On the Role of Keratinocyte Growth Factor
for Thymic Epithelial Cell Development
and the Protection
from Graft versus Host Disease

Von

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Summary

The function of the thymus is to provide the physiological microenvironment for the development of T lymphocytes. In this function, thymic epithelial cells play a critical role for the successful establishment and maintenance of the immune system's capacity to distinguish between self and non-self. The cellular and architectural organizations of the thymus provide functionally distinct regions that control the separate and discrete maturational steps in the ordered differentiation from precursor cells to phenotypically mature and functionally competent T cells. The appropriate choice of the T cell antigen receptor (TcR) repertoire is the consequence of interactions with MHC classes I and II molecules on thymic epithelial cells (and other stromal cell types) underscoring the importance of thymic epithelial cells for thymocyte differentiation and selection. However, thymic epithelial cell homeostasis is severely disrupted after conditioning with radio-chemotherapy and allogeneic recognition by donor T cells in the context of allogeneic hematopoietic stem cell transplantation (HSCT). HSCT is the therapy of choice for a number of malignant and non -malignant diseases. The success of allogeneic HSCT depends on the efficiency by which the host's immune system is restored. The thymus is one of the target organs of donor derived T cells and is severely damaged after HSCT. For this reason, the T-cell lineage is not re-generated as rapidly and efficiently as most other hematological lineages, which results in a protracted immune deficiency in the post-transplant period. Recent studies aimed at a better understanding of the regenerative mechanisms demonstrated that the donor-derived peripheral T-cell pool is restored through two independent pathways: (i) expansion of adoptively transferred mature T-cells, and (ii) intrathymic *de novo* generation of T-cells from donor-derived precursor cells. While transfer of mature T-cells during allogeneic HSCT may provide a short-term restoration of immune functions, but is limited by the complication of Graft versus Host Diseases (GVHD), the long-lasting and complete reconstitution of the peripheral T cell pool depends ultimately on new T cell generation.

Keratinocyte growth factor (KGF; a.k.a. Fibroblast Growth Factor 7) was described as a key effector molecule for the protection/restoration of epithelial cells following radio- chemo-therapeutic conditioning; although the precise mechanism of action remain unknown. The presented thesis details the biological effects of KGF on the thymic microenvironment and its functions during postnatal homeostasis of the thymus and during T cell mediated damage in the course of GVHD.

To investigate the molecular and cellular basis for the effect of KGF on function and architecture of thymic epithelial cells, different experimental systems were investigated. The *in*

vitro proliferation capacity of different thymic epithelial cell lines in response to KGF exposure was tested and fetal thymic organ cultures (FTOC) were employed to analyze the effect of KGF in the complex *ex vivo* conditions. Additionally, thymic architecture and function were also investigated following the *in vivo* exposure to KGF. Under these conditions KGF induced the proliferation of thymic epithelial cells. Gene expression analysis of thymic epithelial cells treated *ex vivo* or *in vivo* with KGF revealed the upregulation of specific transcripts for *Wnt* and *Bmp* family members and *IL-7*, i.e. molecules known to regulate thymopoiesis. In consequence, KGF also stimulated an increase in thymic T lymphopoiesis resulting in an increase in size, weight and cellularity. Most importantly, KGF did not alter the architectural organization and the composition of the thymic epithelial subpopulation, thus allowing for a regular expansion and selection of thymocytes. These characteristics identified KGF as an ideal candidate for the preservation/restoration of thymic epithelial cell injury in the presence of acute GVHD. Indeed, when given to recipients of allogeneic T cells in a non-irradiated murine P->F_1 transplantation model, KGF preserved normal thymic size, cellularity, epithelial cell architecture and thymocyte maturation. This normal function and phenotypic appearance of the thymus correlated with a decreased infiltration by donor-derived T cells. In addition, the typical GVHD-induced impairment in cell cycle progression of pro- and pre-T cells was also prevented by KGF. Moreover, the treatment with KGF decreased the number and *in situ* activation of infiltrating allogeneic T cells and drives the disease from an acute into a chronic course.

Taken together, these findings have detailed the role of KGF for thymic epithelial cell biology under physiological growth conditions, tissue maintenance, epithelial cell repair and protection in the presence of thymic GVHD.

Introduction

The thymus is the primary site for the development of T cells of the TcRαβ lineage (reviewed in (1, 2)), whereby intrathymic lymphopoiesis is regulated by an active cross-talk between lymphoid cells and different stromal cells. Hematopoietic stem cells originating from bone marrow home to the thymus via the blood circulation, undergo a process of expansion, maturation and TcR repertoire selection, and finally emigrate to the periphery as mature T cells.

Thymus development

Structure and cellular composition of the thymus

The thymus lies in the upper mediastinum above the heart and is a pyramid-shaped organ formed of two structurally identical lobes that meet in the midline (*Figure I*). A connective tissue capsule surround each lobe, which is comprised of numerous lobules, formed by invagination of connective tissue derived from the capsule. Analysis of the thymic structure at the histological level allows the distinction of three separate areas: The thin subcapsular region, the lymphocyte-rich cortex and the epithelial cell-dense medulla (*Figure II*). In conventional light microscopy, the cortex is separated from the medulla by a clearly visible cortico-medullary junction (CMJ). Strands of connective tissue extend at irregular intervals from the surface of the thymus into the organ proper and, therefore, form septa that carry nerve bundles, blood vessels and the efferent lymphatics that drain the connective tissues of both septa and capsules. Thymocytes develop in physical contact with several types of stromal cells which, combined, create distinct microenvironments composed of epithelial cells and non-epithelial cells such as fibroblasts, macrophages, and dendritic cells. The autochthonous epithelial cells constitute the most abundant population of stromal cells and their function is clearly distinct from that of the bone marrow-derived hematopoietic stromal cells. Combined, the epithelial cells form an integrated cellular network that provides distinct developmental niches for cells of the T lymphoid lineage.
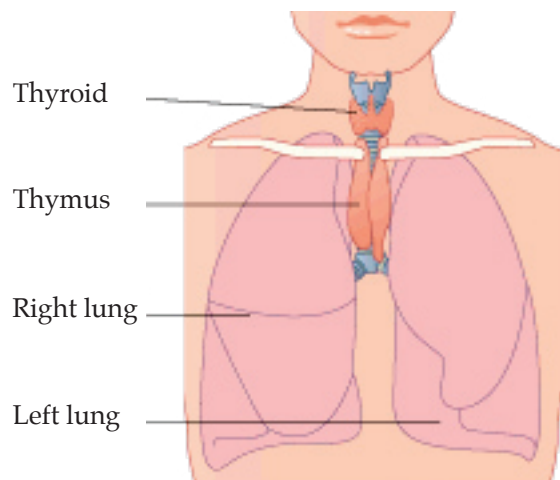

Figure I The thymus is positioned above the heart. Adapted from *www.cancerhelp.org.uk*
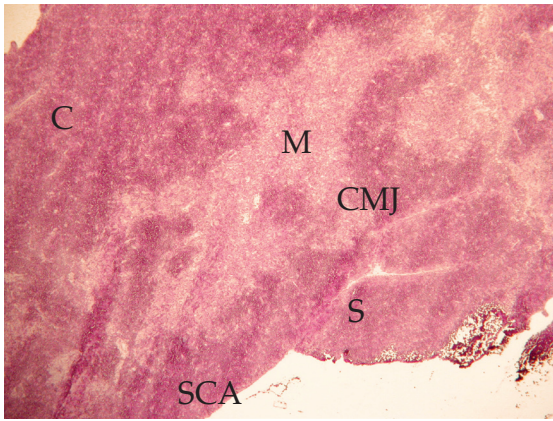
Figure II Thymus section from a healthy mouse. Hematoxilin and Fast Red stain. M: medulla, C: cortex, CMJ: cortico-medullary junction, SCA: sub capsular area, S: septum. (40x)

Using phenotypic characteristics such as cell surface antigen expression and morphology in electron microscopy, the compartment of thymic epithelial cells can be distinguished in cortical and medullary epithelial cells, respectively and several subpopulations therein (see below). The bone marrow-derived dendritic cells are located in the medulla where they primarily are detected in the vicinity of the CMJ. In contrast thymic macrophages can be detected in both cortex and medulla (*2*). B cells are also present in the thymus (*3, 4*). Thymic B cells were thought to be involved in the maintenance of T cell tolerance to self antigens, including those expressed by B cells (*5, 6*). However, it appeared that B cells are not the only cell type responsible for tolerance induction. Recently, several reports have described B lymphopoiesis in the thymus (*7*). In the thymus of young normal mice, CD117^{+}, B220^{low} pro- and pre-B cells are present but disappear with age. B220^{low}, CD5^{+}, B-1 B cells are present in the thymus of older animals (*8*).

Epithelial cells, which are the major component of the thymic stromal cells, generate the three-dimensionally (3-D) organized meshwork architecture peculiar to the thymus. In contrast, in other epithelial organs, e.g. the visceral organs and skin, epithelial cells are placed on the basement membranes and make close contact with adjacent cells forming sheets. The meshwork architecture of the thymus is formed through a marked change of epithelial organization during organogenesis of the thymus anlage. Epithelial cells of the mouse thymus anlage originate in the third pharyngeal pouch endoderm and the third pharyngeal cleft ectoderm. Protrusion of both epithelial layers into the pharyngeal arch mesenchymal region on E9–11 results in formation of the thymus anlage (*9*). Epithelial cells of the thymus anlage develop through interactions of thymic mesenchymal cells (*10-13*) and developing thymocytes (*14-18*); finally generate a 3-D organized meshwork architecture (*19, 20*).

Organogenesis and thymic epithelial cell development

The process of organogenesis consists of several independent stages of maturation that combined assure the correct composition, architectural organization, and function of an organ. In the case of the thymus, the individual maturational steps include the events of positioning, induction, outgrowth, patterning, and differentiation. The end product of this complex process is a fully developed primary lymphoid organ competent to generate mature

thymocytes. Thymic organogenesis can be distinguished into three different phases: the early organogenesis corresponds to events that occur between E9.5 – E11 of mouse development, the phase of late organogenesis occurs between E11.5 and E15, while maturational changes to the thymus organ during late fetal development take place between E15.5 and birth. While each of these phases appears to be regulated by their own set of genetic controls, the formation of the early thymic primordium is furthermore dependent on a coordinated interaction of all three embryonic germ layers, i.e. (i) the endoderm from the epithelial lining of the ventral aspects of the third pharyngeal pouch; (ii) the neuroectoderm-derived neural crest mesenchyme; and (iii) the mesoderm-derived hematopoietic and endothelial cells (reviewed in (21)). The commitment to a thymic epithelial cell fate is exclusively restricted to endodermal endothelium of the ventral aspect of the third pharyngeal pouch by a genetic program yet to be completely defined. While it has been thought that epithelial cells of ectodermal origin (i.e. branchial cleft) would also contribute to the epithelial primordium of the thymus, recent experiments using cell tracking and microsurgery methods have provided ample evidence that ectodermal epithelium does not play a role in the formation of the thymus. Epithelial cells destined to a thymic fate form a bilateral epithelial bud, which eventually detaches from the lining of the third pharyngeal pouch. These primordia migrate by E12.5 to a midline positioned on top of the heart. In late fetal development, the thymus, which by now harbors lymphocytes at stages of early and intermediate cell development, begins to display a distinction into a cortical and into a medullary compartment (reviewed in (22)). These areas have separate functions and hold different populations of thymic epithelial cells (*Figure III*). Recently, a common precursor population for all thymic epithelial cells has been isolated using a cell surface phenotype characterized by the monoclonal antibody MTS24 (23-25) (reviewed in (26)). This antibody stains epithelium of the third pharyngeal pouch as early as E10.5 and
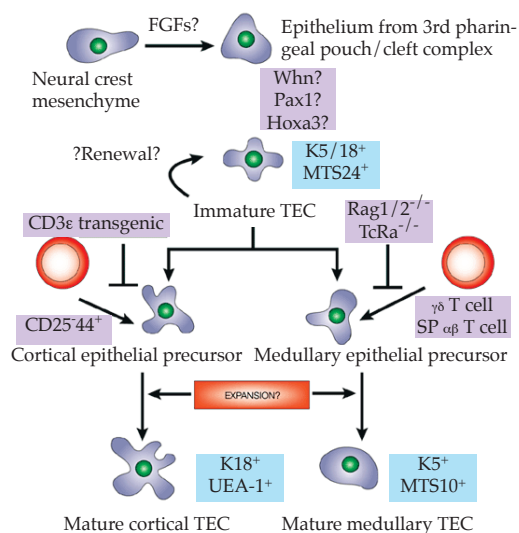
Figure III Thymic epithelial cell development. Several studies have highlighted a number of genes which seem to directly influence the maturational programme of thymic epithelium. In addition studies on genetic mutations in immature thymocytes helped to understand the mechanism that regulate the growth and development of thymic epithelial cells. Adapted from *Anderson et al., Nature reviews Immunology, 2001.*

identifies all epithelial cells that form the thymic primordium at E11.5. The MTS24 antibody recognizes the protein backbone of a glycoprotein yet to be defined at the genetic level. The functional capacity of MTS24+ thymic epithelial cells was demonstrated in cell transfer studies where MTS24+ cells from E15.5 embryos were isolated by flow cytometry and engrafted

under the kidney capsule of syngeneic recipients. Here, these precursor cells gave rise to a structurally complete and functionally competent thymus supporting regular thymopoiesis. While positivity for the MTS24-recognized epitope identifies epithelium giving rise to the thymus, positive staining with this antibody occurs also in other anatomical locations; this epitope is thus not exclusive for epithelial precursors involved in thymic organogenesis. The presence of MTS24$^+$ thymic epithelial cells within the developing thymus is drastically reduced by E15.5 but can still be detected in thymic tissue of adult mice. Here, MTS24$^+$ cells are located in the outer aspects of the medulla closed to the CMJ where they occasionally form small clusters. To further characterize the phenotype of MTS24$^+$ cells, sections of thymic tissue were analyzed at E11.5 and E12.5 for the expression of cytokeratins (CK). It appears from these studies that both CK5 and CK8/18 are co-expressed by a majority of MTS24$^+$ thymic epithelial cells (*24*). This result further supports the notion that MTS24$^+$ cells operate as a common precursor cell to all thymic epithelial cells as the expression of CK8/18 serves as a typical marker for cortical thymic epithelial cells while the expression of CK5 is typically localized to medullary epithelial cells. Thus, the co-expression of specific cellular markers early in thymic ontogeny and their segregation into distinct cellular compartments define distinct subpopulation of epithelial cells and at later developmental stages has been taken as indication for a common precursor to both cortical and medullary epithelial cells ((*23, 24, 27, 28*) and reviewed in (*26*)). The subdivision of thymic epithelial cells into cells with a cortical and medullary phenotype, respectively, is already apparent by E15.5. Using intracytoplasmic markers such as cytokeratins, reactivity to the monoclonal antibody MTS10 and binding of the lectin UEA-1 has allowed a further separation of epithelial cells into cortical and medullary subpopulations (*28*). For example, the major cortical subset of thymic epithelial cells is defined by the presence of CK8/18 but the lack of CK5 and CK14 expression. In contrast, the minor subpopulation of cortical epithelial cells is defined by a CK8$^+$CK18$^+$CK5$^+$CK14$^-$ phenotype and these cells are preferentially localized to the CMJ. Cells with a stellate morphology that express CK5, CK14, and MTS10 but fail to stain for CK8/18 characterize the most abundant medullary subpopulation of epithelial cells. In contrast, the minor subpopulation of medullary epithelial cells has a CK8$^+$CK18$^+$CK5$^-$CK14$^-$ phenotype and stains with UEA-1 lectin. Displaying a globular morphology, these cells are usually situated close to the CMJ (*28*) and are thought to play a role in negative thymic repertoire selection.

The maturation of thymic epithelial cells is, at least in part, controlled by molecular cues provided in a paracrine fashion by mesenchymal cells early in development and by developing thymocytes later in organogenesis. The importance of this mesenchymal-epithelial interaction has best been demonstrated in experiments where thymic epithelial cells were grown in the absence of mesenchymal cells (*29*). Under these conditions, thymic epithelial cells failed to proliferate and were incompetent to support early thymocyte development. Recently,

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fibroblasts growth factors have been identified as a source effecting mesenchymal-epithelial interactions (see below). However, thymic epithelial cell differentiation beyond E12 appears to be independent from the presence of perithymic mesenchyme (*30*).

