PART I: GENERAL PRINCIPLES

1

Historical Aspects of Chronic Graft versus Host Disease

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The scientific "discovery" of graft versus host disease (GVHD) mirrors some aspects of this devastating disease. It is based on an accurate observation but an error in interpretation. Similarly, GVHD and in particular chronic GVHD confront patients as a paradox. The symptoms appear just when he or she believes to be cured from the previously diagnosed lethal disease, that is, at engraftment of the healthy donor cells. GVHD can have devastating effects on patients, families, and transplant teams. Conversely, few "man made diseases" have provided so much insight into basic immunobiology as GVHD. It is the hope of this book, to turn the knowledge gained from chronic GVHD into benefit for all patients after a hematopoietic stem cell transplant (HSCT) and for the large population of patients with autoimmune disorders or cancer in general.

THE BEGINNING OF HSCT

The old Greek proverb that "war is the father of all goods" applies to few medical technologies as well as it does to HSCT. The recognition of late bone marrow failure from atomic bomb exposure in Hiroshima and Nagasaki had double consequences. Vast research funds were allotted to find tools to overcome this lethal late complication of high dose radiation exposure. Results came rapidly. As early as 1949, Jacobson showed in his pioneering work that mice survived otherwise lethal total body irradiation when their spleens were shielded during radiation exposure [1]. Humoral, spleen-derived products were believed to be protective. Two years later, Lorenz provided proof that cellular, not humoral factors were responsible [2]. He demonstrated that bone marrow cells from a healthy animal, given as intraperitoneal or intravenous injection, could protect mice from radiation-induced bone marrow aplasia (primary disease). The debate whether humoral or cellular factors were key elements to protect bone marrow from the late sequelae of total body radiation was closed for the next 40 years; bone marrow transplantation was born. It is of interest to note that decades later, combinations of growth factors were proven to be radioprotective after total body irradiation even though this approach has never gained clinical application [3].

Clinicians involved in leukemia treatment quickly grasped the unique possibility given to them by the marrow lethal effects of radiation. For the first time, it became possible to "take away" a diseased bone marrow; total body irradiation was the tool. It was only necessary to replace the diseased bone marrow with normal healthy donor cells. Treatment for acute leukemia, a uniformly lethal disease was at hand. With the report by Ford of successful experimental bone marrow transplants in mice, it was evident that donor type cells indeed did repopulate the whole hematopoietic system [4]. It appeared to be only a question of time to find the adequate dose of total body irradiation, sufficient enough to kill all leukemic cells, but low enough not to induce irreversible organ damage outside the bone marrow. The concept was rapidly explored in humans [5]. Bone marrow transplantation spread as a promising investigational tool and more than 60 bone marrow transplants were reported to the International Bone Marrow Transplant Registry in 1962 [6]. However, frustration with the complications of transplantation tempered the initial enthusiasm and bone marrow transplants came to a near halt for more than a decade. Despite successful engraftment and complete remissions in some patients, no long-term survivors were observed. Early, aplasia-associated complications were high. Even worse, those few patients with successful initial engraftment did not survive long term. The transplants were either rejected, their leukemia returned, or patients died from a unique syndrome occurring after engraftment. They developed a generalized rash, became icteric, and suffered from untractable diarrhea. Similar observations were made in mice. Most animals with a bone marrow transplant did survive the initial total body irradiation–induced aplasia (primary disease) but died days to weeks later from a syndrome of wasting, rash, diarrhea,

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and icterus, the "secondary disease." It did take years of intensive laboratory investigations to recognize the major histocompatibility antigens (human leukocyte antigens; HLA) as the key elements for graft rejection and GVHD and to establish the necessary instruments for supportive care to restart successful transplants. It was only in 1968 [7] when the successful bone marrow transplants from HLA-typed and identical sibling donors were performed for patients with severe combined immune deficiency syndromes that brought back HSCT as a viable treatment option.

