Mice lacking MHC class II molecules. *Cell* 66: 1051.

3. Viville, S., J. Neefjes, V. Lotteau, A. Dierich, M. Lemeur, H. Ploegh, C. Benoist, and D. Mathis. 1993. Mice lacking the MHC class II-associated invariant chain. *Cell* 72: 635.

4. Abhyankar, S., D. G. Gilliland, and J. L. Ferrara. 1993. Interleukin-1 is a critical effector molecule during cytokine dysregulation in graft versus host disease to minor histocompatibility antigens. *Transplantation* 56: 1518.

5. Kakucska, I., Y. Qi, B. D. Clark, and R. M. Lechan. 1993. Endotoxin-induced corticotropin-releasing hormone gene expression in the hypothalamic paraventricular nucleus is mediated centrally by interleukin-1. *Endocrinology* 133: 815.

6. Tilders, F. J., R. H. DeRijk, A. M. Van Dam, V. A. Vincent, K. Schotanus, and J. H. Persoons. 1994. Activation of the hypothalamus-pituitary-adrenal axis by bacterial endotoxins: routes and intermediate signals. *Psychoneuroendocrinology* 19: 209.

7. Xenocostas, A., D. G. Osmond, and W. S. Lapp. 1991. The effect of the graft-versus-host reaction on B lymphocyte production in bone marrow of mice. Depressed genesis of early progenitors prior to mu heavy chain expression. *Transplantation* 51: 1089.

8. Hakim, F. T., S. O. Sharrow, S. Payne, and G. M. Shearer. 1991. Repopulation of host lymphohematopoietic systems by donor cells during graft-versus-host reaction in

unirradiated adult F1 mice injected with parental lymphocytes. *J. Immunol.* 146: 2108.

9. Piguet, P. F., G. E. Grau, B. Allet, and P. Vassalli. 1987. Tumor necrosis factor/cachectin is an effector of skin and gut lesions of the acute phase of graft-vs.-host disease. *J. Exp. Med.* 166: 1280.

10. Copelan, E. A. 1992. Conditioning regimens for allogeneic bone marrow transplantation. *Blood Rev.* 6: 234.

11. Nestel, F. P., K. S. Price, T. A. Seemayer, and W. S. Lapp. 1992. Macrophage priming and lipopolysaccharide-triggered release of tumorr necrosis factor alpha during graft-versus-host disease. *J. Exp. Med.* 175: 405.

12. Kabelitz, D., T. Pohl, and K. Pechhold. 1993. Activation-induced cell death (apoptosis) of mature peripheral T lymphocytes. *Immunol. Today* 14: 338.

13. King, L. B. and J. D. Ashwell. 1993. Signaling for death of lymphoid cells. *Current Opinion Immunol.* 5: 368.

14. Gonzalo, J. A., E. Baixeras, A. Gonzalez-Garcia, A. George-Chandy, N. Van Rooijen, C. Martinez, and G. Kroemer. 1994. Differential in vivo effects of a superantigen and an antibody targeted to the same T cell receptor. Activation-induced cell death vs passive macrophage-dependent deletion. *J. Immunol.* 152: 1597.

15. Liu, Y., and C.A. Janeway. 1990. Interferon γ plays a critical role in induced cell death of effector T cell: a possible third mechanism of self-tolerance. *J. Exp. Med.* 172: 1735.

16. Akbar, A. N., M. Salmon, J. Savill, and G. Janossy. 1993. A possible role for bcl-2 in regulating T-cell memory--a 'balancing act' between cell death and survival.

Immunol. Today 14: 526.

17. Klimpel, G. R., C. R. Annable, M. G. Cleveland, T. R. Jerrells, and J. C. Patterson. 1990. Immunosuppression and lymphoid hypoplasia associated with chronic graft versus host disease is dependent upon IFN-gamma production. *J. Immunol.* 144: 84.

18. Lapp, W.S., M. Mendes, H. Kirchner, and D. Gemsa. 1980. Prostaglandin synthesis by lymphoid tissue of mice experiencing a graft-versus-host-reaction: relationship to immunosuppression. *Cell. Immunol.* 50: 271.