Thymocyte development

The majority of T cells develop in the thymus following an ordered maturational process from an early not yet lineage determined precursor cell to a population of mature T cells. These cells bear an appropriately selected antigen-specific TcR repertoire and are poised to exit the thymus from the medulla to the periphery. Multiple, sequential steps that are phenotypically highlighted by a changing expression of specific cell surface antigens characterize the normal developmental progression during T cell maturation. The earliest immature T cells arise from precursors derived during fetal development from the liver and after birth from the bone marrow. During the very early stages of development when intrathymic vessel formation has not yet occurred, these precursors exit from the perithymic vasculature and enter the thymus anlage through the capsule. After establishment of the blood circulation, high endothelial venules (HEV) at the CMJ provide the site of entry for these hematopoietic precursors. The precise molecular mechanisms by which these cells home to the thymus are presently not known. The use of several cell surface markers allows to distinguish several distinct thymocyte subpopulations (*Figure IV*). Using the expression of the glycoproteins CD4 and CD8, four maturational subpopulations of T cells of the αβ TcR lineage can be recognized: approximately 5 % of the cells express neither CD4 nor CD8 and are thus designated double-negative (DN) cells. These DN cells are the immediate precursors to thymocytes that concomitantly express both CD4 and CD8 (defined as double-positive (DP) cells). DP cells constitute the most abundant subpopulation as these cells make up for approximately 80 % of all thymocytes. Later in development, thymocytes express (together with a complete T cell antigen receptor, TcR) either only CD4 (CD4-single positive, CD4 SP cells ~10 %) or only CD8 (CD8 SP cells, ~5 %) and constitute the population of mature intrathymic T cells (*31*). The population of TN thymocytes of the αβ TcR lineage can further be separated using the expression profile of two other cell surface markers, i.e. CD25 (*32*) and CD44 (*33, 34*). The most immature thymocytes within this population express only CD44 and are designated TNI. Upon further maturation, these cells acquire the cell surface marker CD25 and therefore progress to the TNII stage. Subsequently these cells lose CD44 (TNIII) and then also CD25 (TNIV). Immune-histological studies have nicely demonstrated that TNI and TNII cells are located in the deeper aspects

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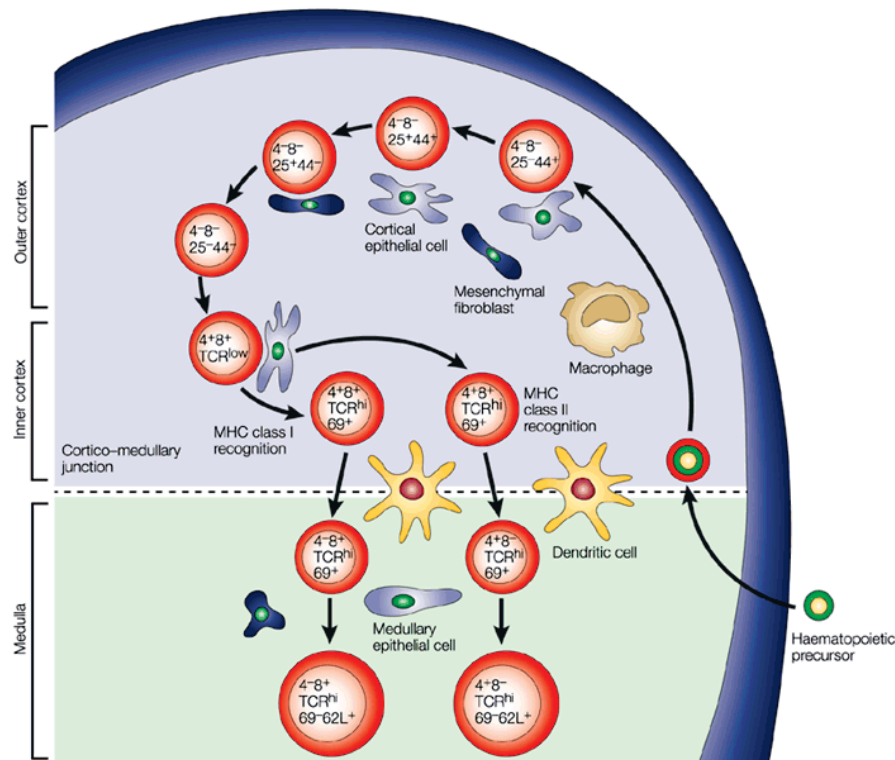
Figure IV T-cell development in the adult thymus. Thymus is organized in distinct compartment where thymocytes enter in contact with different stroma cell types and differentiation can be followed according the expression of cell-surface markers. Figure adapted from *Anderson et al., Nature reviews Immunology, 2001*.

of the thymus and close to the CMJ, while the TNIII cells accumulate in the subcapsular area of the cortex. Moreover, CD25 is within the thymus not a unique marker for TN cells as regulatory CD4$^+$ T cells also express CD25 at the end of their intrathymic development (reviewed in (*35*)).

In the course of the last few years, several additional cell surface markers have been used to define the phenotype of the most immature thymocytes. One study (*36*) designates these cells as early thymic precursors (ETP) and characterized them as cells negative for CD4, CD8, CD3, CD25, CD11b, CD19b, GR1, NK1.1, TcRαβ, TcRγδ, and IL-7R (CD127) but positive for CD44 and c-kit (CD117). ETPs are, therefore, considered as part of the previously defined DNI subpopulation of thymocytes. The acquisition of CD25 (i.e. the transition to the DNII phenotype) is paralleled by the D-J rearrangement of the TcRβ locus. Moreover, these cells begin to express the surrogate α-chain of the pre-TcR, a molecule known as pTα. While only a subpopulation of TNI cells stain positive for CD127, all of the TNII cells express this α-chain of the IL-7 receptor. Once progressed to the TNIII stage of the intrathymic T cell development, the cells are now also competent to express the complete pre-TcR on their cell surface, downmodulate CD117 expression and are rendered low positive to negative for the cell surface marker CD127. Signaling via the pre-TcR is a critical prerequisite for the downregulation of CD25 expression and thus allows the progression to a TNIV phenotype, which characterizes

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a short-lived subpopulation of immature thymocytes. Upon further maturation, developing thymocytes acquire within hours the cell surface expression of CD4 and CD8 independent of thymic epithelial stromal support (reviewed in (*1, 37*)). Double-positive thymocytes rearrange their TcRα locus and, if successful, express a functional αβTcR on their cell surface ((*38, 39*)). These cells are now rendered susceptible to positive and negative thymic selection: a complex process that assures the correct selection of TcR specificities: cells with a potentially harmful (i.e. self reactive) specificity are deleted by programmed cell death (apoptosis) while thymocytes bearing a TcR able to recognize foreign antigens in the context of self-MHC are stimulated to full maturation (*40*). These events occur within a relatively short window of time as the life span of DP cells estimate to approximately 3-4 days, regardless whether these cells are subjected to apoptosis as a consequence of negative selection and neglect, or whether they have undergone positive selection. The TcR engagement at the DP stage leading to positive selection induces upregulation of the TcR concomitant with an eventual downmodulation of either CD4 or CD8 as a consequence of the MHC-restriction of the selected antigen receptor. Thymocytes that recognize an antigen in the context of MHC class I develop into CD8 SP cells while cells bearing a TcR specific for antigens presented by MHC class II attain a CD4 SP phenotype. The maturational transition from DP to single-positive (SP) thymocytes is gradual, consists of various intermediate stages, and is paralleled by the physical translocation from the cortex to the medulla. In detail, positively selected DP thymocytes first downmodulate both CD4 and CD8 before re-expression of these glycoproteins occurs. Because CD4 re-expression is relatively faster when compared to that of CD8, thymocytes with a CD4^{low} CD8 ^{low} TcR^{intermediate} phenotype give first rise to CD4^{high} CD8 ^{low} TcR^{intermediate} thymocytes (which account for approximately 4 % of all thymocytes). It is only at this stage of development that the maturing thymocytes activate a lineage-specific program that results in a selective suppression of CD4 or CD8 synthesis dependent on the specificity of the selected TcR. In the case of positive selection of thymocytes bearing a TcR restricted for MHC class II molecules, CD4^{high} CD8^{low} TcR^{intermediate} cells stop to express CD8 and upregulate the cell surface expression of their antigen receptor to obtain the CD4^{high} CD8^{-} TcR^{high} phenotype. In contrast, thymocytes which have successfully engaged the complex of antigen MHC class I, maintain their CD8 re-expression while turning off CD4 synthesis, thus giving rise via an intermediate stage characterized by a CD4^{low} CD8^{low} TcR^{high} phenotype to mature CD8^{high} TcR^{high} thymocytes. Mature SP thymocytes reside in the medulla between several days to up to two weeks prior to their emigration into the periphery. SP thymocytes are distinguished from mature peripheral T cells by their expression of heat-stable antigen (HSA, CD24) and the concomitant lack of the non-classical MHC class I molecule Qa-2 on the cell surface (reviewed in (*2*)). While there is generally no reentry of naive T cells from the periphery to the thymus, a fraction of activated peripheral T cells appear to regain the capacity to home again to the thymic medulla (*41*).

Keratinocyte growth factor

Keratinocyte growth factor (KGF) is a member of the family of fibroblast growth factors. Also known as fibroblast growth factor-7 (FGF-7), KGF is a heparin-binding molecule that displays mitogenic activity, affects changes in cell morphology and controls the differentiation of several epithelial cell types. The KGF cDNA encodes a protein of 194 amino acids, which bears potential N-linked glycosylation sites (*42*). The area of highest homology between FGF-7 and other FGF family members is within the carboxy-terminal two/third of the KGF coding sequence.

KGF is expressed by mesenchymal cells and is typically detected during development in several anatomical sites including the ureteric bud, the urogenital sinus, the kidney rudiment, the external genitalia, the mammary glands, the thyroid and the thymus anlage (*43*). The most potent inducer of KGF expression in fibroblasts is the proinflammatory cytokine IL-1 (*44*), which, in addition to TNF-α and IL-6 (*45*), plays a dominant role in the events leading to wound healing. However, in addition to mesenchymal cells, other cell types are also competent to express KGF. For example T cells of the γδ lineage isolated from either the skin or the intestine secrete KGF (*46*). Similarly, thymic T cells belonging the αβTcR lineage but representing different stages of development also express KGF under physiological conditions (*47*). In contrast to expectations based on the wide tissue expression of KGF, mice rendered completely deficient for the expression of this factor were described to develop normally but for the exception of having a greasy fur. The overt absence in these mice of developmental defects, of alterations in epithelial cell growth and of deficiencies in wound healing argues for the presence of other factors that signal in parallel and thus compensate for the loss of KGF (*48*). Indeed, there is substantial redundancy for KGF signaling as both *in vitro* studies with cell lines and *in vivo* investigations of gene targeted mice (see below) concluded that practically all KGF functions can also be effected by FGF-10 signaling which occurs via a receptor binding both KGF and FGF-10.

Further insights into the biological role of KGF have been obtained by transgenesis experiments. Overexpression of KGF in skin fibroblasts resulted in a hypertrophy of the epidermis and hypoproliferation of keratinocytes (*49*), while overexpression of KGF directed to epithelial cells of the developing embryonic lung disrupted normal pulmonary morphogenesis (*50*). In these mice, the lung parenchyma was cystic and displayed numerous dilated saccules lined by columnar epithelial cells. Although mesenchymal cells are maintained in the transgenic mice where KGF was expressed under the transcriptional control of the surfactant protein C promotor, the architectural relationship between mesenchyme and epithelial cells was severely

altered. Yet in other transgenic mice hepatic overexpression of KGF resulted in high serum levels and caused changes in several tissues known to express the KGF specific receptor (*51*). The reported pathological changes included hepatocyte and biliary epithelial hyperplasia, pancreatic ductal hypoplasia, enhanced proliferation of bronchial epithelial and pneumocyte II cells, and kidney hypoplasia with cystic dilatations of the cortico and medullary collecting duct systems. Finally, overexpression of KGF under the control of the human insuline promotor resulted in the emergence of hepatocytes within the islets of Langerhans and caused the proliferation of intra-islet duct cells (*52, 53*). Thus, the precise temporal and spatial control of KGF expression is dominant in normal organogenesis as a consequence of mesenchymal-epithelial signaling.

The observation that KGF promotes proliferation of all epithelial cells along the mouse digestive tract initiated studies to use recombinant KGF in murine models of gastrointestinal injury as induced by radiation (*54, 55*), chemotherapy (*55*), or a combination of both (*55*). Treatment with KGF before and after injury resulted, in this model, in a remarkable reduction in the mortality rate when compared to appropriate controls (*54*). This finding was paralleled by both lesser weight loss following radiation and/or chemotherapy and faster weight gain during recovery (*55*). These clinically relevant changes may be accounted for by the trophic effect of KGF on gastrointestinal epithelium as the mucosal thickness increased significantly and crypt survival in the small intestine was enhanced by severalfold. Taken together, these findings have suggested that KGF may have a substantial therapeutic potential to lessen epithelial side effects secondary to injury by chemo-radiotherapy.

KGF receptor: FgfR2IIIB

The receptors for Fgfs (FgfR) represent a family of transmembrane molecules which are encoded by four separate genes (FgfR1-FgfR4) and composed of either two or three Ig-like loops in the external domain, a transmembrane segment and a ligand-activated cytoplasmic tyrosine kinase domain. The two membrane proximal Ig-loops form the ligand-binding domain; receptor diversity is increased by alternative splicing which generates two distinct receptor forms for FgfR1, FgfR2 and FgfR3. Alternative splicing of the Ig-loop III generates two separate isoforms (IIIb and IIIc) that display distinct ligand-binding properties and tissues distributions (*56-58*). The FgfR2IIIb isoform constitutes the receptor where KGF binds preferentially and is typically, if not exclusively expressed on epithelial cells (*43, 59*). Thus, KGF exhibits a paracrine mode of action, as it is usually produced by cells of mesenchymal origin but acts on epithelial cells (*10-13, 43, 59-65*). In addition to KGF, the FgfR2IIIB is also activated by Fgf1, Fgf3, Fgf10, albeit each of these molecules displays a different affinity for this receptor. It is, therefore, not surprising, that mice deficient for KGF fail to display any

developmental pathology (*48*). To further investigate the biological role of FgfR2IIIb and to determine its developmental importance, receptor transgenic and gene-targeted mice have been generated. For example expression of a dominant negative FgfR2IIIb receptor in lung epithelium resulted in a complete block of airway branching and epithelial maturation leading to two undifferentiated tubes below the bifurcation of the trachea (*66*). In another model, the FgfR2 gene was altered in such a way that expressed receptors were devoid of the entire Ig-like loop III (*67*). About a third of the mutant mice failed to form the chorioallantoic fusion junction and the remaining mice did not have the labyranthine portion of the placenta secondary to a lack of trophoblast cell proliferation. Moreover, these mice did not develop limb buds thus providing direct genetic evidence that FgfR2 signalling is essential for limb formation. Loss of the IIIc ligand-binding exon, the transmembrane domain and the kinase I domain of FgfR2 revealed that signalling through such a receptor was not possible and led to alteration in development at E4.5 to E 5.5 prior to the formation of the egg cylinder (*68*). To gain insight into a potential role of FgfR2IIIc signalling during development beyond implantation, fusion chimeras were constructed using homozygous mutant embryonic stem cells and wild-type tetraploid embryos. Although chimeric mice survived until the end of a regular gestation, the absence of normal FgfR2 activity did cause severe developmental defects in limb bud formation and lung development (*69*).

KGF and the thymus

Several cell types of different developmental origin are responsible for the thymic production of KGF, including mesenchymal cells and thymocytes. However, it appears to be a matter of debate which specific subpopulation of maturing thymocytes expresses KGF. In a recent study by Erickson and colleagues (*47*), KGF transcription was detected by RT-PCR only in DP and CD4 SP thymocytes. In contrast, work by Min and colleagues (*70*) reported that KGF mRNA was present in all four major thymocytes subpopulations (TN, DP, CD4 SP and CD8 SP), as well as in thymic stroma cells. Nevertheless, the precise phenotypic nature of the latter cell was not further specified. In contrast, it is unanimously accepted that thymic epithelial cells (as defined by the presence of MHC class II but the absence of CD45) express the specific receptor for KGF, designated FgfR2IIIb (*71*). This observation is further corroborated by the detection of FgfR2IIIb expression on epithelial cells at all stages of thymic development as investigated by in situ hybridization (*47*). Interestingly, the strongest signals for FgfR2IIIb-specific transcripts were detected in the medulla as well as the subcapsular region, two distinct areas that have previously been noted to share several phenotypic markers when analyzed by immunohistochemistry.

To detail a role for KGF in the formation of distinct compartments within the thymic

microenvironment, fetal thymic organ cultures (FTOC) were analyzed. Erickson and colleagues (*47*) tested whether the formation of the medulla is regulated by KGF, assuming that this factor would be provided by phenotypically mature thymocytes. Such a hypothesis is in keeping with observations that mice deficient in mature SP thymocytes also lack a regularly shaped medulla. Using T cell-depleted fetal thymic tissue from E16, *ex vivo* treatment with KGF failed, however, to generate a proper medullary foci, present in the control group, but the medullary compartment was more diffusely organized. These results, therefore, indicate that the *in situ* presence of KGF cannot replace yet unknown signals provided by developing thymocytes able to induce the regular medullary formation. However, *in vivo* treatment of Rag-deficient mice with KGF resulted in an increase in size of the otherwise diffuse medullary areas (*47*). The molecular and cellular mechanisms that account for this obvious discrepancy in the biological role of KGF *in vitro* versus *in vivo* treatment for the patterning of a thymic medulla has not been further addressed. It is, however, conceivable that KGF's capacity to form a medulla is either dependent on a stimulation of extrathymic factors that act in concert with KGF or that KGF's biological activities dependent on the concomitant presence of immature thymocytes. Further experiments will need to be carried out to address these points. *In vitro* exposure for 48 hours of unmanipulated fetal thymic tissue to KGF resulted in a dose-dependent reduction in thymic cellularity and in a maturational impairment of CD4 SP thymocytes. Thus, KGF in the dosages given (10 ng/ml), during prolonged culture (15 days) and in the experimental system analyzed will also affect lymphoid development, albeit via an indirect mechanism as thymocytes do not express FgfR2IIIb (*47*).