THE CONCEPT OF GVHD

The first clinical and experimental HSCT were performed at the same time that solid organ transplantation was being developed. Rejection, a host versus graft reaction, was recognized as the first obstacle to success. Simonson in 1953 observed interstitial pyroninophilic lymphoid cells in the renal cortex as early as 6 days after the renal transplant. He postulated that kidney-derived donor cells reacted against host antigens in the renal circulation and brought up for the first time the idea of an immune response generated by the graft [8]. Today it is evident that he described the beginning of rejection. Still, his idea that a transplant could react against the recipient, that a graft versus host reaction could occur in parallel to a host versus graft reaction, was novel, revolutionary at that time, and highly controversial. A few years later, he could prove that GVHD indeed could occur. He injected lymphocytes into newborn mice and into chicken embryos. Both were immunologically naïve and unable to reject the transplanted living lymphocytes. Both, newborn mice and chicken embryos, died soon after the injection of the lymphocytes. These results were proof that his graft generated immunity hypothesis of GVHD was correct [9]. Two facts helped confirm this. First, it was recognized, that the phenomenon of chicken embryo death had been described more than 40 years earlier by Murphy [10]. He had observed lymphoid infiltrates in the spleen of chicken embryos when they were injected at day 7 into the allantois with adult chicken bone marrow or spleen tissue. No such reaction was observed after implantation of goose bone marrow. Chicken embryos were unable to reject chicken spleen cells but were capable of rejecting xenogeneic goose cells. The phenomenon could therefore only be explained as a graft versus host reaction. Second, several transplantation phenomena suddenly had a uniting explanation. Secondary disease after bone marrow transplantation and total body irradiation, acute killing effect, homologous disease, runting disease, parabiosis intoxication, parent versus hybrid effect, or embryo disease all had the same immunologic basis (Figure 1.1) [11–18]. It was Billingham who brought the puzzle together and who stipulated in 1966 the three basic requirements for GVHD [19]: (1) immunocompetent transplanted cells, (2) antigens in the host, which can be recognized by the transplanted cells but are lacking in the donor, and (3) sufficient time of complete engraftment to mount the immune response (security of tenure). These requirements remain valid till date.

THE CONCEPT OF CHRONIC GVHD

In all early clinical transplants before 1968, rejection and acute GVHD were the sole immunological complications. There were no long-term survivors. In the late seventies, several centers observed some peculiar clinical findings in some bone marrow transplant recipients who survived several months after the transplant [20]. These findings occurred when patients began to recover from their acute GVHD. The clinical syndrome of chronic GVHD became clear with the description of four patients from the bone marrow transplant programme at the National Institute of Health in Bethesda, MD, three after a bone marrow transplant for severe aplastic anemia, one for acute myeloid leukemia. [21–23] All had had acute GVHD, grade II to IV. Three patients showed skin changes that were very similar to known autoimmune disorders but different in each: one had scleroderma-like, one lupus erythematodes-like, and one lichen planus-like lesions. Furthermore, all four had a clinical syndrome indistinguishable from Sjögren's disease with a sicca syndrome, oral vasculitis, reduced Schirmer's test, reduced parotid flow, and characteristic lesions in lip biopsies. Furthermore, three patients had restrictive lung disease with reduced CO diffusion capacities and one had combined restrictive and obstructive lung disease. One patient showed liver pathologies similar to primary biliary cirrhosis. These observations came as a surprise at that time. It could not be a chance phenomenon – there were too few long-term survivors for these autoimmune-like changes to be due to chance. It was clear that the phenomenon was related to the transplant procedure and chronic GVHD was brought up as the most likely explanation. Similar observations were reported from other transplant centers and the concept of chronic GVHD in man was established [24–27] (Figure 1.2).

POLITICAL EFFECTS OF CHRONIC GVHD IN THE LATE SEVENTIES

The clarity of the syndrome and its high frequency, the clear correlation with the transplant procedure, and the lack of any meaningful preventive or therapeutic procedure had devastating effects on some transplant programmes. Most importantly, the deterrent pictures of some survivors through a "man-made disease" did have their impact. They did coincide with times, when immediate transplant-related mortality was still higher than survival [23]. This high mortality together with the portrayed idea that all survivors would develop severe complications with high morbidity and mortality was one reason that the bone marrow transplant programme at the National Institutes of Health in the United States was brought to an immediate stop in 1975 and remained closed for more than a decade.