19. Hoffman, R. A., J. M. Langrehr, S. M. Wren, K. E. Dull, S. T. Ildstad, S. A. McCarthy, and R. L. Simmons. 1993. Characterization of the immunosuppressive effects of nitric oxide in graft vs host disease. *J. Immunol.* 151: 1508.

20. Kaplan, H.S., and B.H. Rosston. 1959. Studies on a wasting disease induced in F1 hybrid mice injected with parental strain lymphoid cells. *Stanford Med. Bull.* 17: 77.

21. Lapp, W.S., T. Ghayur, M. Mendes, M. Seddik, and T.A. Seemayer. 1985. The functional and histological basis for graft-versus-host-induced immunosuppression. *Immunol. Rev.* 88: 107.

22. Witherspoon, R.P., D. Matthews, R. Storb, K. Atckinson, M. Cheever, H.J. Deeg, K. Doney, J. Kalbfleisch, D. Noel, and R. Prentice. 1984. Recovery of in vivo cellular immunity after human marrow grafting. Influence of time postgrafting and acute graft-versus-host disease. *Transplantation* 37: 145.

23. D'Andrea, A. D., E. Bayever, K. M. Haines, R. W. Wilmott, R. A. Polin, C. S. August, and S. D. Douglas. 1986. Serial evaluation of lymphocyte function in bone marrow-grafted patients. *Diagnostic Immunol.* 4: 281.

24. Seddik, M., T.A. Seemayer, and W.S. Lapp. 1980. T cell functional defect

associated with thymic epithelial cell injury induced by a graft-versus-host reaction. *Transplantation* 29: 61.

25. Seemayer, T.A., W.S. Lapp, and R.P. Bolande. 1977. Thymic involution in murine graft-versus-host reaction. Epithelial injury mimicking human thymic dysplasia. *Am. J. Pathol.* 88: 119.

26. Bonifacino, J. S., S. A. McCarthy, J. E. Maguire, T. Nakayama, D. S. Singer, R. D. Klausner, and A. Singer. 1990. Novel post-translational regulation of TCR expression in CD4+CD8+ thymocytes influenced by CD4. *Nature* 344: 247.

27. Nakayama, T., C. H. June, T. I. Munitz, M. Sheard, S. A. McCarthy, S. O. Sharrow, L. E. Samelson, and A. Singer. 1990. Inhibition of T cell receptor expression and function in immature CD4+CD8+ cells by CD4. *Science* 249: 1558.

28. Nakayama, T., L. E. Samelson, Y. Nakayama, T. I. Munitz, M. Sheard, C. H. June, and A. Singer. 1991. Ligand-stimulated signaling events in immature CD4+CD8+ thymocytes expressing competent T-cell receptor complexes. *Proc. Natl. Acad. Sci. USA* 88: 9949.

29. Kornbluth, M., E. You-Ten, J. Desbarats, S. Gamache, A. Xenocostas, and W.S. Lapp. 1991. T cell subsets in the thymus of graft-versus-host immunosuppressed mice. Sensitivity of the L3T4$^+$Lyt-2$^-$ subset to cortisone. *Transplantation.* 51: 262.

30. Beschorner, W.E., K.A. Kelly, A.D. Hess, and G.W. Santos. 1987. Cyclosporine and the thymus: influence of irradiation and age on thymic immunopathology and recovery. *Cell. Immunol.* 110: 350.

31. Hollander, G.A., B. Widmer, and S.J. Burakoff. 1994. Loss of normal thymic repertoire selection and persistence of autoreactive T cells in graft vs host disease. *J. Immunol.* 152: 1609.

32. Schwinghammer, T. L. and E. J. Bloom. 1993. Pharmacologic prophylaxis of acute graft-versus-host disease after allogeneic marrow transplantation. *Clin. Pharm.* 12: 736.

33. Carella, A. M., E. Gaozza, A. Congiu, P. Carlier, S. Nati, M. Truini, A. Castellaneta, and M. Viale. 1991. Cyclosporine-induced graft-versus-host disease after autologous bone marrow transplantation in hematological malignancies. *Ann. Hematol.* 62: 156.