Hematopoietic stem cell transplantation and GVHD

In recent years, hematopoietic stem cell transplantation (HSCT) has become the therapy of choice for an expanding number of malignant and non-malignant diseases. The transfer of hematopoietic stem cells (HSC) from a donor to a genetically non-identical recipient is referred to as allogeneic HSCT. In contrast, the cell transfer between two genetically identical individuals is defined as syngeneic HSCT, while autologous HSC involves the use of the patient's own HSC. Autologous HSCT has been successfully used in conjunction with chemotherapy for solid organ tumors (breast cancer and neuroblastoma, among others) and malignancies affecting mature cells of the lymphoid lineage (including non-Hodgkin lymphoma, Hodgkin's disease, etc.), while allogeneic HSCT is favored for the therapy of different forms of leukemia (including acute and chronic myeloid leukemia and acute lymphoid leukemia), and for the treatment of
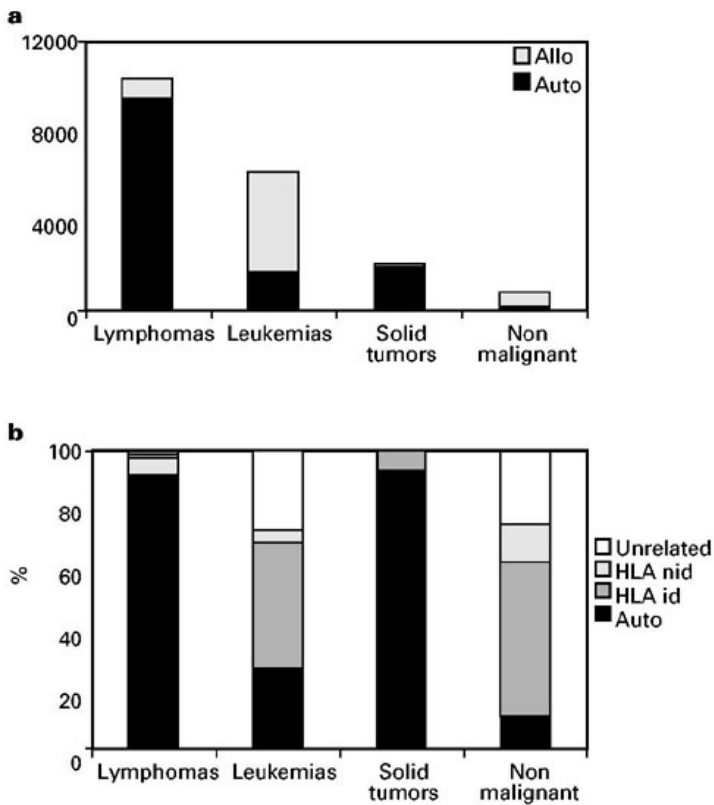
Figure V Main indication and donor type of HSC in europe 2001. a) Absolute numbers of HSCT by donor type and main indication. b) Relative proportion of donor type by main indication. Adapted from *Gratwohl at al., 2002, Bone Marrow Transplantation*.

diseases affecting the functional production of hematopoietic cells. As demonstrated in *Figure V* (adapted from (*72*)), close to 20'000 HSCT have been carried out in Europe in the course of the year of 2001. One third of these transplants concerned the engraftment of allogeneic cells while 2/3 entailed the transfer of autologous cells. In the same year, 281 HSCT were performed in Switzerland alone and in comparison to the European practice, fewer of these transplants (28 %) used allogeneic cells.

Stem cell engraftment in the absence of transplant-related complications critically determines the outcome of HSCT. In particular, graft-versus-host disease (GVHD), pulmonary dysfunction and veno-occlusive disease constitute major complications of allogeneic HSCT. In contrast, the recurrence of the original disease (an event also known as relapse) constitutes the most important complication of autologous HSCT. From an immunological point of view, a transient and frequently prolonged immunodeficiency follows both allogeneic and autologous HSCT and constitutes a particular medical challenge, as this complication correlates with the occurrence of disease relapse and life-threatening bacterial, viral or fungal infections. Initially caused by chemotherapy and/or total body irradiation that are used as pre-transplant conditioning regimen (see below), the slow recovery of the different hematopoietic cell lineages following HSCT constitutes a major reason for the prolonged persistence of a marked immunodeficiency. Importantly, lymphocyte recovery is significantly delayed in the presence of GVHD when compared to allogeneic recipients of an HSCT without this complication. Thus, GVHD significantly contributes to an extended and often exacerbated state of immunodeficiency (*73*). However, the precise cellular and molecular mechanisms that account for this GVHD-associated damage are still unknown. Their elucidation necessitates an increased understanding of the pathophysiological processes leading to this debilitating disorder.

The basic requirements for GVHD

In the absence of effective immunosuppression, the outcome of most allogeneic HSCT will be complicated to various degrees by GVHD. Three independent requirements have been identified for GVHD to occur: (i) the graft contains a sufficient number of immunologically competent cells; (ii) donor and host are genetically different in that important transplantation isoantigens are present in the latter but lack in the former; and (iii) the host immune system is incapable of mounting an effective anti-graft response. Given these conditions, GVHD can develop in different clinical settings where tissues harboring immunocompetent cells are transferred across relevant histocompatibility differences (*74*).

Genetic basis of acute GVHD

The major histocompatibility antigens encoded by the major histocompatibility complex (MHC) genetic loci exert the biggest impact on the immune system's recognition of antigenic differences between donor and host (*75*). The MHC constitutes a highly polymorphic, multi-gene and multi-allelic complex central to both cell-mediated and humoral immunity. The MHC molecules are cell surface proteins that present antigenic peptides to T cells and thus control the activation of effector cells of the adaptive immune system. Two different classes of MHC molecules have been defined based on structural and functional characteristics: Class I molecules are composed of the MHC-encoded polymorphic chain that harbors the antigen binding site and the invariant chain designated β2-microglobuline. The complete MHC class I complex is expressed on most nucleated cells. The MHC class II molecules are composed of two chains that are both encoded within the MHC locus. Combined, these two proteins form the antigen-binding site. Since MHC class II cell surface expression is particularly induced on skin and intestinal epithelium in the course of inflammation, it is not surprising that these anatomical sites serve as specific targets for alloantigen recognition during acute GVHD (*76, 77*). Recognition of the allogeneic MHC is followed by ligation of adhesion molecules on T cells to their corresponding receptors on target cells. In order for the activation to occur, additional costimulatory signals are required as provided for example by interactions of CD28/B7, CD4/MHC class II and CD8/MHC class I antigen (*78-83*). In consequence the correctly stimulated lymphocyte begin to acquire the cell surface expression of molecules such as CD25 (IL-2R), CD69 and CD71, which serve as ideal markers for recent cell activation. In parallel, primed T cells initiate the transcription of cytokines, which are essential for the differentiation, proliferation and amplification of the T cell (*84-86*).

Minor histocompatibility antigens (miH) represent genetically polymorphic peptides derived from intracellular proteins that are presented by either the host's or the donor's MHC molecules (*87*). Genetic polymorphism of endogenous cellular proteins represents the miH:

these molecules are critical in matched-sibling allogeneic HSC graft. T cells do not recognize antigen alone, but in conjunction with the MHC of the antigen presenting cells (*88*). In this context, miH constitute a relevant immunological difference in the transplantation of MHC-matched allogeneic tissues. Following their activation, donor T cells mediate the tissue destruction known clinically as GVHD (see below).

Clinical and experimental studies have provided ample evidence that the severity of GVHD directly correlates with the number of donor T cells transfused (*89*). Based on these observations, T cell depletion (TCD) from donor bone marrow has prevented the development of both experimental and clinical GVHD (*90*). It is, however, of note that the presence of donor T cells in the allogeneic bone marrow graft exerts also the beneficial effect of an anti-tumor activity (also known in hematopoietic malignancies as graft-versus-leukemia (GvL)-effect) (*91*). Thus, depletion of T cells from the bone marrow inoculums as a measure to prevent GVHD increases the risk of leukemic relapse to a level similar to that observed in autologous HSCT. In keeping with the conditions for the occurrence of GVHD, this complication can also occur under conditions where recipients remain immunocompetent. Specifically, MHC heterozygous recipients will not reject tissues derived from donors that are homozygous for one of the recipient's haplotypes. Such a condition of a P->F_1 (parent into an F_1 generation) transplantation is usually only given under experimental conditions. Given these experimental circumstances, the allogeneic donor T cells will recognize the recipient and will mediate an immune response leading to GVHD.

Clinical, histopathological and immunological characteristics have been used to differentiate the course of GVHD into an acute and chronic disease phases. For practical purposes, GVHD occurring in allogeneic recipients within the first 100 days after transplantation is referred to as acute GVHD, while chronic GVHD is defined as a disease typically apparent only 100 days post-transplantation. However helpful such a distinction may be, this"100-Days" rule is not always easily applied in clinical practice as acute GVHD (aGVHD) may indeed occur within days or may only be apparent as late as 1–2 months after HSCT. Moreover, clinical and histological changes considered characteristic for chronic GVHD (cGVHD) might develop as early as 40–50 days post-transplant and overlap with aGVHD. Hence, the time of disease onset constitutes only an arbitrary criterion to separate two pathophysiologically distinct forms of GVHD. Acute and chronic GVHD are, however, better separated in experimental models of HSCT. Chronic GVHD is characterized in the Parental -> F_1 murine transplantation models by a reduction of cytotoxic lymphocyte (CTL) activity, the persistence of recipient T and B cells, a polyclonal B cell activation whit abnormal Ig secretion, the production of anti-ssDNA antibodies and an immune mediated damage to the kidney, immunopathologically reminiscent of glomerulonephritis (*92-97*). In contrast acute GVHD is highlighted by anti-host

CTL activity, an increase in donor CD8$^+$ T cells, and NK cells, splenomegaly and lymphoid hyperplasia. Later in consequence of acute GVHD: splenic hypoplasia, cachexia, anaemia, general and specific immune suppression and epithelial cell damage are observed (*98*).

GVHD affects multiple organ systems and can thus account for severe morbidity and mortality following allogeneic HSCT. Principal targets of GVHD are the epithelial cells of skin, liver, gastrointestinal tract and thymus and the observed severity of pathology correlates with the number of T cells transfused with the donor HSC inoculum. The ensuing damage causes in the target organs programmed cell death of parenchyma cells and leads thus to a loss of organ-specific functions. In the skin, both epidermis and hair follicles are frequently destroyed in the course of GVHD, leading to alopecia and inflammatory changes of the integument. In the liver, the small bile ducts are usually profoundly affected in GVHD, leading from segmental disruptions of the hepatic architecture to a complete failure of liver functions. Intestinal crypt destruction results in mucosal ulcerations that may either be locally defined or diffuse (*99-103*). Finally, thymic epithelial cells of both cortex and medulla appear to be targets of the process of alloantigen recognition, although a precise analysis of the subpopulation most affected by GVHD have not yet been reported. In addition to these T cell-mediated pathologies typical for GVHD, non-specific defects have also been observed to occur during a later phase of the disease, i.e. the development of a severe wasting syndrome, known as cachexia, generalized erythroderma with bullos formation and liver pathology marked by high bilirubin level.

The complex immunopathological events leading to acute GVHD are currently understood to be composed of an afferent and an efferent phase. The afferent phase of acute GVHD is caused by the immunosuppressive and therapeutic measures taken to prepare the recipient for HSCT. Known as conditioning, these treatments usually consist of total body irradiation (TBI) and/or high-dose chemotherapy. Both of these interventions cause also extensive damage to host tissues including intestine, skin, liver, and thymus. The ensuing pathology triggered by conditioning is mediated by inflammatory cytokines such as IL-1, TNF-α, GM-CSF, and INF-γ that is secreted by damaged host tissue (*104, 105*). These inflammatory molecules mediate the upregulation of adhesion molecules and cause the enhanced expression of MHC molecules on the respective target tissues of GVHD. These molecular changes increase, in turn, the allogeneic recognition of host tissue by donor T cells and thus lead to the efferent phase of GVHD, which is characterized by the activation of donor T cells upon recognition of host tissue antigens (*106-109*). Models of GVHD using defined inbred mouse strains have established that donor CD4$^+$ T cells induce GVHD in the context of donor/host MHC class II differences while CD8$^+$ T cells recognize in this context disparities between MHC class I molecules (*104, 110*). Under conditions where MHC identity exists between donor and recipient, differences in miH may suffice to trigger allorecognition via CD8$^+$ cells leading to GVHD (*107, 110*). Activated donor T

cells exert their effect both via the release of proinflammatory cytokines as well as by a direct cytotoxic activity (see below).

At least two independent signaling pathways need to be successfully engaged in order for T cells to be fully activated. The first signal is mediated as a consequence of the recognition of the MHC-peptide complex via the T cell antigen receptor (designated signal 1). The second signal is produced by the binding of antigen-unspecific co-stimulatory molecules on T cells to their respective ligands on antigen-presenting cells (see above) (110).

One of the molecular events necessary for successful T cell activation is the expression and secretion of IL-2. This cytokine controls the clonal expansion of activated donor T cells and contributes to the differentiation of these cells into effector cells that secrete soluble mediators of inflammation (111, 112). For example the provision of IL-2 and IFN-γ primes donor as well as residual host mononuclear phagocytes to produce TNF-α, IL1 and nitric oxide (108, 113). The additional release of inflammatory cytokines by mononuclear phagocytes may, however, occur only in the presence of a second triggering signal such as bacterial endotoxins (LPS). These microbial products gain access through a damaged intestinal mucosa and activate gut-associated lymphocytes and macrophages. The concentration of LPS needed to trigger a robust response by macrophages is significantly lowered if the macrophages have been previously primed by IFN-γ. Thus IFN-γ and LPS work synergistically in priming of naive macrophages to contribute to infiltration damage via their release of TNF-α and NO (114, 115). Thus, the tissue destruction during GVHD is generated by two independent mechanisms that act efficiently in concert: (i) direct cell-mediated cytotoxicity effected by both cytotoxic T lymphocytes (CTL) and natural killer (NK) cells; (ii) provision of soluble inflammatory mediators. Moreover, there is increasing evidence that a differential activation of either "T helper 1" cells (Th1) or "T helper 2" cells (Th2) will preferentially determine either of the two detailed pathways leading to phenotypically distinct forms of GVHD (see below) (108, 116).

Immunodeficiency as a consequence of GVHD

Although immunodeficiency following HSCT may be caused by several mechanisms, research in recent years has started to provide detailed insight into the cellular and molecular pathogenesis of this disorder. Following the conditioning-related elimination of the peripheral T cell pool, both extrathymic and thymic pathways of T cell development contribute to the reconstitution of the peripheral T cell compartment following HSCT (117-119). The first pathway concerns the clonal expansion of mature donor-derived T cells whereas the second pathway relies on the thymic output of *de novo* generated, HSC-derived mature T cells. While the mechanisms of peripheral donor T cell expansion fail to renew the T cell antigen receptor repertoire, it is the process of thymic T cell generation that assures the maintenance of a

broad specificity of the T cell antigen receptor repertoire over time. The relative contribution to each of these different pathways depends on thymic function and the number of donor-derived mature T cells transfused with the HSC inoculums (*120, 121*). The presence of GVHD affects either pathway and thus exacerbates and prolongs the state of immune deficiency in the post-transplant period (*122, 123*). In addition, the rapid disappearance of host lymphoid cells during GVHD in a non-conditioned experimental model of HSCT is a consequence of an elimination of these host cells by mechanisms involving either perforin- or Fas-mediated killing (*123-125*).

In the presence of GVHD, there is an obvious lack of the infused mature donor T cells bearing specificity for antigen other than alloantigens to functionally contribute to peripheral T cell function. The precise pathogenesis for this phenomenon is not yet known. However, depletion and functional loss of donor T cells have as a likely explanation been proposed. For example, activation-induced cell death of allo-reactive donor T cells may lead to bystander apoptosis of grafted T cells devoid of host reactivity (*124*). Alternatively, but not mutually exclusive, is a mechanism by which non-host specific donor T cells fail to respond to specific antigenic or mitogenic stimuli secondary to their severely skewed T cell antigen receptor repertoire following homeostatic expansion in a lymphopenic host (*124*). Moreover, antigen-non-specific suppression of both B- and T cell reactivity may be the consequence of an exposure to suppressive cytokines (e.g. IFN-γ, TNF-α) and immunosuppressive molecules (e.g. NO) provided by the tissue of conditioned recipients (*108, 126-128*). As previously mentioned, the production of these soluble mediators is upregulated in the course of GVHD as a consequence of donor T cell activation. In addition, enhanced levels of IL-1-triggered glucocorticoid secretion may also contribute in this context to peripheral T cell suppression (*115*). While it may be attractive to assume that increased levels of serum glucocorticoid concentrations may have a deletional effect on immature thymocytes, recent data derived from experiments in the mouse provide evidence to the contrary since thymic pathology observed during acute GVHD is mediated by a mechanism of apoptosis independent of glucocorticoids (*129*). Finally, the disruption of the peripheral microenvironmental niches necessary for the expansion of post-thymic T cells may constitute another factor contributing to the immunodeficiency in the presence of GVHD (*120*).

The reconstitution of a normal T cell antigen receptor repertoire determines the success of allogeneic bone marrow transplantation and is critically dependent not only on the clonal expansion of mature donor-derived T cells, but also on the swift re-establishment of a regular thymic function. However, two separate mechanisms account for the loss of regular thymic function in the presence of GVHD. First, a decreased self-renewal capacity of the most immature intrathymic T cell precursors has been observed in the presence of GVHD and may persist for

several months after HSCT engraftment. Since alloreactive T cells and HSC derive from the same donor, mechanisms other than the direct recognition of alloantigens will have to account for this functional alteration. Second, a direct damage to the thymic microenvironment impairs T cells maturation and selection because the thymus serves as a direct target of GVHD.

Thymic pathology in GVHD

The thymus has been identified as a primary target for GVHD for almost 30 years (*122*). A comprehensive understanding of the molecular and cellular basis of GVHD-mediated pathology to the thymus is, however, still missing. Different animal models have unequivocally demonstrated a common pattern of structural changes to the thymic tissue as a consequence of allo-antigen disparity between host and donor in HSCT: decrease in thymic size, depletion of the cortex, loss of the CMJ, medullary epithelial cell necrosis, and loss of Hassall's bodies (*130, 131*).