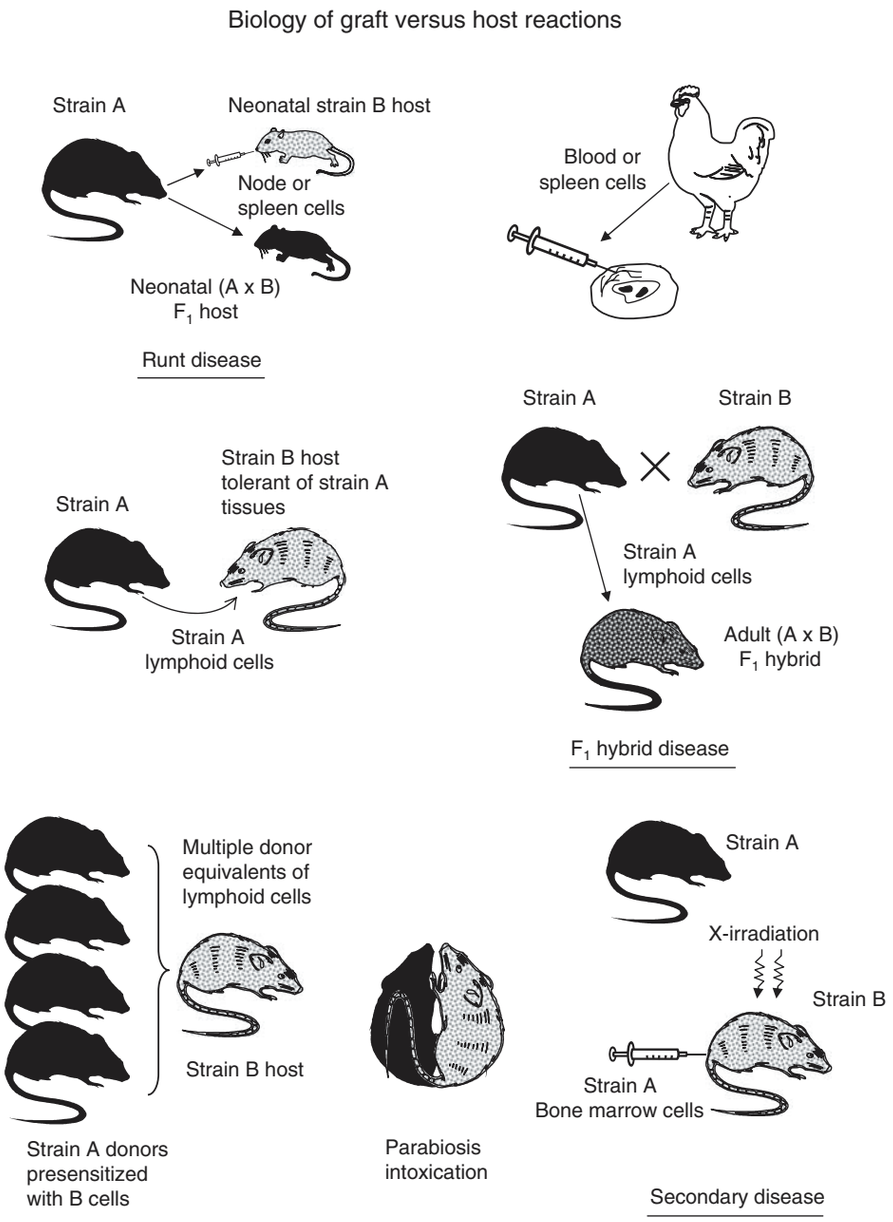

Figure 1.1 Various types of graft versus host reactions in animals, according to a drawing by van Bekkum [28].

Explanations to the illustrations:
Runt disease occurs when a neonatal animal, either strain B or offspring of strain A and B, a so called F_1 hybrid, is injected with lymphoid cells from a lymph node or spleen of type A. The neonatal animal and the neonatal F_1 hybrid cannot reject parental cells but is recognized as foreign by the parental cells. The same occurs when a neonatal (tolerant) animal of strain B is tolerized with a skin graft from an adult strain A and later injected with strain A lymphoid cells.

Adult chicken organ graft on embryo effect is observed, when adult lymphoid cells are injected into the allantois of an egg. The egg cannot reject the adult lymphoid cells; these cells recognize "the egg" as foreign and mount a reaction.

F_1 hybrid disease can occur, similar to runt disease in neonatal mice, but as well in adult F_1 A × B animals, if they are injected with either strain A or B adult lymphoid cells. An F_1 hybrid cannot reject A or B cells but is itself recognized as foreign by either parental A or B cells.

Sensitized multiple donor reaction was observed, when high numbers of lymphoid cells from multiple A animals, presensitized with strain B cells, were injected into strain B animals. The B animal cannot reject too high an amount of donor cells (high dose tolerance) but is recognized as foreign by the A cells.

Parabiosis intoxication can be observed when animals are connected with their blood circulation and one animal recognizes the other as foreign without being rejected.

Secondary disease was observed when animals survived the immediated sequelae of total body irradiation but died after engraftment of donor cells.

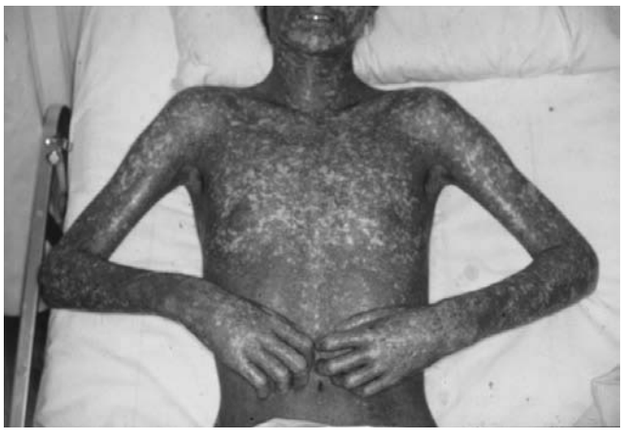

Figure 1.2 Severe chronic graft versus host disease in one of the first-described patients [22]. See Plate 1 in the color plate section.