34. Weshler, Z., R. Breuer, R. Or, E. Naparstek, M. R. Pfeffer, E. Lowental, and S. Slavin. 1990. Interstitial pneumonitis after total body irradiation: effect of partial lung shielding. *Br. J. Haematol.* 74: 61.

35. Herve, P., M. Flesch, P. Tiberghien, J. Wijdenes, E. Racadot, P. Bordigoni, E. Plouvier, J. L. Stephan, H. Bourdeau, and E. Holler. 1992. Phase I-II trial of a monoclonal anti-tumor necrosis factor alpha antibody for the treatment of refractory severe acute graft-versus-host disease. *Blood* 79: 3362.

36. Heney, D., D. R. Norfolk, J. Wheeldon, C. C. Bailey, I. J. Lewis, and D. L. Barnard. 1991. Thalidomide treatment for chronic graft-versus-host disease. *Br. J. Haematol.* 78: 23.

37. Bianco, J. A., F. R. Appelbaum, J. Nemunaitis, J. Almgren, F. Andrews, P. Kettner, A. Shields, and J. W. Singer. 1991. Phase I-II trial of pentoxifylline for the prevention of transplant-related toxicities following bone marrow transplantation. *Blood* 78: 1205.

38. Clift, R. A., J. A. Bianco, F. R. Appelbaum, C. D. Buckner, J. W. Singer, L. Bakke, W. I. Bensinger, R. A. Bowden, G. B. McDonald, and M. Schubert. 1993. A randomized controlled trial of pentoxifylline for the prevention of regimen-related toxicities in patients undergoing allogeneic marrow transplantation. *Blood* 82: 2025.

39. Parlevliet, K. J. and P. T. Schellekens. 1992. Monoclonal antibodies in renal transplantation: a review. *Transplant International* 5: 234.

40. Barnes, P. J. and I. Adcock. 1993. Anti-inflammatory actions of steroids: molecular mechanisms. *Trends Pharmacol. Sci.* 14: 436.

41. Paliogianni, F., S.S. Ahuja, J.P. Balow, J.E. Balow, and D.T. Boumpas. 1993. Novel mechanism for inhibition of human T cells by glucocorticoids. Glucocorticoid inhibit signal transduction through IL-2 receptor. *J. Immunol.* 151: 4081.

42. Hatakeyama, M., T. Kono, N. Kobayashi, A. Kawahara, S.D. Levin, R.M. Perlmutter, and T. Taniguchi. 1991. Interaction of the IL-2 receptor with the src-family kinase p56lck: identification of novel intermolecular association. *Science.* 252: 1523.

43. Sauer, J., G. K. Stalla, O. A. Muller, and E. Arzt. 1994. Inhibition of interleukin-2-mediated lymphocyte activation in patients with Cushing's syndrome: a comparison with hypocortisolemic patients. *Neuroendocrinology* 59: 144.

44. Brezinschek, H.P., M. Gruschwitz, R. Sgonc, S. Moormann, M. Herold, M.E. Gershwin, and G. Wick. 1993. Effects of cytokine application on glucocorticoid secretion in an animal model for systemic scleroderma. *J. Autoimmunity* 6: 719.

45. Wick, G., Y. Hu, S. Schwartz, and G. Kroemer. 1993. Immunoendocrine communication via the hypothalamo-pituitary-adrenal axis in autoimmune disease. *Endocrine Rev.* 14: 539.

46. Schlaghecke, R., E. Kornely, J. Wollenhaupt, and C. Specker. 1992. Glucocorticoid receptors in rheumatoid arthritis. *Arthritis & Rheumatism* 35: 740.

47. Gonzalo, J.A., A. Gonzalez-Garcia, C. Martinez-A., and G. Kroemer. 1993. Glucocorticoid-mediated control of the activation and clonal deletion of peripheral T

cells in vivo. *J. Exp. Med.* 177: 1239.

48. Heim, L.R., R.A. Good, and C. Martinez. 1966. Influence of adrenalectomy on homologous disease. *Proc. Soc. Exp. Biol. Med.* 122: 107.

49. Zacharchuk, C.M., M. Mercep, P.K. Chakraborti, S.S. Simons, Jr., and J.D. Ashwell. 1990. Programmed T lymphocyte death. Cell activation- and steroid-induced pathways are mutually antagonistic. *J. Immunol.* 145: 4037.