Moreover, a slow regeneration of the thymic architecture has been observed in non-lethal models of GVHD that are dependent or independent of irradiation for preconditioning. The reparative mechanism required for thymic reconstitution may need as much as 6 months to complete. However, this repair is not necessarily paralleled by a regained regular function, an impairment may last for an extended period of time (*131-134*).

The mechanisms by which conditioning and GVHD induce the profound changes to thymic architecture and function have not yet been fully elucidated. It is conceivable that the following candidate mechanisms, either alone or in concert, may contribute to the observed pathology.

(i) Thymic epithelial cell function is severely affected by the damaging effects of γ-irradiation (*135*). In addition to impaired thymic stromal function (*136*), γ-irradiation also upregulates Fas (*137*) cell surface expression, enhances extrathymic T cell development (*138*), and triggers the release of inflammatory cytokines (*116*).

(ii) The thymic infiltration of donor-derived allo-reactive T cells and the enhancement of host NK activity constitute common features of acute GVHD and may be responsible for the direct cytotoxic destruction of thymic stromal tissue (*139-141*). Indeed, acute GVHD has been associated with changes in the composition and architectural organization of thymic stromal cells (*71*).

(iii) A profound decrease of thymocytes (in particular DP cells) has been noted in acute GVHD. Two independent mechanisms may account for this apparent lack: (i) DP cells and their immediate precursors (TN cells) display a decreased capacity to enter into cell cycle, and (ii) DP thymocytes undergo programmed

cell death at an increased frequency in the presence of GVHD (*142*).

Both of these mechanisms may be a consequence of altered thymic epithelial cell function. Thus, reduced proliferative capacity and an increased susceptibility to apoptosis account for the reduced number of thymocytes.

(iv)   GVHD affects thymic T cell antigen repertoire selection and is associated with the emergence of T cell clones with a "forbidden" T cell antigen receptor specificity. Moreover, the loss of normal thymic selection and the consequential persistence of autoreactive T cells have been related to the transition of acute GVHD to a more chronic form of the disease (*143, 144*).

A growing number of reports have identified the importance of a regular thymic function and the selection of a broad repertoire of T cell antigen receptor specificities for the successful outcome of HSCT (*118, 145-147*). While these parameters may clearly constitute an important goal for clinical practice, the therapeutic measures to attain this aim have yet to be fully be defined. It is, therefore, informative to analyze in depth the cellular and molecular mechanisms that interfere with normal thymic T cell development in the presence of GVHD, as this understanding will provide insight into novel strategies to prevent, reduce or repair GVHD-associated pathology to the immune system.

Aim of the thesis

The aim of this thesis is to detail the biological role of KGF on thymic epithelial cell homeostasis and function under physiological conditions and in instances of epithelial cell damage secondary to GVHD. For this purpose different models have been employed to detect the molecular events triggered by KGF signaling. In particular I studied the following issues: I) identification of the cells within the thymic microenvironment that express the receptor specific for KGF, FgfR2IIIb, II) analysis of the response to KGF signaling by thymic epithelial cells at the cellular and molecular level, III) assessment of the role of KGF in the prevention/repair of tissue damage to thymic epithelial cells in the course of GVHD.

Materials and Methods

Animals

C57BL/6 (B6, H-2^b), and [C57BL/6 x DBA/2] F$_1$ (B6D2F$_1$, H-2^{bxd}) mice were obtained from Iffa Credo (Charles River, France), and from a breeding colony at the University Hospital, Basel, Switzerland. The C57BL/6 congenic mouse strain B6.SJL-Ptprc^{a}Pep3^b/BoyJ (Ly5.1) was obtained from The Jackson Laboratories, ME, USA. Mice were housed in a pathogen-free facility. All animals were kept in accordance with Swiss guidelines and regulations. Female mice used for this study were between 4 and 10 weeks of age.

Reagents

For four-color flow cytometric (FACS) analyses, the following moAbs (conjugated to biotin, FITC, PE or CyChrome) were used: anti-CD3 (clone 145-2C11), anti-CD8 (53-6.7), anti-CD4 (RM4-5), anti-TCRβ (H57-592), anti-CD44 (clone IM7), anti-CD25 (PC61), anti-Ly5.1 (CD45.1; A20), and anti-CD16/CD32 (2.4G2) (Pharmingen, San Diego, CA), Streptavidin-Tricolor (Caltag, Burlingame, CA) and Streptavidin-Cy5 (Zymed Laboratories, San Francisco, CA). FITC-conjugated anti-BrdU moAb (3D4) was purchased from Becton Dickinson (Mountain View, CA). For immunohistology, polyclonal rabbit anti-cytokeratin-5 Ab (Progene GmbH, Heidelberg, Germany), biotinylated mouse anti-cytokeratin-18 moAb (Ks 18.04, Progene GmbH, Heidelberg, Germany), biotinylated UEA-1 lectin (Vector Laboratories, Lausanne, Switzerland), MTS10 (Pharmingen, San Diego, CA), rat anti-MTS24 (supernatant, generous gift of R. Boyd, Melbourne, Australia), polyclonal rat anti-FGFR2 Ab (unpublished; a generous gift from Dr. Sabine Werner, Zürich, Switzerland) and rat IgG2a anti FGFR2IIIb (R&D System, Minneapolis), biotinylated anti-CD80-moAb (16-10A1, Pharmingen, San Diego, CA), were used. The medullary thymic epithelial cell line mTEC2-3 was a gift from Dr. M. Kasai (Tokyo, Japan).

Graft-versus-Host Disease induction

The transplantation model used has been previously described in detail (*142*). In brief, acute GVHD was induced by transplantation of non-irradiated B6D2F$_1$ mice (Ly5.2; H-2^{bxd}) with 25 x 10^6 unseparated parental C57BL/6 splenocytes (Ly5.2$^+$; H-2^b), or with congenic B6.CD45.1 (Ly5.1$^+$; H-2^b) cells. Donor cells were administered in a volume of 400 μl HBSS (Gibco) via tail vein injection. Syngeneically transplanted mice (B6D2F$_1$–>B6D2F$_1$) served as non-GVHD controls and received 25 x 10^6 donor splenocytes.

KGF treatment

KGF treatment in normal mice

C57BL/6 mice were injected i.p. for a period of 3 days with HBSS (Hank's balanced salt solution) or recombinant human KGF (rhKGF; solubilized in HBSS) at a dose of 5 mg/kg per day. rhKGF was produced in E.coli and had an ED50 of 40.02 ng/ml (kindly provided by Amgen, Thousand Oaks, CA, USA).

KGF treatment in the GVHD setting

B6D2F$_1$ mice were injected i.p. for a period of day –3 to +3 after induction of GVHD (day 0) with HBSS (Hank's balanced salt solution) or recombinant human KGF (rhKGF; dissolved in HBSS) at a dose of 5 mg/kg per day.

Flow cytometric analysis

Cell surface marker analysis

Cells suspension from different organs (0.5-1x10^6) were washed, resuspended in 2% FCS/PBS/sodium azide and incubated for 15 min at 4°C with unlabeled 2.4G2 moAb to prevent unspecific binding of the Fcγ receptors. For three-color flow cytometry, cells were first stained with fluorochrome- and biotin-conjugated moAbs and were subsequently labeled with Streptavidin-fluorochrome-conjugated (BD-Pharmingen, Basel or Zymed Laboratories, San Francisco, CA). To exclude dying cells from the analysis 7.5 μg/ml of Propidium Iodid was added to the ready to be analyzed cells. Analysis was performed using a two-laser FACS Calibur (Becton Dickinson, Mountain View, CA).

Analysis of cell proliferation in vivo

Mice were injected i.p. with 5'-bromo-2'-deoxyuridine (BrdU; 1 mg in 0.2 ml PBS; Sigma, Buchs, Switzerland) three and one hours for thymocytes analysis, 48 and 24 hours for thymic epithelial cells analysis before the initiation of the experimental analysis. Thymi were isolated and DNA-synthesizing cells were detected by four-color flow-cytometry, as described previously (*142*). Thymocytes (1x10^6) were stained with a mixture of CyChrome-conjugated anti-TcRβ, -CD8, -CD4 moAbs, PE-conjugated anti-CD44 moAb and Biotinilated anti-CD25 moAb for 30 min on ice. Cells were then washed and incubated with Streptavidin-Cy5 for 15 min. Cells were subsequently permeabilized with ice-cold 0.15M NaCl/95% ethanol for 30 min at 4°C and fixed for another 30 min at room temperature in 1% Paraformaldehyde/PBS

with 0.01% Tween. Cells were then treated with 50 U/ml DNAse I in a 0.15 M NaCl/4.2mM MgCl$_2$ -buffer for 10 min at room temperature. After washing, cells were stained with FITC-conjugated-anti-BrdU moAb for 30 min at room temperature. Washed cells were analyzed using a FACScalibur (Becton Dickinson, Franklin Lakes, NJ, USA).

Detection of donor/host chimerism

To discern donor-derived T cells (Ly5.1$^+$) from host T cells (Ly5.2$^+$), thymocytes and splenocytes were isolated from transplanted and control mice and stained with anti-CD45.1 moAb.

Cell separation

Freshly isolated thymocytes were stained with the appropriate moAbs and then sorted into CD4$^-$CD8$^-$, CD4$^+$CD8$^+$, CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ subpopulations with the use of a FACS-Vantage cell sorter (Becton Dickinson, Franklin Lakes, NJ, USA). For thymic epithelial cells isolation thymic lobes were separated and small cuts were made. Thymic lobes were then stirred two times in fresh IMDM-2 (IMDM+2% FCS) for 30 min in ice. The thymic lobes were transferred in new tubes containing Collagenase IV 1mg/ml+ DNAse 10mg/ml in IMDM-2 and digested at 37°C for 15 min; every 7 min the suspension was mechanically disturbed. After mechanical disruption of the lobes done with a cut glass pipette the supernatant containing thymocytes was discarded and new Collagenase IV 1mg/ml+ DNAse 10mg/ml in IMDM-2 was given to the thymi. After three round of digestion all supernatant was carefully discarded and fresh Collagenase IV 1mg/ml+ DNAse 10mg/ml in IMDM-2 was used for the last digestion round. Thymic lobes were suspended using needle with different diameters and the cell suspension was carefully washed in FACS buffer with 5 mM EDTA before starting the staining procedure. Adherent cells were stained with a combination of anti-IA^b (MHC class II) and CD45 moAbs. IA$^+$CD45$^-$ cells were sorted on a FACSvantage (Becton Dickinson, Franklin Lakes, NJ, USA).

Early thymic emigrants detection

C57BL/6 anesthetized mice were injected in one thymic lobe with 10 μl of FITC (Sigma, Buchs, CH) 125 μg/ml diluted in PBS (Stock solution 1mg/ml). 16 hours later the mice were sacrificed; thymus, lymph nodes and spleen were dissected and cell suspension was analyzed for the presence/absence of FITC$^+$ lymphocytes (CD3$^+$, CD4$^+$ or CD8$^+$).

Histopathology and immunohistology

For detection of FgfR2 and CD80 surface expression, thymi were isolated and embedded

in OCT (Tissue-Tek, Sakura Finetec, Netherlands). Frozen samples were cut into 6 μm thick sections, fixed with 4% paraformaldehyde/PBS and stained with biotinylated anti-FgfR-2 or anti-CD80 antibodies for one hour. After washing, sections were incubated with streptavidin-conjugated horseradish peroxidase. Sections were then incubated with AEC and counterstained with hemalaun. For analysis of thymic morphology, sections were stained with hematoxylin and eosin.

Panels of antibodies and lectins have previously been used to characterize different thymic epithelial cell subsets. In brief, thymic epithelial cell subsets were identified using combinations of anti-cytokeratin 18 moAb and UEA-1 lectin, polyclonal anti-cytokeratin 5 antibody and the epithelial cell-specific MTS-10 antibody. The particular staining protocol was adapted from Klug and coworkers (28) and is listed in more detail in *Table 1*. Two- and three-color immunoflourescent sections were analyzed using a confocal microscope (Carl-Zeiss AG, Feldbach. Switzerland).

TEC subpopulations	CK18	UEA-1	CK5	MTS10
Major cortical TEC	+			
Minor cortical TEC and CMJ	+		+	
Major medullary TEC			+	+
Minor medullary TEC	+	+		

Table 1. Phenotypic analysis of TEC using antibodies again cytokeratins (CK) and the cell surface molecule MTS10 as well as binding of the lectin UEA-1.

Fetal thymic organ culture (FTOC) analysis

Fetal thymic organ culture

From C57BL/6 mice embryo E15.5, thymic lobes were isolated and put in culture on Track-Etch Membrane (Nucleopore #PC MB 13 mm, 0.8 mm pores, boiled in water for 30 min and kept sterile) in presence of 2'-deoxyguanosine (2-dG) at 1.35 mM in IMDM-10 (10% FCS). The fetal thymic lobes were incubate for 6 days in presence of 2-dG, 37°C, 7.5% CO_2; complete thymocytes depletion is achieved. After this point the lobes were washed and starved 24h in IMDM-1 (1%FCS), after starvation the lobes were stimulated with different rhKGF concentration for 24h or longer: than washed. After stimulation I performed different type of analysis:

Proliferation assays

I add ^{3}H-Thymidine for different times and lobes were then digested with 1xTrypsin/EDTA at 37°C for 30 min. Cells were harvested onto a DNA filter (Printed Filtermat A, Wallac) using a Micro 96™ harvester (Skatron Instrument Inc.). After amplification with scintillation fluid, the signal from ^{3}H-labeled DNA was detected with a beta counter (C1205 Beta Plate-Counter™, Wallac).

Reconstitution and phenotype analysis

Fetal liver stem cells were isolated from fetal liver, day 14.5 fetuses. 2.5-5x10^4 fetal liver cells in IMDM-10 were incubated in a Terasaki-plate well together a depleted fetal lobe for 24 hours. After this period the lobes were incubated back on a Track-Etch Membrane in IMDM-10 for at least 20 days and then thymocytes phenotype and thymic epithelial phenotype were analyzed using flow cytometry.

Stimulation and RNA extraction

Depleted thymic lobes were washed and starved o/n in IMDM-1, than stimulated with different factors at different concentrations: - KGF 100 ng/ml (Amgem, Thousand Oaks, CA, USA), - NFkB inhibitor (PS1145) 10 µM (*), - FTase inhibitor (L-778,123) 1 µM (*), - FTase inhibitor (L-779,575) 1 µM (*), - GGTase inhibitor (L-841,491) 10 nM (*), - Fgf20 100 ng/ml (*), - p53 inhibitor 10 µM (*). (*: Generous gift of B. R. Blazar, Minneapolis). Stimulation was carried out for 24 hours and than RNA was isolated.

Isolation of mouse tissue RNA and cDNA synthesis and PCR

RNA isolation

Frozen thymic tissues or frozen cell suspensions were suspended in 1 ml TRI-reagent (Molecular Research Center Inc.) and homogenized with a Polytron homogenizer (Kinematica PT 1200) for approximately 30 sec with increasing speed and all samples were incubated 10 min at room temperature. To extract the aqueous phase, 100 µl (1:10) bromochloropropanol (Molecular Research Center Inc.) was added. Samples were shaken for 10 sec and incubated for another 10 min at room temperature. The samples were centrifuged at top speed in a tabletop microcentrifuge (Eppendorf) at 4°C for 10 min. The aqueous phase was transferred to another Eppendorf tube and precipitated for 1 hour at room temperature with an equal volume of isopropanol (Sigma, Buchs). The samples were centrifuged again at top speed at 4°C for 30 min. The liquid was carefully aspirated and the pellet was resuspended in 1 ml 75%

ethanol, vortexed and centrifuged at top speed at 4°C for 10 min. The liquid was aspirated as much as possible and the pellet was dried. The pellet was then dissolved in 30 μl H_2O and the total RNA concentration was measured with a Gene-Quant machine II (Pharmacia Biotec).

cDNA synthesis from total RNA

To obtain cDNA, total RNA samples (4 μg) were mixed with 18 μl H_2O containing 500 μM dNTP, 10 mM DTT, 1 μl DNAse I and 1 $μl$ of 1x1st strand buffer. This mixture was incubated for 30 min at 37°C. After the incubation, the oligo dT_{20} primer was added at a final concentration of 500 nM and the mixture was heated for 5 min at 70°C. The samples were quickly centrifuged at room temperature. Thereafter, 200 units of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase was added and the samples were incubated at 42°C for 1 h. The reaction was quenched for 5 min at 95°C and then the samples were diluted in aliquots at a concentration of 20 ng/μl and stored at –70°C. All buffers are from Gibco BRL, Basel, CH.

Polymerase chain reaction (PCR)

For end-point PCR analysis of FgfR2IIIb mRNA expression, total RNA was isolated from whole thymic tissue, freshly isolated thymic epithelial cells, established thymic epithelial cell lines or thymocyte subpopulations (where indicated) were isolated. After reverse transcription, the cDNA was amplified for 38 cycles.

For quantitative PCR analysis, total RNA was isolated from unseparated thymic tissue at day 13 after transplantation. RNA was reverse transcribed and the resulting cDNA was amplified in a total volume of 25 $μl$ buffer containing 20 ng cDNA, 1 x SYBR Green ™ PCR Master Mix (PE Biosystems, Rotkreuz, Switzerland), and 300 nM forward and reverse primer each. Primers for real-time PCR were designed according to published mouse RNA sequences. As an internal reference for thymic epithelial cells, the gene for the epithelial V-like antigen (EVA) (*148*) was amplified, whereas GAPDH or S18rRNA was used as an internal reference for all cells. The cycle conditions were: 50°C for 2 min followed by 95°C for 10 min, after which 40 cycles of amplification were carried out (95°C for 15 sec, 60°C for 1 min). The PCR reaction was performed in a GeneAmp 5700 SDS Real Time PCR machine (AB Applied Biosystems). Samples were analyzed in triplicates, and the result was averaged.