CHRONIC GVHD AND AUTOIMMUNITY

The close similarity of the clinical syndrome of chronic GVHD and some clinical entities of autoimmune disease brought up the debate on whether chronic GVHD is an entity by its own or simply an autoimmune disease, triggered by the host versus graft–graft versus host interaction [29]. Chronic GVHD in animals, termed also as chronic allogeneic disease was already known [30] and Fialkow had shown in some elegant mice experiments in F_1 hybrid mice with chronic GVHD that host helper T cells were stimulated and were responsible for the production of autoantibodies. He believed chronic graft versus host to be a true host-derived but graft-triggered autoimmune process [31]. Still, in contrast to his mice experiments where Fialkow did find autoantibodies, none of the autoantibodies that are typical in Sjögren's disease, lupus erythematodes, or primary biliary cirrhosis were found in those four initial chronic GVHD patients [21, 22]. Obviously, the F_1 hybrid model could not explain sufficiently the human disease. The debate on the pathophysiology of chronic GVHD is ongoing.

CHRONIC GVHD AND GRAFT VERSUS LEUKAEMIA EFFECTS

As early as the first experiments by Ford, who documented the cellular replacement of donor by recipient hematopoiesis after an allogeneic HSCT, the impact of GVHD on residual leukemia was described [4]. The clinical relevance was realized by G. Mathé in 1964 when he described a patient with acute leukemia, treated for his severe pancytopenia and fever by infusions of granulocytes from a donor with chronic myeloid leukemia. The patient recovered from his neutropenia, showed defervescence, and cleared his bone marrow from all leukemic blasts. In parallel, he developed a skin rash, icterus, and diarrhea. A few weeks later all clinical signs of graft versus host disease disappeared and his leukemia returned [32]. The formal correlation between acute and, even more so chronic GVHD, and control of leukemia was reported by Weiden [33] in 1979 in his seminal paper. The finding of a clear correlation between graft versus host disease and graft versus leukemia or graft versus tumor effects have since been confirmed repeatedly. Still, it remains a matter of debate whether the beneficial effects of increased tumor control outweigh the detrimental effects of the increased transplant-related mortality, which are invariably associated with acute and chronic GVHD [34].

CHRONIC GVHD AS LATE ALTERED IMMUNITY?

The recognition of chronic GVHD as a clinical syndrome similar to known autoimmune disorders suggested a new therapeutic approach to autoimmune disorders. If HSCT, by its transfer of hematopoietic stem cells, could induce an autoimmune-like syndrome, it should be possible to eradicate the hematopoietic (including the immune) system of a patient with a severe autoimmune disease by HSCT from a healthy donor. Debate over this concept was passionate. It was known that patients with severe aplastic anemia could be cured with a transplant from a twin donor. It was intensively debated whether they would need conditioning for treatment of their disease. If it were a true stem cell defect only, stem cells alone would be sufficient; if it were an autoimmune disease, some conditioning for eradication of the disease would be required [35]. HSCT for aplastic anemia paved the way for the concept of HSCT in autoimmune diseases. The high transplant-related mortality was prohibitive for any clinical trials in other less immediately fatal autoimmune disorders for a long time. Experimental studies in animals, however, proved the concept to be valid. They prepared the way to where we stand today [36–38].

The original work of Fialkow has been taken up again. Today, autoimmune disorders are viewed as the consequence of environmental effects and chance phenomena on a particular genetic background, resulting in skewed immune response. Increasing findings suggest today that patients after successful allogeneic HSCT might follow a similar pattern. HSCT changes the genetic background and gives an exogenous stimulus. HSCT patients indeed can have a skewed immune reconstitution and can develop a late altered immune syndrome [36–39]. It remains open and it will be fascinating to see in the future whether such late altered immunity and chronic GVHD are distinct entities or just quantitatively different subsets of the same basic underlying immune reaction.

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2

The Pathophysiology of Acute Graft versus Host Disease

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ACUTE GVHD PATHOPHYSIOLOGY: A THREE-STEP MODEL

Acute graft versus host disease (GVHD) results from complex interactions between donor T cells and host tissues in an inflammatory milieu. The pathophysiology of acute GVHD can be considered as a three-step process involving both the innate and adaptive immune systems (Figure 2.1). GVHD pathophysiology can be summarized in a three-step process. In step 1, the conditioning regimen (irradiation, chemotherapy, or both) leads to the damage and activation of host tissues, especially the intestinal mucosa. This allows the translocation of lipopolysaccharide (LPS) and other inflammatory stimuli from the intestinal lumen to the circulation, stimulating the secretion of the inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) from host tissues. These mediators increase the expression of major histocompatibility complex (MHC) antigens and adhesion molecules on host antigen-presenting cells (APC)s, enhancing the recognition of MHC and minor histocompatibility antigens (mHA) by mature donor T cells. Donor T-cell activation in step 2 is characterized by a predominance of T-helper type 1 subset (Th1) cells and the secretion of interferon-gamma (IFN-γ), which activates mononuclear phagocytes. Regulatory T cells (Treg) limit the proliferation and clonal expansion of activated donor T cells. In step 3, effector functions of activated mononuclear phagocytes are triggered by the secondary signal provided by LPS and other stimulatory molecules that leak through the intestinal mucosa damaged during steps 1 and 2. Activated macrophages, along with cytotoxic T lymphocyte (CTL), secrete inflammatory cytokines that cause target cell apoptosis. CD8+ CTL also lyse target cells directly. Damage to the GI tract in this phase, principally by inflammatory cytokines, amplifies LPS release and leads to the "cytokine storm" characteristic of severe acute GVHD. This damage results in the amplification of local tissue injury, and it further promotes an inflammatory response.