50. Iseki, R., M. Mukai, and M. Iwata. 1991. Regulation of T lymphocyte apoptosis. Signals for the antagonism between activation- and glucocorticoid-induced death. *J. Immunol.* 147: 4286.

51. Ellner, J.J. 1986. Immune dysregulation in human tuberculosis. *J. Lab. Clin. Med.* 108: 142.

52. Margolis, M.L., and B.L. Van Uitert. 1985. Anergy in tuberculosis. *Biomed. Pharmacother.* 39: 292.

53. Sarma, G. R., C. Immanuel, G. Ramachandran, P. V. Krishnamurthy, V. Kumaraswami, and R. Prabhakar. 1990. Adrenocortical function in patients with pulmonary tuberculosis. *Tubercle* 71: 277.

54. Immanuel, C., G. R. Sarma, P. V. Krishnamurthy, G. Ramachandran, and V. Kumaraswami. 1992. Salivary cortisol in the assessment of adrenocortical function in patients with pulmonary tuberculosis. *Ind. J. Med. Res. Infect. Diseases* 95: 1.

55. Kling, M. A., M. A. Demitrack, H. J. Whitfield,Jr., K. T. Kalogeras, S. J. Listwak, M. D. DeBellis, G. P. Chrousos, P. W. Gold, and H. A. Brandt. 1993. Effects of the glucocorticoid antagonist RU 486 on pituitary-adrenal function in patients with anorexia nervosa and healthy volunteers: enhancement of plasma ACTH and cortisol secretion in underweight patients. *Neuroendocrinology* 57: 1082.

56. Malozowski, S., S. Muzzo, R. Burrows, L. Leiva, L. Loriaux, G. Chrousos, J. Winterer, and F. Cassorla. 1990. The hypothalamic-pituitary-adrenal axis in infantile malnutrition. *Clin. Endocrinol.* 32: 461.

57. Drott, C., G. Svaninger, and K. Lundholm. 1988. Increased urinary excretion of cortisol and chatecholamines in malnourished cancer patients. *Ann. Surgery* 208: 645.

58. Mizoguchi, H., J.J. O'Shea, D.L. Longo, C.M. Loeffler, D.W. McVicar, and A.C. Ochoa. 1992. Alterations in signal transduction molecules in T lymphocytes from tumor-bearing mice. *Science.* 258: 1795.

59. Daynes, R. A., A. W. Meikle, and B. A. Araneo. 1991. Locally active steroid hormones may facilitate compartmentalization of immunity by regulating the types of lymphokines produced by helper T cells. *Res. Immunol.* 142: 40.

60. Heinzel, F. P., M. D. Sadick, S. S. Mutha, and R. M. Locksley. 1991. Production of interferon gamma, interleukin 2, interleukin 4, and interleukin 10 by CD4+ lymphocytes in vivo during healing and progressive murine leishmaniasis. *Proc. Natl. Acad. Sci. USA.* 88: 7011.

61. Gelpi, C., M. A. Martinez, S. Vidal, A. Alguero, C. Juarez, J. A. Hardin, and J. L. Rodriguez-Sanchez. 1990. Different strains of donor parental lymphoid cells induce different models of chronic graft-versus-host disease in murine (Balb/c x A/J)F1 hybrid hosts. *Clin. Immunol. Immunopathol.* 56: 298.

62. Fast, L. D. 1990. Identification of a single non-H-2 gene regulating graft-versus-host disease response. *J. Immunol.* 144: 4177.

63. DeFranco, D., D. Bali, R. Torres, R. A. DePinho, R. P. Erickson, and S. Gluecksohn-Waelsch. 1991. The glucocorticoid hormone signal transduction pathway in mice homozygous for chromosomal deletions causing failure of cell type-specific inducible gene expression. *Proc. Natl. Acad. Sci. USA.* 88: 5607.

64. Sher, E. R., D. Y. Leung, W. Surs, J. C. Kam, G. Zieg, A. K. Kamada, and S. J. Szefler. 1994. Steroid-resistant asthma. Cellular mechanisms contributing too inadequate response to glucocorticoid therapy. *J. Clin. Invest.* 93: 33.