Primers used

Mip-1b: sense TGCTCGTGGCTGCCTTCT,

anti-sense CAGGAAGTGGGAGGGTCAGA;

Mip-1a: sense TTTTGAAACCAGCAGCCTTTG,

anti-sense TCTTTGGAGTCAGCGCAGATC;

TECK: sense CAGCACAGGATCAAATGGAATG,

anti-sense GGTTGCAGCTTCCACTCACTT;

IL7: sense GGGAGTGATTATGGGTGGTGAG,

anti-sense TGCGGGAGGTGGGTGTAG;

Aire: end point PCR sense GGTTCTGTTGGACTCTGCCCTG,

anti-sense TGTGCCACGACGGAGGTGAG;

Granzyme B: sense GACCAGCTCTGTCCTTGGCA,

anti-sense ATGTCAGTTGGGTTGTCACAGC;

CD80: sense GAGTCTGGAACCCATCTGCA,

anti-sense GAAGCGAGGCTTTGGGAAAC;

KGFR: sense CACTCGGGGATAAATAGCTC,

anti sense GTCCTTCTCTGTGGCATCAT.

IL7: real time sense GAAGACCCAGCGCAAAGTAGA,

anti-sense CACGCCTGGTCCATCCAT

IL7R: real time sense AGGCCTCCTGTGTATCAAG,

anti-sense GACATGGTGACATACGCTTCT

Shh: real time sense   GGAACTCACCCCCAATTA,

anti-sense TGCTCGACCCTCATAGTGTA

Wnt4: real time sense ACCCAAAAACCAAACCAAAACA,

anti-sense GTGGCTGCAGGTTCCACTG

Wnt5b: real time sense AGGAAGTTGGCTGCACACG,

anti-sense CCGCTTTGGAAGATGTTGGT

Wnt10b: real time sense TCCTCCACTACAGCCCAGAACCT,

anti sense TTATCCATTCCCGCCCTTCCT

BMP2: real time sense ATGACTGGATCGTGGCACCT,

anti sense BMP2 CAGCATGCCTTAGGGATTTTG

BMP4: real time sense AAGAACTGCCGTCGCCATT,

anti sense TGGCATGGTTGGTTGAGTTG

AIRE: real time sense CCAGTGAGCCCCAGGTTAAC,

anti sense GACAGCCGTCACAACAGATGA

FoxN1: real time sense AACAGGCAATGAGGCAGGTG,

anti sense TAGAGAGTGCTGAGGTGCATGTC

The transcript expression was normalized to:

S18rRNA: real time sense CGCTATTGGAGCTGGAATTACC,

anti sense GCCCTGTAATTGGAATGAGTCC

GAPDH: real time sense ACCATGTAGTTGAGGTCAATGAAGG,

anti sense GGTGAAGGTCGGTGTGAACG

EVA: sense GTGCCGCCTGCTCGTC,

anti-sense CCGAACATCTGTCCCGTTGA

Statistical analysis

Groups were compared by one-way ANOVA. Where ANOVA revealed a significant difference, the Bonferroni/Dunn post hoc test was performed. The overall statistical significance level was set to 5%.

For 2 groups comparison where the data were not "Gaussian"-distributed the Mann-Whitney test was used.

StatView™ from SAS Institute Inc. was used for statistical analysis.

Results

The effect of KGF on thymic epithelial cells

Thymic epithelial cells are crucial for the complex events of T cell development as these cells control both proliferation and differentiation of thymocytes. This capacity is shared to various degrees with other stromal cells such as dendritic cells, macrophages and thymic fibroblasts. Damage by radiation, chemotherapy, or T cell-mediated allo-recognition may, therefore, severely affect both composition and function of the thymic stromal cells and may promote changes in normal thymic function. Thus, the protection of the thymic stroma in general and of thymic epithelial cells in particular may specifically prevent the loss of thymic function in the context of HSCT.

In view of this documented protective role for epithelial cells of the intestine and the lungs, KGF may also exert a beneficial effect on thymic epithelial cells. In a first series of experiments, the effect of KGF was, therefore, tested on thymic epithelial cells of unchallenged, healthy mice. In a second series of experiments, a murine transplantation model was investigated where the transfer of haploidentical T cells results in severe thymic pathology affecting both lymphoid and stromal cells.

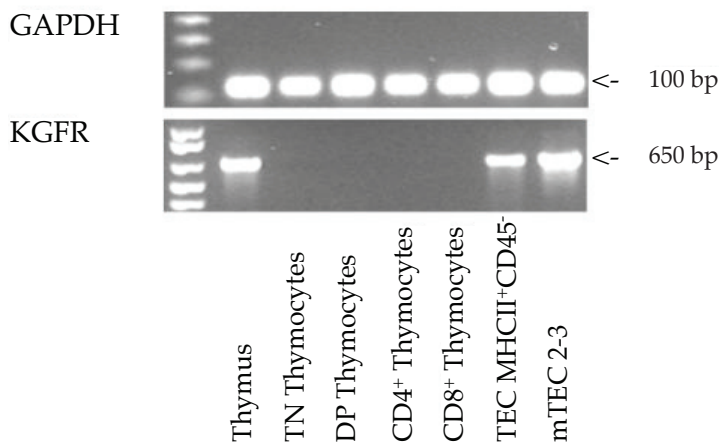*The receptor for KGF, FgfR2IIIb, is expressed on thymic epithelial cells but not on thymocytes*

Figure 1 Detection of FgfR2IIIb specific transcripts in unseparated thymic tissue, purified thymocytes at distict stages of development, freshly isolated thymic epithelial cells and an established medullary epithelial cell line.

A beneficial effect of KGF on thymic T cell development may either be effected directly through binding of KGF to its specific receptor expressed on thymocytes or, alternatively, may be accomplished indirectly by stimulation of thymic stromal cells. To define the thymic target cell(s) of KGF activity, thymocytes at all maturational stages and thymic epithelial cells were analyzed by end-point RT-PCR for transcripts

specific for FgfR2IIIb, also known as KGF receptor (KGFR). As demonstrated in *Figure 1*, transcripts for FgfR2IIIb were exclusively detected in freshly isolated thymic epithelial cells (as defined by the cell surface expression of MHC class II and by the lack of CD45 positivity) and in an established thymic medullary epithelial cell line (mTEC 2-3) ((*149*)). In contrast, none of the purified thymocyte subpopulations harbored mRNA specific for FgfR2IIIb. The selective detection of transcripts for the receptor in thymic epithelial cells but not cells of hematopoietic origin was further ascertained using immunohistology. For this purpose, a polyclonal antibody was used that had been raised against YDINRVPEEQMTFKDLVS peptide and that specifically recognizes all splice variants of FgfR2 (S. Werner, personal communication). As
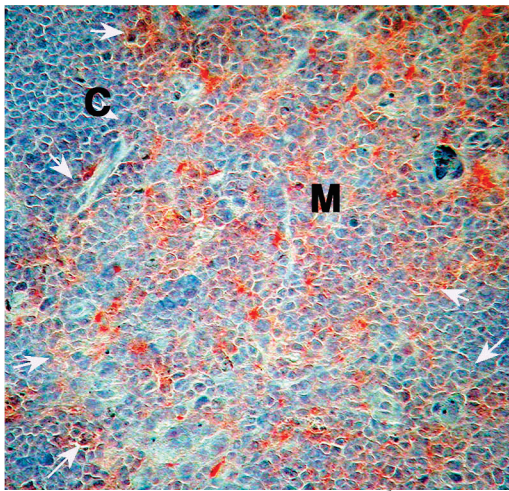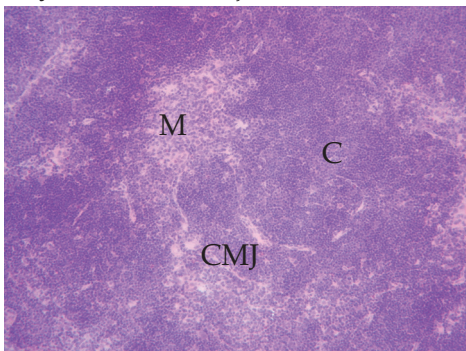

Figure 2 Expression of FgfR2 on thymic section. Arrow: expression of FgfR2 on cortical epithelial cells. C: cortex. M: medulla.

demonstrated in *Figure 2*, epithelial cells but not thymocytes stained positively with this antibody that also recognizes the FgfR2IIIb splice variant. Specific signals were abundant in the medulla where thymic epithelial cells form a tight cellular meshwork. In contrast, positive staining was sparse in the cortex, a finding, which is in keeping with the relatively low abundance of epithelial cells in this anatomical compartment. Taken together, these results demonstrate that FgfR2IIIb is selectively expressed on epithelial cells within the thymus.

The cellular composition and architecture of the thymic epithelial cell compartment has been noted to be critical for normal function. It is for this reason that the influence of KGF on the thymic stromal composition and organization was investigated in treated mice. Thymic epithelial cells can be phenotypically differentiated into four distinct subpopulations based on binding of UEA-1 lectin and the use of antibodies that characterize cell surface antigen designated MTS-10, cytokeratins (CK) 5 and 18: (i) major cortical thymic epithelial cells (CK18+, CK5-, UEA-1-, MTS-10-), minor cortical thymic epithelial cells (CK18+, CK5+), major medullary

Thymus: Saline injected mouse   Thymus: KGF injected mouse
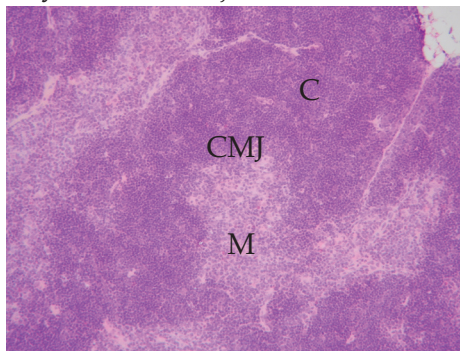

Figure 3 KGF treatment does not modify thymic architecture of cortex and medulla. HE staining of thymic sections; C:cortex, M: medulla, CMJ: cortico-medullary junction.

thymic epithelial cells (CK5+, MTS-10+), and minor medullary thymic epithelial cells (CK18+, UEA-1+) ((28)) (see *Table 1*). For the phenotypic analysis of the thymic epithelial stroma, mice were injected with KGF for three consecutive days at a dose of 5 mg/Kg/day and thymic tissue was removed two
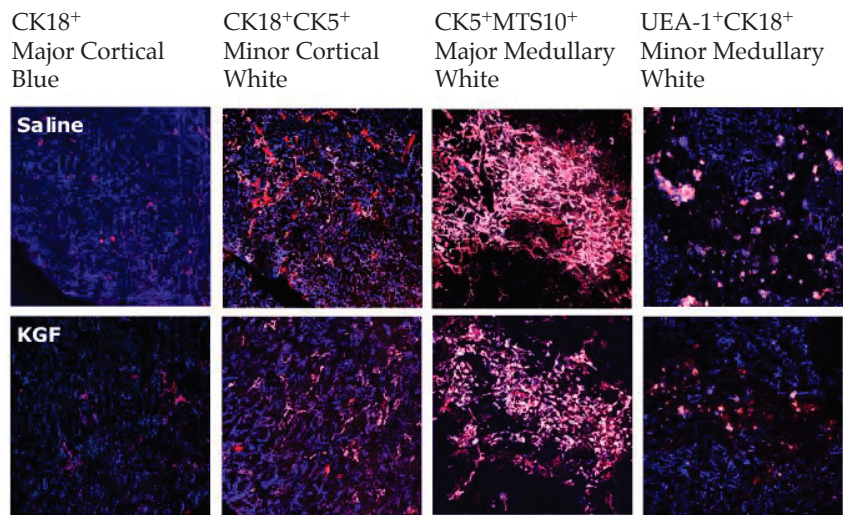

Figure 4 KGF does not alter the phenotype nor composition of thymic epithelium.

weeks later, frozen in OCT and processed for immunohistology. Comparing thymic tissues of mice treated with KGF or, alternatively, with HBSS (Saline control), revealed the same results in the make-up and architectural organization of the epithelial compartment (*Figure 3*). Thus, *in vivo* treatment with KGF did not qualitatively alter the epithelial stroma and its microenvironmental organization (*Figure 4*). However, thymic cellularity had doubled in response to KGF treatment (see below) although the relative distribution of thymocytes into the four distinct subpopulations characterized by the differential expression of CD4 and CD8 remained unaffected (*Figure 5*). Taken together, these results demonstrate that *in vivo* treatment with KGF increases thymic cellularity in a proportional fashion affecting both stromal as well as lymphoid cell lineages.
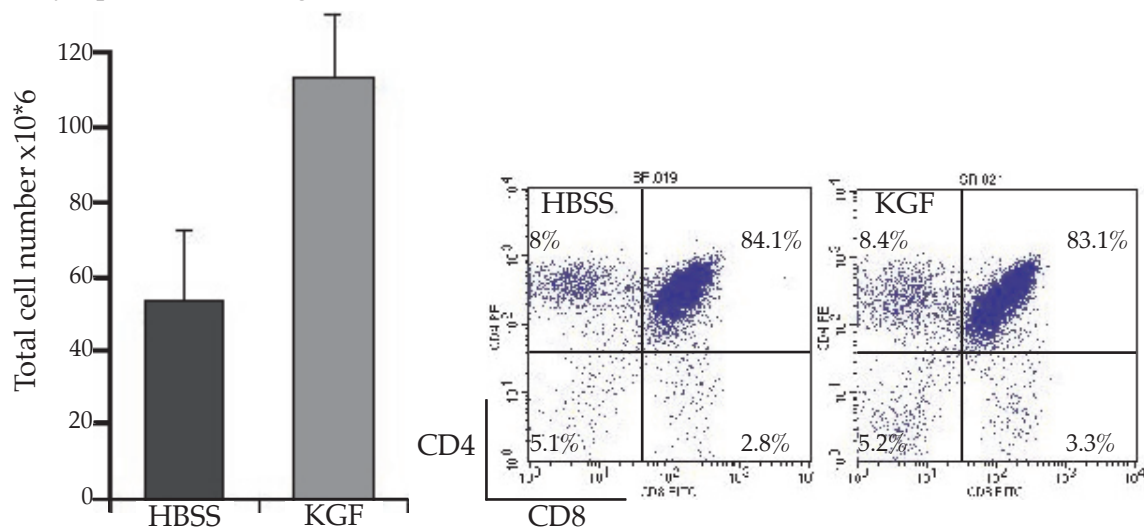

Figure 5 KGF doubles the thymic cellularity in 15 days, but does not alter the phenotype nor composition of CD4/CD8 thymocytes.

Next, the FgfR2IIIb expression on thymic epithelial cells was analyzed by confocal microscopy
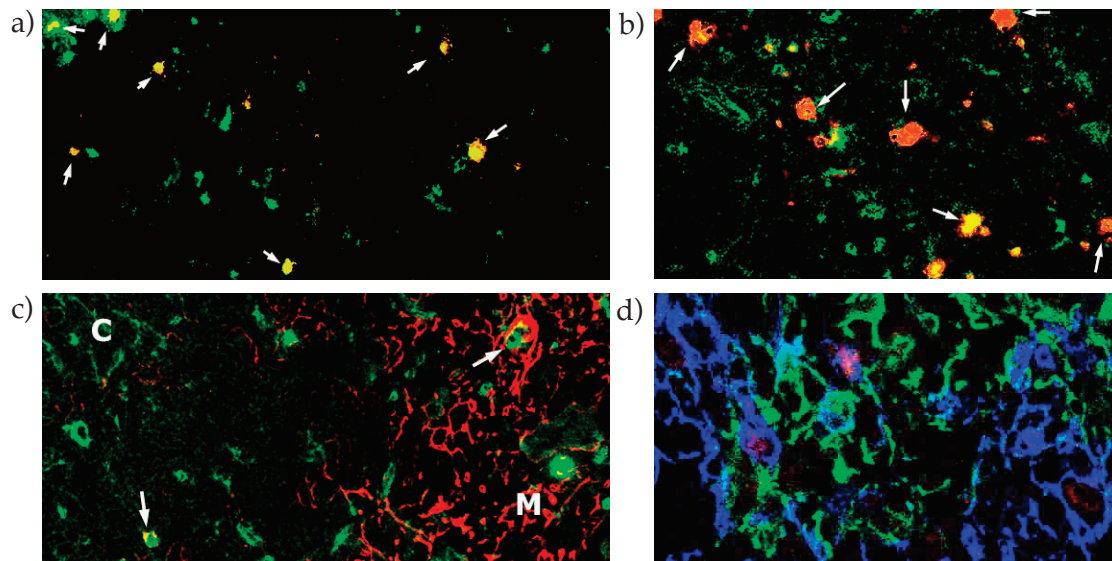
Figure 6 Expression pattern of FgfR2IIIb in the thymus. a) All MTS24+ cells are positive for FgfR2IIIb. Red: MTS24; green: FgfR2IIIb; yellow: colocalization. b) All UEA-1+ cells are positive for FgfR2IIIb. Red: UEA-1; green: FgfR2IIIb; yellow: colocalization. c) Only few epithelial cells of the medulla are positive for both CK5 and FgfR2IIIb; in addition few cortical cells also express both markers. Red: CK5; green: FgfR2IIIb; yellow: colocalization. d) At the CMJ minor cortical epithelial cells express FgfR2IIIb. Green: CK5; Blue: CK18; red: FgfR2IIIb; pink-white: colocalization.

and related to the expression of epithelial cell-specific markers detailing either a cortical or a medullary phenotype. As demonstrated in *Figure 6*, the expression of FgfR2IIIb could be co-localized to MTS24$^+$ cells, a population of epithelial cells known for its potential to serve as a precursor to all thymic epithelial cell types. In addition, all UEA-1$^+$ cells expressed also the KGF receptor (*Figure 6b*). In contrast, only a small proportion of CK5$^+$ medullary epithelial cells were also positive for the presence of FgfR2IIIb, and among those, an even smaller fraction of cortical epithelial cells stained positively for the expression of this receptor (*Figure 6c and d*). Finally, a small population of minor cortical epithelial cells expressing concomitantly the cell surface markers CK5 and CK18 were positive for FgfR2IIIb expression. Taken together, the receptor specific for KGF is expressed on medullary epithelial cells and on a small subpopulation of major and minor cortical epithelial cells. Moreover, all cells with the phenotype of the putative thymic epithelial stem cell also express FgfR2IIIb.