It should be noted from the outset that all these steps do not carry equal weight in the pathogenesis of acute GVHD. The pivotal interaction occurs in step 2, where host APCs activate allogeneic donor T cells. The subsequent cytokine cascade is clearly important, but blockade of individual cytokines may not reverse established GVHD when other cellular effectors such as CTL are present. GVHD can also occur when no conditioning of the host has occurred (e.g., transfusion associated GVHD).

Step 1: Effects of Hematopoietic Cell Transplantation Conditioning

The first step of acute GVHD starts before donor cells are infused. Prior to hematopoietic cell transplantation (HCT), a patient's tissues have been damaged, sometimes profoundly, by underlying disease and its treatment, infection and transplant conditioning. These important effects help explain a number of unique and seemingly unrelated aspects of GVHD. For example, a number of clinical reports have noted increased risks of GVHD associated with advanced stage leukemia, certain intensive conditioning regimens, and histories of viral infections [1]. Total-body irradiation (TBI) is particularly important in this process because it activates host tissues to secrete inflammatory cytokines, such as TNF-α and IL-1 [2], and it induces endothelial apoptosis that leads to epithelial cell damage in the gastrointestinal (GI) tract [3]. Injury to the gut is transient and self-limited after autologous HCT. However, after allogeneic HCT, further damage by GVHD effectors amplifies GI and systemic GVHD by allowing the translocation of microbial products such as LPS into systemic circulation. This scenario helps to explain the increased risk of GVHD associated with intensive conditioning regimens. The overall risk of GVHD after reduced intensity regimens is similar but usually occurs later due to the lower level tissue injury involved. The relationship between conditioning intensity, inflammatory cytokines, and GVHD severity have been confirmed by animal models [4] and by clinical observations [1].

Step 2: Donor T-cell Activation and Cytokine Secretion

Donor T-cell Activation

Donor T-cell activation occurs during the second step of acute GVHD. Murine studies have demonstrated that host

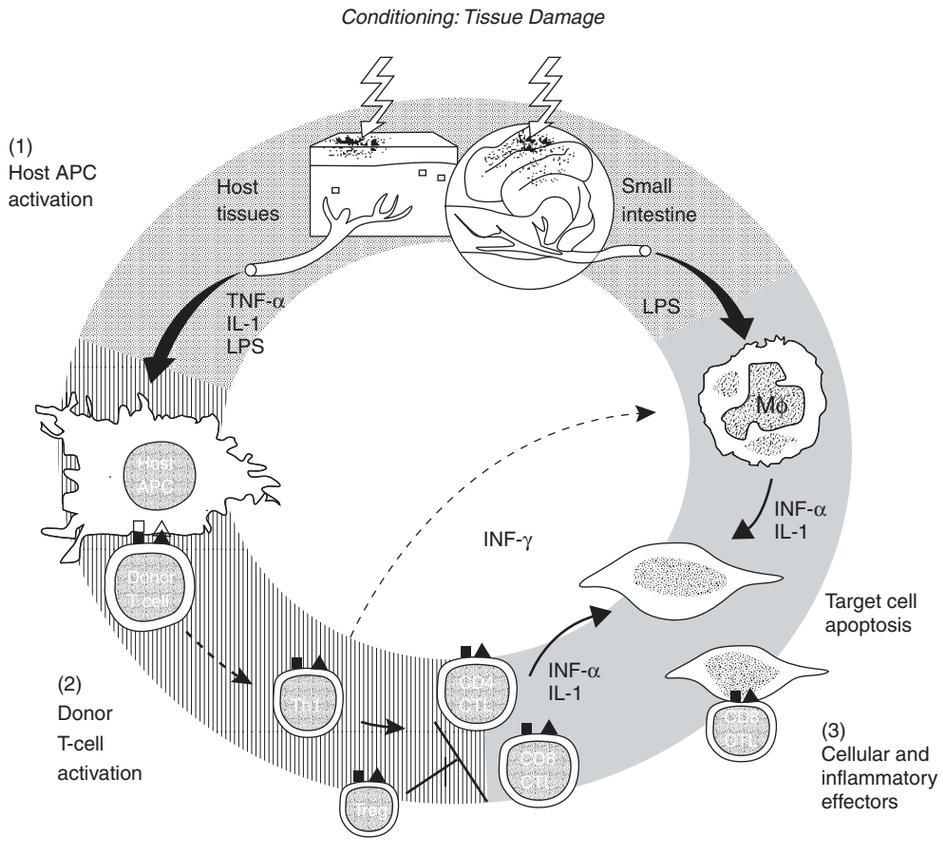

Figure 2.1 The pathophysiology of acute graft versus host disease (GVHD).

The three steps are (1) tissue damage to the recipient by the radiation/chemotherapy pretransplant conditioning regimen, (2) donor T-cell activation and clonal expansion by host antigen-presenting cells (APCs), and (3) cellular and inflammatory factors. This schema underscores the importance of mononuclear phagocytes and other accessory cells to the development of GVHD after complex interactions with cytokines secreted by activated donor T cells.

CTL, cytotoxic T lymphocyte; GI, gastrointestinal; IL, interleukin; IFN-γ, interferon-gamma; LPS, lipopolysaccharide; MHC, major histocompatibility complex; mHA, minor histocompatibility antigens; Treg, regulatory T cells; Th1, T-helper type 1 subset; TNF-α, tumor necrosis factor-alpha.

APCs alone are both necessary and sufficient to stimulate donor T cells [5, 6]. Donor T cells migrate to secondary lymphoid organs, such as Peyer patches, lymph nodes, and spleen where they first encounter host APCs [7]. In murine models of GVHD to MHC differences between donor and host, robust donor T-cell proliferation is observed in the spleen as early as day 3 after HCT, preceding the engraftment of donor stem cells [5, 7].

After allogeneic HCT, both host- and donor-derived APCs are present in secondary lymphoid organs. T-cell receptors (TCRs) of donor T cells can recognize alloantigens either on host APCs (direct presentation) or donor APCs (indirect presentation). During direct presentation, donor T cells recognize either the peptide bound to allogeneic MHC molecules or the foreign MHC molecules themselves [8]. During indirect presentation, T cells respond to the peptides generated by degradation of the allogeneic MHC molecules that are presented on self MHC [9]. In GVHD to mHA, direct presentation is dominant because APCs derived from the host, rather than from the donor, are critical [6].

Several laboratories have identified that naïve ($CD62L^+$) T cells cause experimental GVHD whereas memory ($CD62L^+$) do not, although memory stem cells may be involved [10, 11]. In the majority of HLA-identical HCT, GVHD is induced by mHA, which are peptides derived from polymorphic cellular proteins that are presented on the cell surface by MHC molecules [12]. Because the genes for these proteins are located outside of the MHC, two siblings often will have many different peptides in the MHC groove. In this case, different peptides presented by the same MHC are recognized by donor T cells and lead to GVHD. In mice, the actual number of so-called "major minor antigens" that can potentially induce GVHD is likely to be limited. Several mHA are encoded on the male-specific Y chromosome and are associated with an increased risk of GVHD when

male recipients are transplanted from female donors [13]. mHA with tissue expression limited to the hematopoietic system are potential target antigens of graft-versus-leukemia (GVL) reactivity [14], and separation of GVHD and GVL by using CTLs specific for such antigens is an area of intense research [15].

Adhesion molecules mediate the initial binding of T cells to APCs. TCR signaling after antigen recognition induces a conformational change in adhesion molecules, resulting in higher affinity binding to the APC [16]. Full T-cell activation also requires costimulatory signals provided by APCs in addition to TCR signals. Two primary costimulatory pathways signal through either CD28 or TNF receptors. The best-characterized costimulatory molecules, CD80 and CD86, deliver positive signals through CD28 that lower the threshold for T-cell activation and promote T-cell differentiation and survival, while signaling through CTLA-4 is inhibitory [17].

The most potent APCs are dendritic cells (DCs); however, the relative contribution of DCs and other semiprofessional APCs to the development of GVHD, such as monocytes/macrophages and B cells, remains to be elucidated. Donor APCs may also amplify GVHD, but in the skin, Langerhans cells of host origin appear to be essential for the activation of donor T cells [17, 18]. Signaling through Toll-like receptors (TLRs) and through other innate immune receptors such as NOD may act as "danger signals" and activate host APCs, amplifying the donor T-cell response [19, 20]. DCs can be matured and activated during HCT by (1) inflammatory cytokines; (2) microbial products such as LPS and the dinucleotide CpG entering systemic circulation from intestinal mucosa damaged by conditioning; and (3) necrotic cells that are damaged by recipient conditioning. All of these stimuli may be considered "danger signals" [19] and may make the difference between an immune response and tolerance [21]. When T cells are exposed to antigens in the presence of an adjuvant such as LPS, the migration and survival of T cells are dramatically enhanced in vivo [22]. The effect of age in enhancing allostimulatory activity of host APCs may also help explain the increased incidence of acute GVHD in older recipients [23]. The elimination of host APCs by activated natural killer (NK) cells can prevent GVHD [24]. This suppressive effect of NK cells on GVHD may have relevance in humans. HLA class I differences driving donor NK-mediated alloreactions in the graft-versus-host (GVH) direction mediate potent GVL effects and produce higher engraftment rates without causing severe acute GVHD [24, 25].