KGF induces in vitro the proliferation of a thymic epithelial cell line

KGF has been noted to act as a mitogen on keratinocytes (*150*). To assess a similar effect of KGF on thymic epithelial cells and to correlate a possible increase in thymic cellularity to KGF-mediated cell proliferation, established thymic epithelial cell lines were first tested *in vitro* for their proliferative response to KGF. For this purpose, the thymic epithelial cell lines TEM 2.3 and C6 were grown in 96 well plates to semi-confluence and subsequently serum-starved for 24 hours before exposure for an additional 24 hours to KGF at various
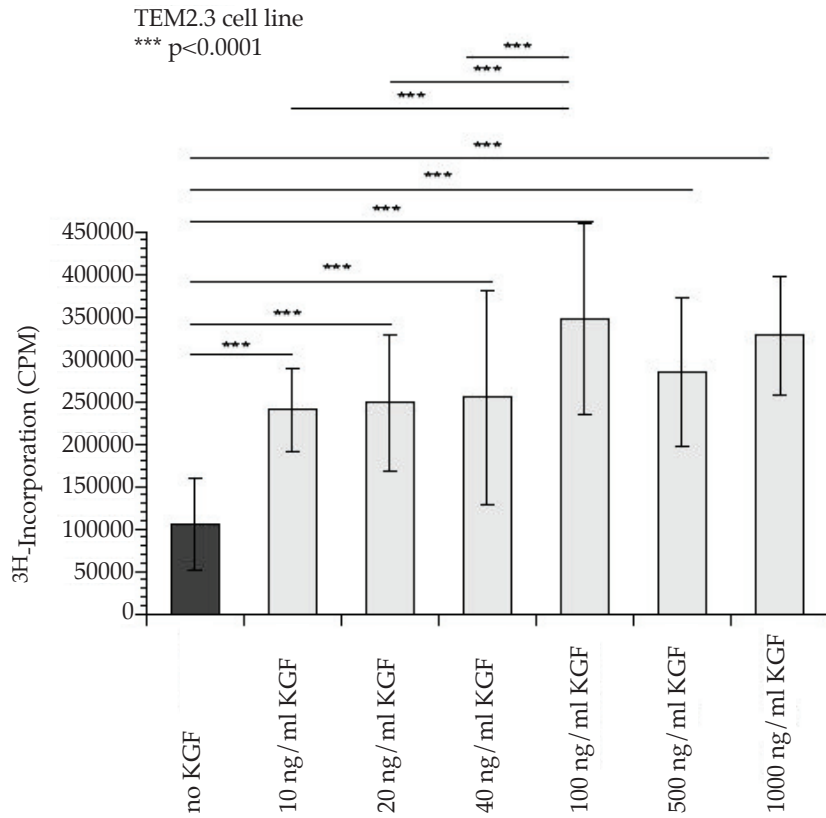
Figure 7 The thymic medullary cell line, TEM2.3, proliferate *in vitro* in response to KGF. Statistic analysis denote a significant increase in proliferation of KGF stimulated cells (grey bars) in comparison to un-treated cells (black bar). Statistic: Anova, post-hoc-test: Bonferroni. ***p<0.0001

concentrations. Proliferation was measured by ^{3}H-Thymidine incorporation. After 24 hours of KGF stimulation, the two medullary thymic epithelial cell lines demonstrated an increased proliferation rate, although a difference in this response was noted when comparing the two
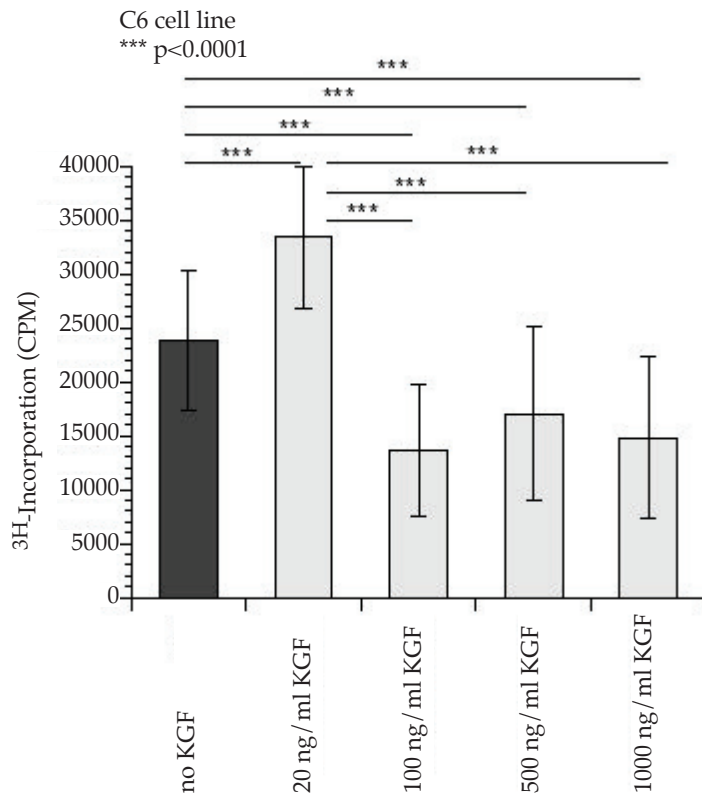

Figure 8 The thymic medullary C6 cell line, proliferate *in vitro* in response to KGF. Statistic analysis denote a significant increase in proliferation of KGF stimulated cells (grey bars) in comparison to untreated cells (black bar). Statistic: Anova, post-hoc-test: Bonferroni. ***p<0.0001

cell lines. TEM 2.3 cells responded best to KGF at a concentration of 100 ng/ml (*Figure 7*), while C6 cells proliferated at 20 ng/ml, but failed to do so at higher concentrations (*Figure 8*). The KGF-mediated increase in proliferation was already apparent 24 hours after stimulation.

KGF induces ex vivo the proliferation of fetal thymic stromal cells and inhibits the reconstitution with thymocytes of lymphoid cell-depleted fetal lobes

To assess the biological effect of KGF on thymic epithelial cells on a more physiological *ex vivo* setting, stromal cell proliferation was measured in fetal thymic organ cultures (FTOC). To this end, fetal thymic lobes were removed from embryos at day 14.5 of gestation and cultured on a floating membrane for 6 days in the presence of 1.25 mM 2-deoxyGuanosin (2-dGuo) to deplete thymocytes. Following this treatment, fetal lobes were grown for two days in the absence of serum whereby KGF at a concentration of 100 ng/ml was added for the last 24 hours of culture. Finally, the medium was exchanged and proliferation was measured by ^{3}H-Thymidine incorporation at 48, 72, and 96 hours after pulsing. For a more reliable measurement, individual lobes were digested with collagenase IV, which generated a single cell suspension of 90 % epithelial cells and 10 % mesenchymal cells. In keeping with the results observed with thymic epithelial cell lines, primary stromal cells exposed to KGF displayed an increased proliferation as early as 72 hours after stimulation with KGF when
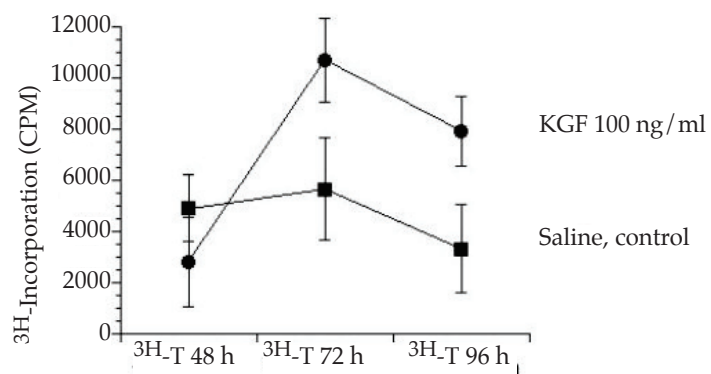

Figure 9 KGF induces proliferation of fetal thymic stromal cells in thymocytes depleted lobes from E14.5 embrios cultured *ex vivo*.

compared to unstimulated cells (*Figure 9*, n=8). Comparable results were also obtained from day 15.5 embryos thymic lobes (data not shown).

Since *in vivo* treatment with KGF resulted in an increased thymocyte cellularity, it was next determined whether treatment with KGF of 2-dGuo exposed thymic stromal cells resulted in a faster kinetic in lymphoid repopulation. To this end, FTOC with lobes from mice at day 15.5 of gestation were depleted for 6 days with 2-dGuo, washed, serum-starved overnight and then stimulated for 48 hours with different concentrations of KGF (50, 100, and 500 ng/ml). Subsequently, the single lobes were reconstituted overnight in Terasaki plates, using hematopoietic precursor cells derived from fetal liver of embryos at day 14.5 of development. Reconstituted lobes were then transferred to floating membranes and grown in complete medium (IMDM + 10% fetal calf serum) for 20 days. At the end of culture, four individual lobes were

pooled, a single cell suspension was generated by collagenase IV/DNAse and the cells were analyzed for cell surface expression using flow cytometry (*Figure 10*). A robust reconstitution of the lymphoid compartment was observed in lobes reconstituted cultured in the absence of KGF. There, the distribution of the thymocyte subpopulations (as defined by CD4 and CD8 expression) revealed an abundance of DP thymocytes but also the presence of TN and SP cells. The over-representation of TN and CD8 SP cells in comparison to age-corrected thymic tissue has already been seen previously (*151*) and has been explained by the
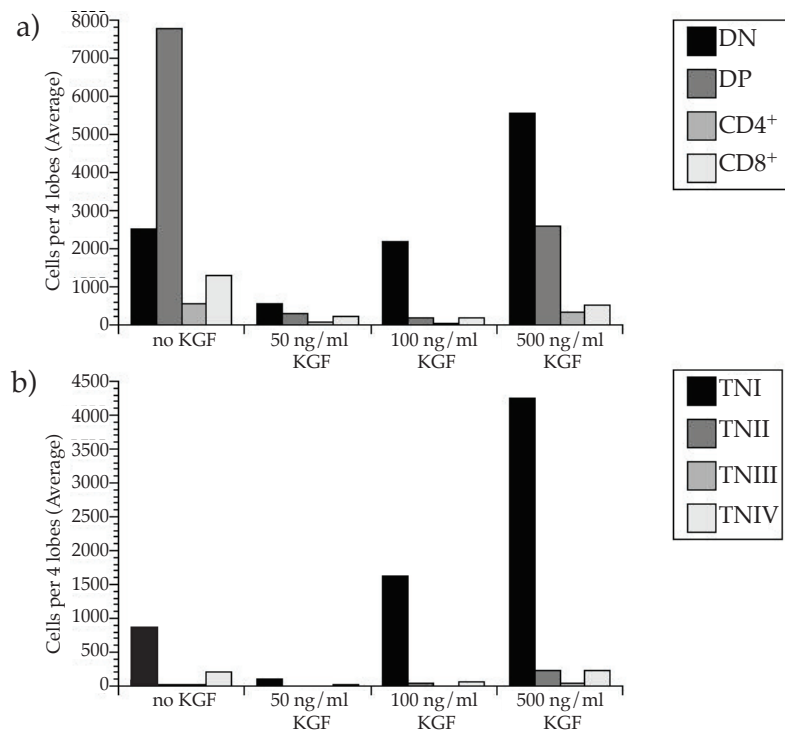

Figure 10 The repopulation and differentiation of the lymphoid lineage in 2-dGuo treated thymic lobes repleted with fetal liver cells, is inhibited in the presence of KGF. *Representative experiment, each bar is the average of 2 samples and each sample is the pool of 4 lobes.* a) Separation of thymocytes subpopulation according to CD4 and CD8 expression. b) Separation of CD4$^-$CD8$^-$CD3$^-$ thymocytes into developmental stages TNI-IV according to CD25 and CD44 expression.

constraints of the experimental system used. In contrast, thymic lobes pre-treated with KGF displayed a dramatically different pattern in lymphoid reconstitution. Here, intrathymic T cell development was partially blocked at the TNI stage and the progression to the maturational cell stages of DP and SP were in consequence severely altered in a dose-dependent manner. The increase of TNI cells was in direct relationship to the concentration of KGF used. In contrast, the suppression in the emergence of DP thymocytes was less effective in instances where higher KGF concentrations were used while changes in the absolute number of SP thymocytes was low in the presence of KGF and only mildly increased in cultures supplemented with the highest KGF concentrations. Taken together, these results demonstrate that KGF exerts a mitogenic effect on thymic stromal cells. This stimulatory effect appears to change the conditions for fetal support of fetal thymopoiesis in a fashion that leads to accumulation of the most immature intrathymic T cell precursors and a partial block in their developmental progression to more mature stages.

KGF induces in vivo proliferation of thymic epithelial cells

The proliferative response of adult thymic epithelial cells was also investigated in mice treated for three consecutive days either with KGF or HBSS (the latter serving as a control group).
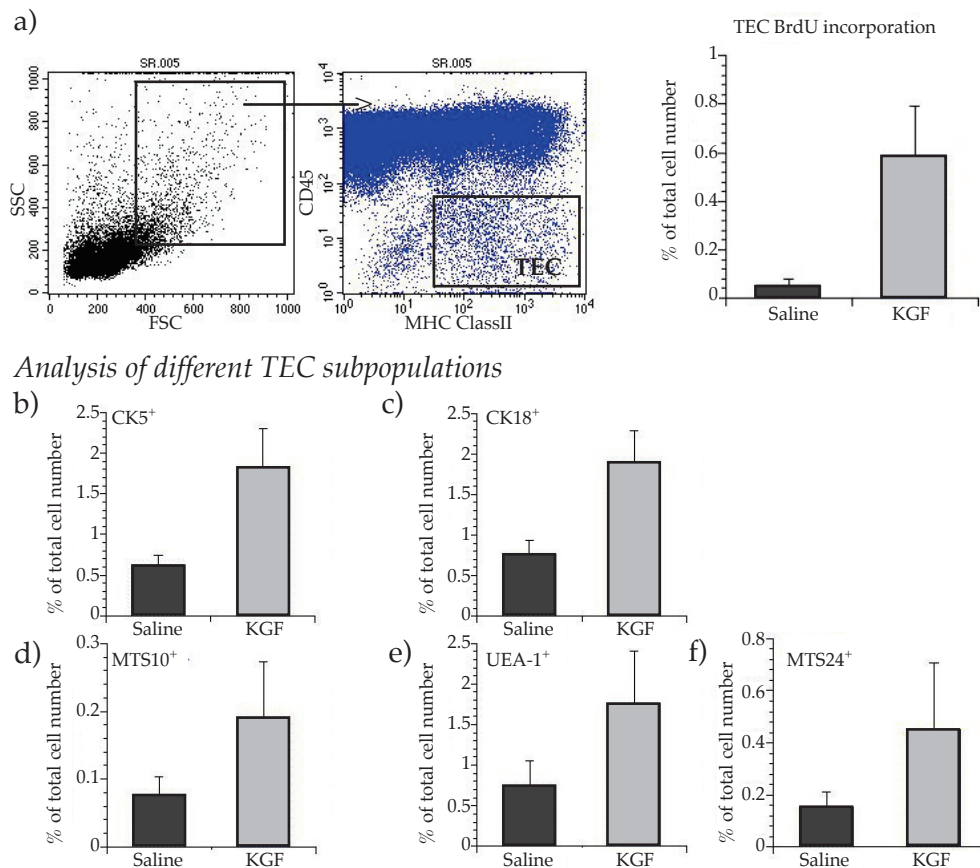

Analysis of different TEC subpopulations

Figure 11 a) KGF induces the proliferation of adult thymic epithelial cells. Staining for TEC and BrdU incorporation in TEC compartment. b)-f) KGF increase the relative cell numbers for different TEC subpopulations. b) CK5. c) CK18. d)MTS10. e) UEA-1. f) MTS24.

The injected mice were also pulsed i.p. with 1 mg BrdU on the second and third day of KGF treatment. Ninety-six hours after initiation of treatment, mice were sacrificed and the thymus was removed for further analysis by flow cytometry. To this end, the tissue was enriched for epithelial cells with a sequence of enzymatic digestion (as detailed in the "methods" section) and a single-cell suspension was prepared. Thymic epithelial cells were identified as large cells that express MHC class II molecules but are devoid of CD45 (see *Figure 11a*). An increased number of all mature thymic epithelial subpopulations was noted when stimulated by KGF (*Figure 11*). Thus, KGF given on three consecutive days to adult mice results in an increased proliferation (BrdU incorporation) of all thymic epithelial cells (CD45^{+} MHC Class II^{+}, *Figure 11a*) and all subpopulations tested, including the rare population of MTS24^{+} cells, which are thought to serve as precursors to all cortical and medullary epithelial cells, show increased

cellularity (*Figure 11b-d*).