Cytokine Secretion by Donor T Cells

T-cell activation involves multiple, rapidly occurring intracellular biochemical changes, including the rise of cytoplasmic free calcium and activation of protein kinase C and tyrosine kinases [26]. These pathways in turn activate transcription of genes for cytokines, such as IL-2, IFN-γ, and their receptors. Cytokines secreted by activated T cells are often classified as Th1 (secreting IL-2 and IFN-γ) or T-helper type-2 subset (Th2) (secreting IL-4, IL-5, IL-10, and IL-13) [27]. The role of Th17 cells, a recently described functional T-cell subset, has not yet been clarified in GVHD [28]. Several factors influence the ability of DCs to instruct naive CD4+ T cells to secrete Th1 or Th2 cytokines. These factors include the type and duration of DC activation along with the DC/T-cell ratio and the proportions of DC subsets present during T-cell interactions [29]. Differential activation of Th1 or Th2 cells has been evoked in the immunopathogenesis of GVHD and the development of infectious and auto-immune diseases. Although this dichotomy has many exceptions, in both settings activated Th1 cells (1) amplify T-cell proliferation by secreting IL-2; (2) lyse target cells by Fas/Fas ligand (FasL) interactions; (3) induce macrophage differentiation in the bone marrow by secreting IL-3 and granulocyte macrophage colony-stimulating factor (GM-CSF); (4) activate macrophages by secreting IFN-γ and by their CD40-CD40 ligand interactions; (5) activate endothelium to induce macrophage binding and extravasation; and (6) recruit macrophages by secreting monocyte chemoattractant protein-1 (MCP-1) [30].

During step 2 of acute GVHD pathophysiology, IL-2 has a pivotal role in both controlling and amplifying the immune response against alloantigens. IL-2 induces the expression of its own receptor (autocrine effect) and stimulates proliferation of other cells expressing the receptor (paracrine effect). IL-2 is secreted by donor CD4+ T cells in the first several days after GVHD induction [31]. In some studies, the addition of low doses of IL-2 during the 1st week after allogeneic bone marrow transplantation (BMT) enhanced the severity and mortality of GVHD [32, 34]. The precursor frequency of host-specific IL-2 producing cells predicts the occurrence of clinical acute GVHD [33, 34]. Monoclonal antibodies (MABs) against IL-2 or its receptor can prevent GVHD when administered shortly after the infusion of T cells [35, 36], but this strategy was only moderately successful in reducing the incidence of severe GVHD [37]. Cyclosporine (CSP) and tacrolimus dramatically reduce IL-2 production and effectively prevent GVHD. IL-15 is another critical cytokine in initiating allogeneic T-cell division *in vivo* [38] and may also be important in clonal T-cell expansion.

IFN-γ is another crucial cytokine that can be implicated in the second step of the pathophysiology of acute GVHD. Increased levels of IFN-γ are associated with acute GVHD [39, 40] and a large proportion of T-cell clones isolated from GVHD patients produce IFN-γ [41]. In animals with GVHD, IFN-γ levels peak between days 4 and 7 after transplantation before clinical manifestations are apparent. Experimental data demonstrate that IFN-γ modulates several aspects of the pathophysiology of acute GVHD. First, IFN-γ increases the expression of numerous molecules involved in GVHD, including adhesion molecules, chemokines, MHC antigens, and Fas, resulting in enhanced antigen presentation and the recruitment of effector cells into target organs [42]. Second, IFN-γ alters target cells in the GI tract and skin so that they are more vulnerable to damage during GVHD; the administration of anti-IFN-γ MABs prevents GI GVHD [43] and high levels of both IFN-γ and TNF-α correlate with the most intense cellular damage in the skin [44]. Third, IFN-γ mediates GVHD-associated immunosuppression seen in several experimental HCT systems in part by the induction of nitric oxide (NO) [45–47]. Fourth, IFN-γ

primes macrophages to produce proinflammatory cytokines and NO in response to LPS [48]. At early time points after HCT, IFN-γ may paradoxically reduce GVHD by enhancing Fas-mediated apoptosis of activated donor T cells [49, 50].

Both cell-mediated and inflammatory cytokine GVHD effector mechanisms can sometimes be inhibited if donor T cells produce less Th1 cytokines [51]. Furthermore, cell mixtures of Th2 donor cells with an otherwise lethal inoculum of allogeneic bone marrow and T cells also protect recipient mice from LPS-induced lethality, demonstrating the ability of Th2 cells to modulate Th1 responses after allogeneic transplantation [52]. Polarization of donor T cells toward a Th2 phenotype by pretreating HCT donors with granulocyte colony-stimulating factor (G-CSF) also results in less severe GVHD [53]. It should be noted, however, that systemic administration of Th2 cytokines IL-4 or IL-10 as experimental prophylaxis of GVHD was either ineffective or toxic [54, 55].

On the other hand, administration of Th1 cytokines can also reduce GVHD. High doses of exogenous IL-2 early after BMT protects animals from GVHD mortality [56]. It has been suggested that IL-2 mediates its protective effect via inhibition of IFN-γ [39]. But injection of IFN-γ itself can prevent experimental GVHD [57] and neutralization of IFN-γ results in accelerated GVHD in lethally irradiated recipients [58]. These paradoxes may be explained by the complex dynamics of donor T-cell activation, expansion, and contraction. Activation-induced cell death (AICD) is a chief mechanism of clonal deletion and is largely responsible for the rapid contraction of activated T cells following an initial massive expansion [59]. Thus, the complete absence of IFN-γ may result in an unrestrained expansion of activated donor T cells, leading to accelerated GVHD. Similarly, administration of IFN-γ inducing cytokines, such as IL-12 or IL-18, protects mice from GVHD in a Fas dependent fashion [50]. Thus, moderate amounts of Th1 cytokines production after donor T-cell expansion may amplify GVHD; extremes in production (either low or high), particularly during T-cell expansion, may hasten the death of activated donor T cells, aborting T-cell expansion and reducing GVHD.

Regulatory T Cells

In both humans and mice, Treg deficiency results in immune dysregulation and autoimmunity, as characterized by the human IPEX syndrome resulting from loss of function mutations of FOXP3 [60]. A similar condition occurs in scurfy mice, a strain that lacks the transcription factor FOXP3 [61]. FOXP3 appears to function as a master control gene for the development and function of natural Treg, which normally constitute ~5% to 10% of the circulating $CD4^+$ T-cell population. Tregs suppress both innate and the adaptive immune functions [62–64] by producing inhibitory cytokines (IL-10 and TGF-B) as well as by cell contact dependent inhibition of APC function and direct cytotoxicity against antigen-presenting B cells [63–65].

Additional purified $CD4^+CD25^+$Treg populations can suppress the proliferation of conventional T cells and prevent GVHD [66, 67]. Tregs do not themselves induce GVHD and the small numbers of Treg cells present in a graft appear to be overwhelmed by the large number of conventional donor T cells present. The use of calcineurin inhibitors (CNI) and thymic injury from GVHD may also interfere with the development of adequate numbers of Treg cells that can control GVHD [66, 68–70]. NK1.1+ T cells (NKT) may also possess regulatory function, and both peripheral blood and marrow NKT cells can prevent GVHD by their IL-4 secretion [71].

Step 3: Cellular and Inflammatory Effectors

The pathophysiology of acute GVHD culminates in the generation of multiple cytotoxic effectors that contribute to target tissue injury. Significant experimental and clinical data suggest that soluble inflammatory mediators act in conjunction with direct cell-mediated cytolysis by CTL and NK cells to cause the full spectrum of deleterious effects seen during acute GVHD. As such, the effector phase of GVHD involves aspects of both the innate and adaptive immune response and the synergistic interactions of components generated during step 1 and step 2.

Cellular Effectors

The Fas/FasL and the perforin/granzyme (or granule exocytosis) pathways are the principal effector mechanisms used by CTLs and NK cells to lyse their target cells [72, 73]. Following recognition of a target cell through TCR-MHC interaction, CTLs secrete perforin and insert it into the target cell membrane forming "perforin pores" that allow granzymes to enter the cell and induce apoptosis through various downstream effector pathways [74]. Ligation of Fas results in the formation of the death-inducing signaling complex and the subsequent activation of caspases [75]. A number of ligands on T cells also possess the capability to trimerize TNF-α receptor (TNFR)-like death receptors (DR) on their targets, such as TNF-related apoptosis inducing ligand (TRAIL:DR4,5 ligand) and TNF-like weak inducer of apoptosis (TWEAK:DR3 ligand) [76, 77].

The involvement of these pathways in GVHD has been tested by utilizing donor cells that are genetically deficient in each molecule. Lethal GVHD occurs even in the *absence* of perforin dependent killing demonstrating that the perforin/granzyme pathway plays a significant, but not exclusive, role in target organ damage. CD4+ CTLs preferentially use the Fas/FasL pathway during acute GVHD, while CD8+ CTLs primarily use the perforin/granzyme pathway, consistent with other conditions involving cell-mediated cytolysis [17].

Fas is a TNF-receptor family member that is expressed by many tissues, including GVHD target organs. Inflammatory cytokines such as IFN-γ and TNF-α can increase the expression of Fas during GVHD [78]. FasL expression on donor T cells is also increased during GVHD [79, 80]. Elevated serum levels of soluble FasL and Fas have also been observed in some patients with acute GVHD [81, 82]. The Fas/FasL pathway is particularly important in hepatic GVHD, consistent with the marked sensitivity of hepatocytes to Fas-mediated cytotoxicity in models of murine hepatitis [83]. Fas-deficient recipients