Taken together, this series of *in vivo*, *ex vivo*, and *in vitro* experiments could unequivocally demonstrate that KGF exerts a proliferative effect on fetal and adult thymic epithelial cells. This activity is in keeping with the expression pattern of FgfR2IIIb exclusively on thymic epithelial cells. In view of this biologically relevant effect on thymic epithelial cells, it is conceivable that KGF may be beneficial to protect or correct damages to thymic epithelial cells in the course of allogeneic bone marrow transplantation.

Gene expression profiling of thymic epithelial cells stimulated with KGF

In order to begin to elucidate the genetic program activated by and accounting for the effects of KGF signaling, adult thymic epithelial cells exposed *in vivo* to KGF were analyzed for the expression of specific genes previously noted to play an important role in thymic epithelial cell development and function. For this purpose, mice were treated with a single dose of KGF (15 mg/Kg) or HBSS (as a control) and sacrificed 24 hours later. Epithelial cells were enriched after digestion of thymic tissue and sorted by flow cytometry to purity (>95 %), using MHC class II cell surface expression and the absence of CD45 as selection criteria. Following mRNA isolation and synthesis of cDNA, the presence of transcripts specific for IL-7, Wnt4, Wnt5b, Wnt10b, BMP2, and BMP4 was quantified by real-time PCR. As demonstrated in *Figure 12*, transcripts specific for IL-7 were increased by 16-fold in consequence of KGF stimulation. The increase in transcripts for the different Wnt family members varied considerably: Wnt4 and Wnt10b were upregulated 1.5-
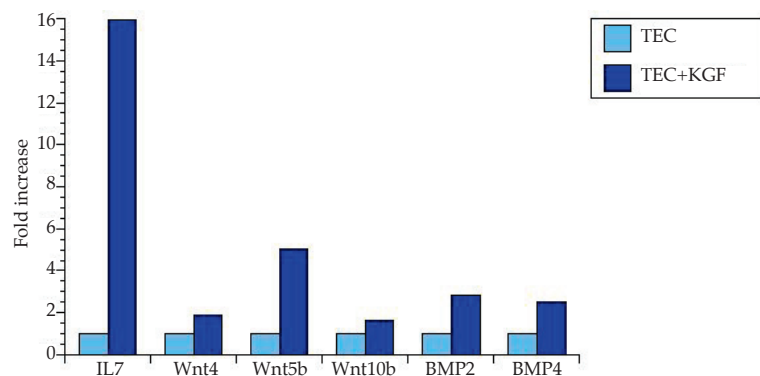

Figure 12 Gene expression profile of adult TEC 24 hours after stimulation with KGF. Value in HBSS-stimulated cells were set to 1 for comparison and data were normalized to the expression of GAPDH.

to 2-fold, while Wnt5b specific transcripts were increased in response to KGF signaling by almost 5-fold. Moreover, a 2- and 2.5-fold increase was also observed for BMP4 and BMP2, respectively. Since IL-7, Wnts and BMPs have previously been implicated in intrathymic T cell maturation, it is not unlikely that changes in the expression of these molecules following KGF signaling may exert a direct influence on thymocyte development. In particular, stimulation via IL-7 has been reported to be important for the survival of TN thymocytes and signaling via Wnt-specific receptors facilitates the transition of TN cells to a DP phenotype (*152*).

Signaling via BMP2 and BMP4 exert an inhibitory effect and block in the progression from DN to DP (*153, 154*), particularly the progression of TNI to TNII. Thus, KGF stimulation of thymic epithelial cells allows for the transcriptional upregulation of several genes that affect intrathymic lymphoid cell development.

To investigate in fetal thymic epithelial cells the effect of KGF signaling on gene expression profiles, specific transcripts were quantified in cells of FTOCs exposed either to KGF or to vehicle alone. To this end, thymic lobes of embryos at day 15.5 of gestation were first cultured in the presence of 2-dGuo for six days to deplete thymocytes and then grown for two days in medium containing only 1 % fetal calf serum. During the last 24 hours of culture, the medium was supplemented with KGF or Fgf-20 in the presence (*Figure 13b*) or absence (*Figure 13a*) of specific biochemical compounds inhibiting defined signaling pathways. Signaling via NFκB was inhibited by PS1145 (10 μM), farnesylation of Ras was prevented by the farnesyl transferase inhibitors L-778.123 (1 μM) and p53 function was blocked by a p53 inhibitor (all these substances were kindly provided by Bruce Blazar, Minneapolis). Following culture, cDNA was generated and analyzed by quantitative PCR for the presence of transcripts specific for IL-7R (IL7 receptor), FoxN1, Wnt4, Wnt5b, Wnt10b, BMP2, and BMP4. As demonstrated in *Figure 13a*, stimulation of fetal stromal cells for 24 hours with KGF resulted in a marked-up regulation of transcripts for IL-7R, Wnt10b, BMP2 and BMP4. In contrast, Fgf-20 signaling resulted only in a limited upregulation of transcripts for IL-7R, Wnt10b, and BMP2 in thymic stromal cells of FTOC when compared to the effect observed with exposure to KGF.

The biochemical pathways involved in KGF-mediated upregulation of IL-7R, Wnt10b, BMP2 and BMP4 were next investigated. Upregulation of IL-7R was not only sensitive to inhibition of Ras farnesylation but was partially blocked by inhibition of p53 (*Figure 13b*). Signaling to upregulate Wnt10b involved NFκB and p53 as the functional inhibition of either these molecules resulted in a block in KGF-mediated transcriptional activation (*Figure 13b*). Transcripts for BMP2 and BMP4 failed to be upregulated in response to KGF in the presence of inhibitors for Ras farnesylation and for p53 (*Figure 13b*). Moreover, blocking of NFκB inhibited also the upregulation of transcripts specific for BMP4 but not for BMP2 (*Figure 13b*). Taken together, these studies revealed, at least for fetal thymic stromal cells, that different intracytoplasmic signaling pathways are used for the transcriptional upregulation of distinct gene products.
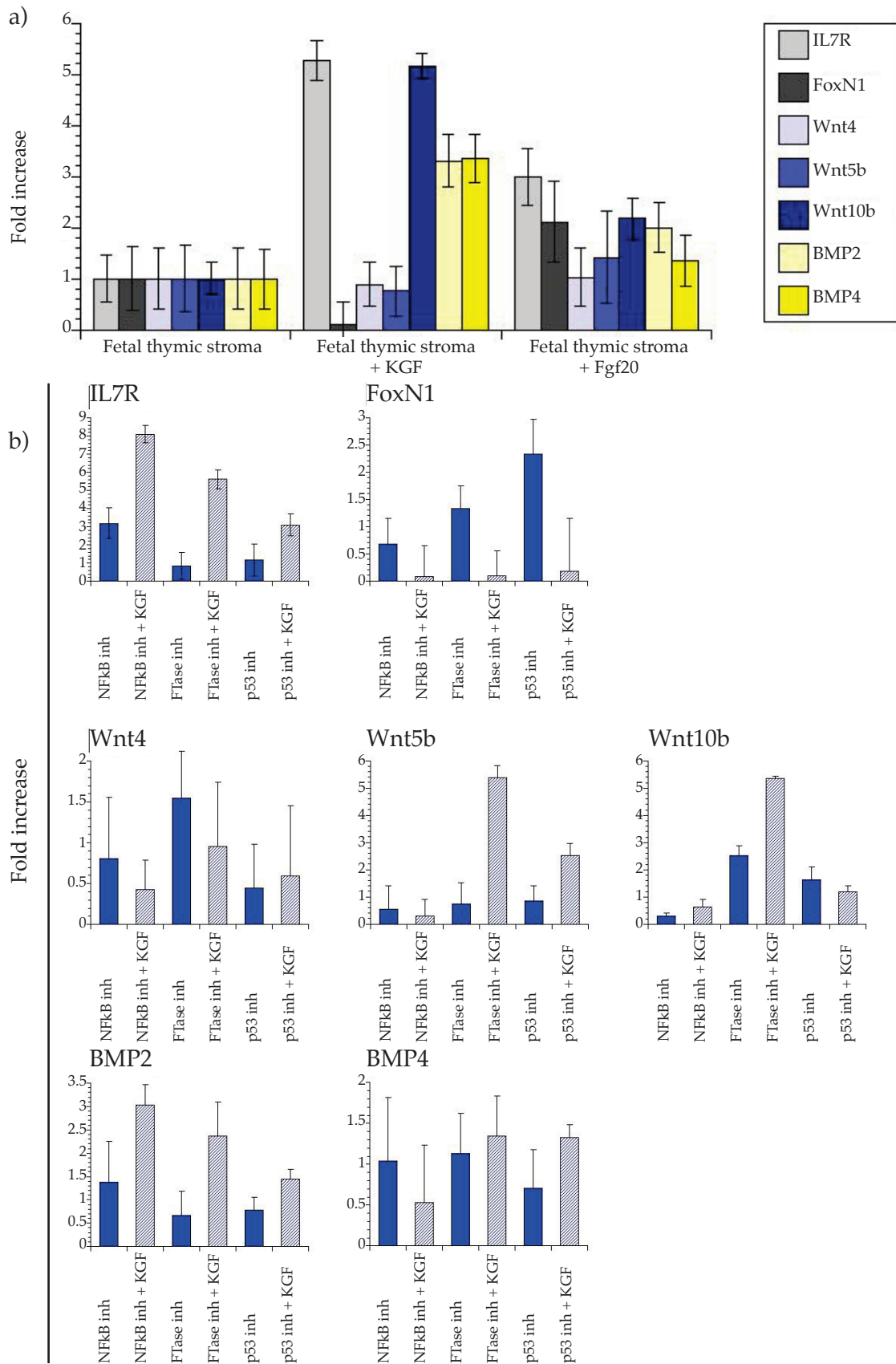
Figure 13 Gene expression profile of fetal thymic stromal cells indicated as fold increase after real time PCR. a) Stroma cells stimulated either with KGF or Fgf20. b) Transcripts analysis after KGF stimulation in combination with NFkB inhibitor, Farnesyl transferase inhibitor (FTase) or p53 inhibitor.

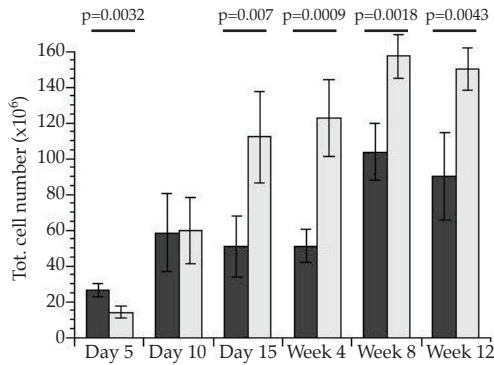# KGF stimulation affects indirectly also thymocyte development

Figure 14 KGF increases thymic cellularity. Mice were injected for 3 consecutive day with KGF (5mg/kg/day, grey bars, n=6, per time point) or HBSS (black bars, n=4, per time point) and analyzed at the indicated times. Statistic: Anova, post-hoc-test: Bonferroni.

In vivo KGF treatment of adult mice resulted in a robust increase in total thymic cellularity. As the overall histological structure of treated thymi remained unchanged and as the analysis of the stromal compartment revealed a proportional increase in the number of thymic epithelial cells, it was conceivable that also cells of the lymphoid compartment had an increase in response to KGF. To address this point directly, female mice C57BL/6 at 4 – 6 weeks of age were injected on three consecutive days with a single dose of KGF (5 mg/Kg/day). Thymocytes cellularity and phenotype were subsequently analyzed at 5, 10, and 15 days as well as 4, 8, and 12 weeks after initiation of treatment. These results were compared

to findings of HBSS injected mice investigated at identical times. As demonstrated in *Figure 14*, injection with KGF resulted in a significant increase in thymocyte cellularity when compared to HBSS injected controls. This change was evident as early as day 15 after treatment and persisted for at least 12 weeks. However, absolute cell numbers were greatly reduced on day 5 in

both KGF and HBSS treated animals when compared to unmanipulated or HBSS injected mice investigated at 10 or more days after the onset of the experiment. This early reduction in cellularity is most likely caused by stress associated with the handling and injection of the mice and thus
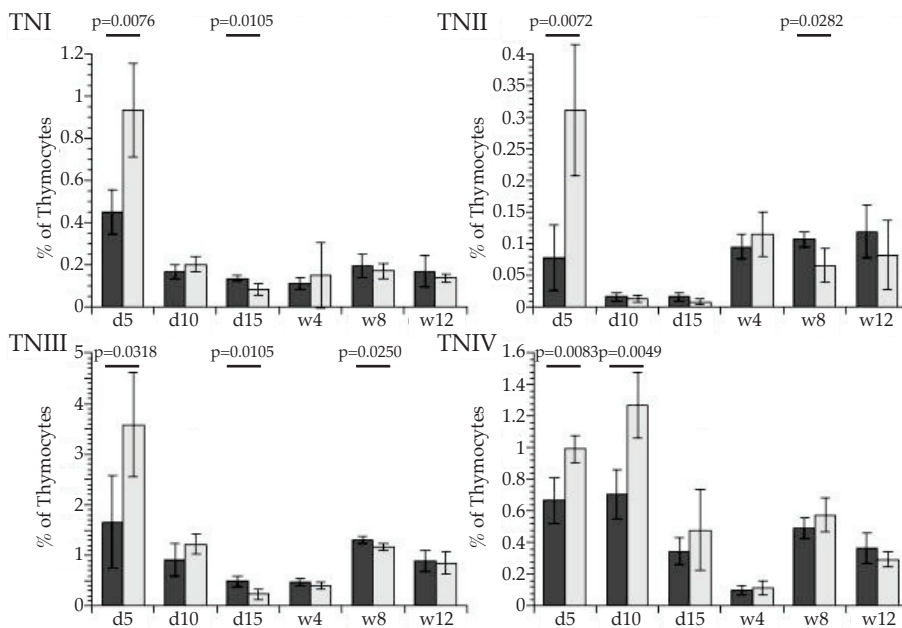
Figure 15 KGF influence TN development shortly after treatment. KGF treated mice: grey bars, HBSS treated mice: black bars. Statistic: Anova, post-hoc-test: Bonferroni.

independent of the biological activity of the components used. Despite the lack of a difference between mice injected with KGF and HBSS, respectively, an ANOVA statistical analysis combined with a Bonferroni Post-hoch test revealed statistically significant difference in thymocyte cellularity at all time point analyzed, excluded day 10, between the two treatment groups (p<0.05 are indicated in the *Figure 14*).

Next, changes in the relative frequency of thymocytes were correlated to specific developmental phenotypes. As demonstrated in *Figure 15*, a difference between the two treatment groups regarding the relative number of thymocytes at distinct maturational stages was only observed on day 5 for all TN subpopulations and on day 10 for TNIV. At all other time points, the measurements of the other thymocyte subpopulations failed to reveal a difference when comparing KGF and HBSS treated mice. These results support the conclusion that KGF treatment causes a proportionally correct expansion of all thymic cell lineages.

The observed increase in cell with the different TN phenotype may have occurred as results of cell proliferation or, alternatively, may be the consequence of a partial maturational block at the
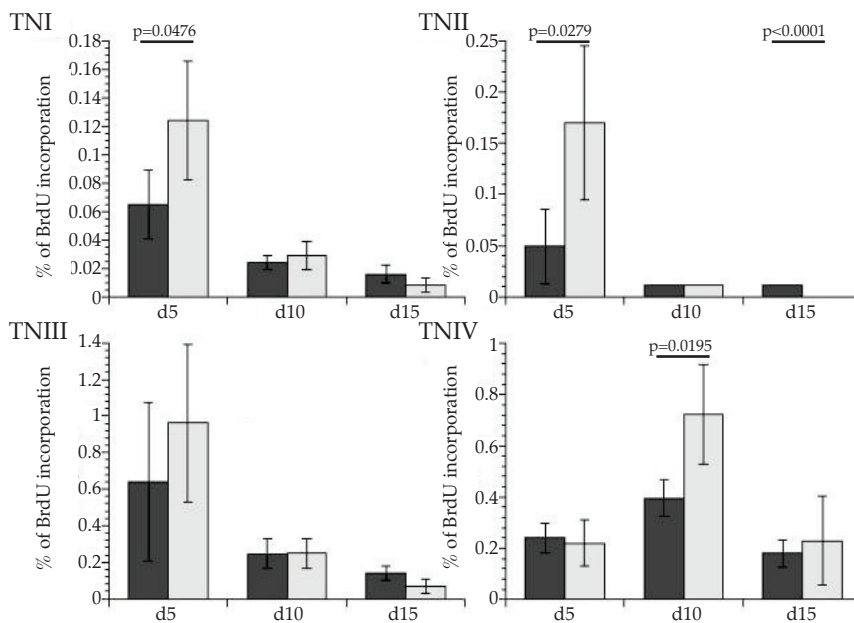
Figure 16 BrdU incorporation as a mesure of cell proliferation of TN thymocytes following *in vivo* exposure to KGF (grey bars) or HBSS (black bars). The data represent the relative percentage of thymocytes with a specific phenotype that have incorporated BrdU. Statistic: Anova, post-hoc-test: Bonferroni.

transition from one to the next developmental stage. To address this issue *in vivo* KGF treated mice (5 mg/kg for 3 consecutive days) were also pulsed with BrdU (1 mg/mouse i.p) 24 hours before sacrifice at the time points: day 5, 10 and 15 after beginning of the treatment. Early after KGF stimulation (i.e. on day 5), the populations of TNI and TNII thymocytes demonstrated statistically significant increases in cell proliferation when compared to HBSS treated mice (*Figure 16*). Following the swift transitions through the subsequent developmental stages, analysis at day 10 after KGF injection revealed only for TNIV cells a difference in proliferation in comparison to HBSS treated mice while differences in cell cycling were not apparent

anymore on day 15 after the initiation of the treatment.
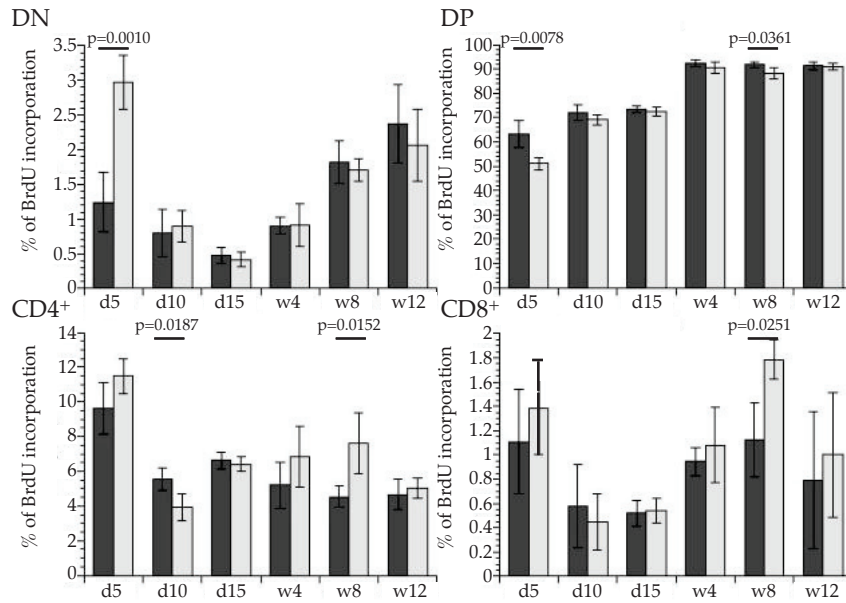

Figure 17 Maturing thymocytes sub-populations analysis in response to KGF stimulation. KGF treatment: grey bars; HBSS treatment: black bars. Statistic: Anova, post-hoc-test: Bonferroni.

The observed relative changes in cell proliferation after injection has to be interpreted in relation to thymic cellularity at the different time points. The absolute cell number for thymocytes and the relative percentage of DP cells is decreased on day 5 following the injections when compared to unmanipulated mice (*Figure 17*). Interestingly, the relative number of DN cells was also increased on day 5 after KGF treatment, when compared to later time points. This increase occurs parallel to stress-related changes and may thus reflect a physiological mechanism of corrective repopulation, possibly initiated by an increased thymopoiesis triggered through the expansion of TNI cells (*Figure 16*). Changes in the relative number of thymocytes of a given SP phenotype was only observed at day 5 after KGF treatment when the number of DP cells decreased in comparison to control mice. An obvious explanation for this finding is presently
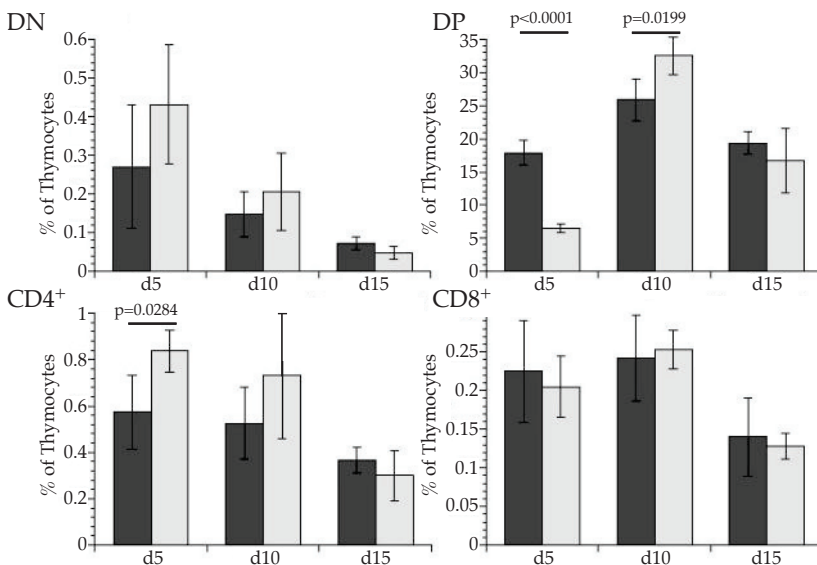

Figure 18 BrdU incorporation analysis of maturing thymocytes. KGF treatment reduce for short term the proliferative capacity of DPs. KGF treated mice: grey bars, HBSS treated mice: black bars. Statistic: Anova, post-hoc-test: Bonferroni.

lacking. However, the absolute thymocyte cell number was comparable between the two treatment groups at day 10 and increased significantly thereafter for the KGF injected animals (*Figure 14*). These dynamic changes were also reflected in the analysis of the thymocytes subpopulations actively cycling. For this purpose, mice treated with either KGF or HBSS were pulsed i.p. with BrdU and analyzed 24 hours later by flow cytometry for the incorporation of this modified uridine base. As demonstrated in *Figure 18* a difference in the BrdU incorporation between the two treatment groups was only observed on day 5 for DP thymocytes. This finding suggest

Figure 19 KGF do not influence the Vβ repertoire of thymocytes. KGF treatment: grey bars; HBSS treatment: black bars.

that thymocyte development occurs normally in KGF treated mice relative to HBSS injected mice but at a steady-state level involving higher total cell numbers.
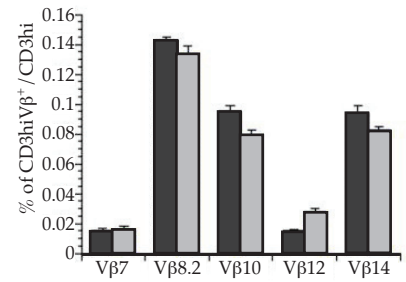
To determine whether thymic selection is altered following treatment with KGF, Vβ chain usage for the T cell antigen receptor was analyzed by flow cytometry (*Figure 19*). To this end, (C57BL/6 x DBA/2) F_1 mice were treated with KGF (5 mg/kg) on 6 consecutive days and analyzed 15 days later. The usage and cell surface density of the Vβ expression was comparable between the KGF and HBSS treated groups of mice, thus excluding the possibility that thymic selection in a microenvironment exposed *in vivo* to KGF would alter thymic selection. However, only further molecular and functional analysis will unequivocally answer the question whether KGF treatment will result in a skewing of the antigen receptor repertoire of treated mice beyond the use of specific Vβ families.

Detection of early thymic emigrants

The increase of thymic cellularity represents a striking finding in KGF treated mice and is clearly coincident with the proliferation of thymic epithelial cells. However, mechanisms independent of a cellular expansion of thymic stroma could contribute to increased thymocyte numbers. For example an identical increase in cellularity may also be achieved secondary to a higher percentage of cells undergoing proliferation or, alternatively, following a dramatic decrease in the exit of mature thymocytes to the periphery. However, both of these explanations have been excluded as possible mechanisms operational in KGF treated mice. Differences in cell proliferation were not observed day 10 following KGF treatment, but a difference in total cellularity between the two treatment groups persisted for at least 12 weeks. A block in the exit of mature thymocytes to the periphery is also unlikely explanation since the relative distribution of the distinct thymocyte subpopulations remained largely normal over the course of 12 weeks and specifically a disproportionate accumulation of single positive thymocytes

Figure 20 Cellularity of a) the thymus, b) spleen and c) lymphnodes in presence or absence of KGF treatment (day 0, 1, 2) and 16 hours after intrathymic injection of FITC. Black bars: saline control (day 8, n=15; day 45, n=10). Grey bars: KGF treated (day 8, n=14; day 45, n=17). Statitsical analysis: Mann-Whitney U.

could not be observed. Because a regular distribution of thymocytes subpopulations had been noted following *in vivo* KGF stimulation, thymopoiesis occurs normally albeit at higher steady-state level when compared to HBSS treated mice.

It is nevertheless still conceivable that thymocyte export and survival in the periphery may be altered in KGF treated mice. To address directly this issue, thymocytes were labeled *in vivo* and their exit as mature T cells was monitored in lymph nodes and spleen. For this purpose, anesthetized naive female C57BL/6 mice (4 weeks of age at the moment of KGF/HBSS treatment) previously treated with either KGF (5 mg/kg on three consecutive days) or HBSS were intrathymically injected with FITC (10 µl of a solution containing 125 µg/ml of FITC

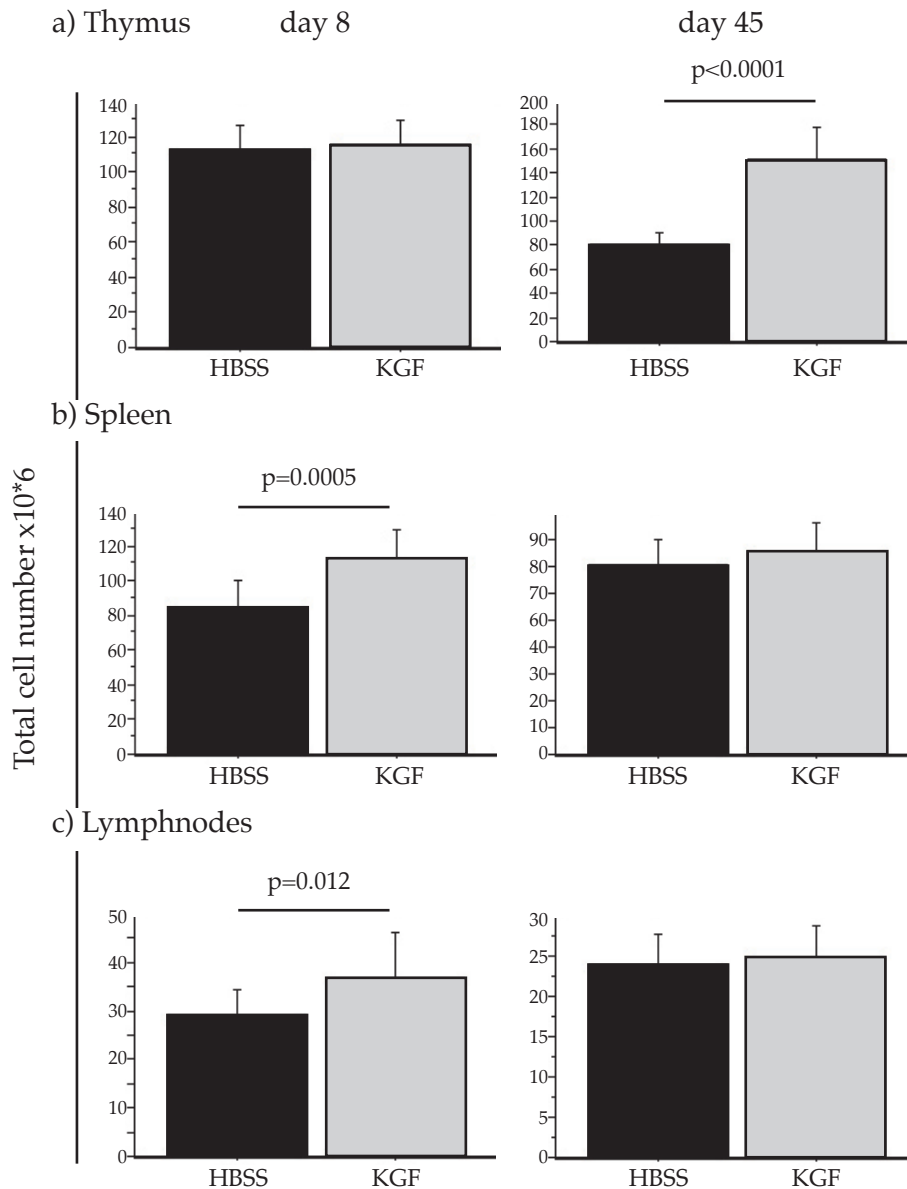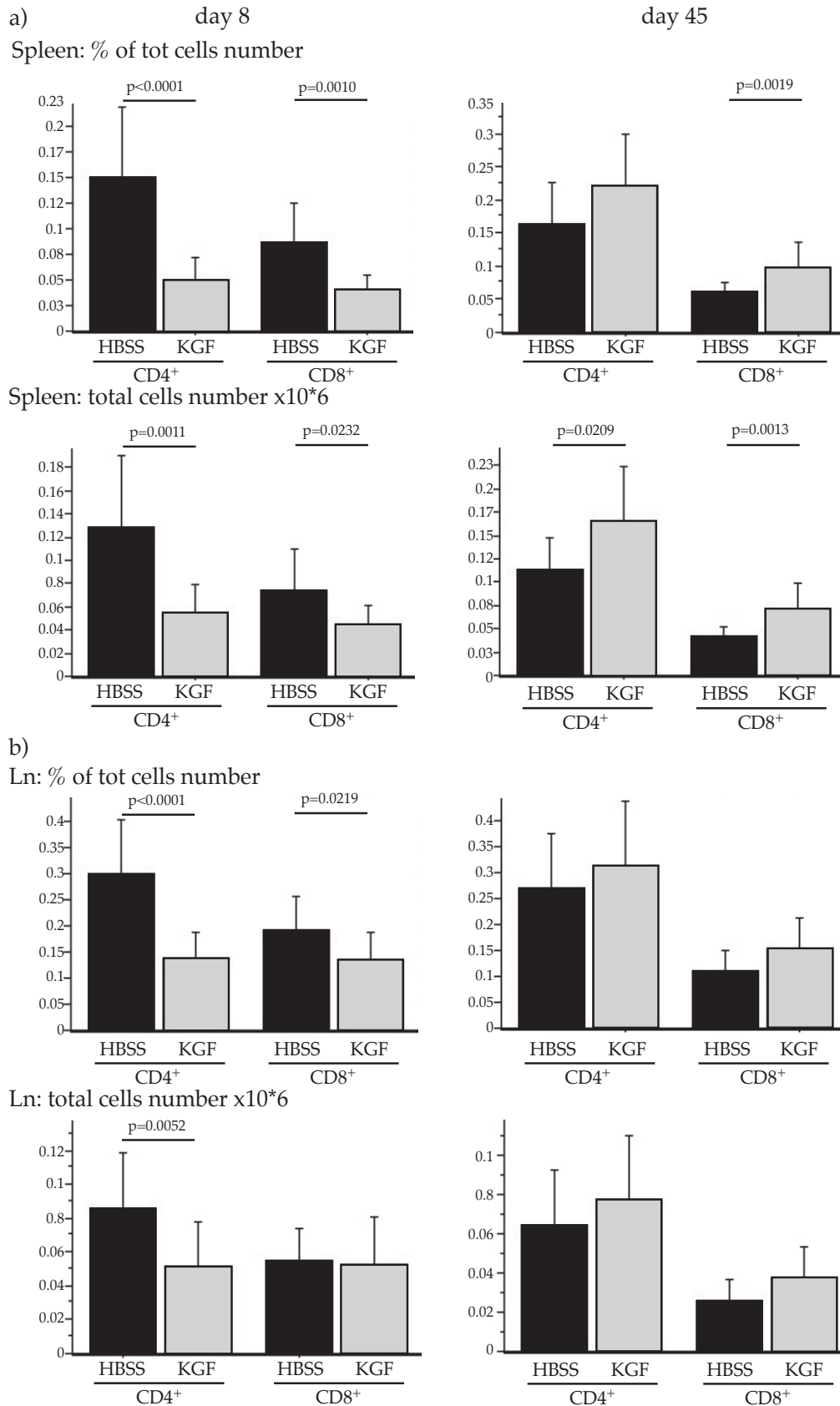*Figure 21* KGF treatment increases long term early thymic emigrants rate. a) spleen and b) lymphnodes in presence or absence of KGF treatment (day 0, 1, 2) and 16 hours after intrathymic injection of FITC. Black bars: saline control (day 8, n=15; day 45, n=10). Grey bars: KGF treated (day 8, n=14; day 45, n=17). Statitsical analysis: Mann-Whitney U.

in PBS) in a single lobe on day 8 and day 45 post KGF/HBSS treatment. These particularly time points were chosen as at day 8 the total thymocytes cellularity is identical between the two treatment groups and at day 45 the thymic cellularity is double in the KGF mice since 20 days and the output has reached a steady-state condition. Mice were sacrificed 16 hours after intrathymic injection and thymus, spleen and lymph nodes were removed for the detection by flow cytometry of CD4 and CD8 FITC positive T cells. As shown in *Figure 20* thymic cellularity is the same at day 8 for both groups and is doubled at day 45 for the KGF treated group. In the spleen and in the lymph nodes the read out is opposite: at day 45 no difference is detectable between the two treatment groups. As demonstrated in *Figure 21*, the relative and absolute numbers of FITC positive CD4 cells was decreased in both spleen and lymph nodes at day 8 after KGF treatment if compared to the HBSS treated group. In contrast, the relative but not the absolute number of CD8 FITC positive cells in the lymph nodes of KGF treated animals were decreased at day 8 after treatment, however in the spleen the relative and the absolute cell numbers resulted decreased in this group. While this finding may be indicative of a KGF induced reduction in thymic export, obvious limitations inherent to this assay allow also other interpretations. For example, it is conceivable that thymic exit is unaffected by KGF treatment but that the distribution and/or survival of newly generated T cells is altered secondary to this treatment. Moreover, either or both of these alternative explanations could hold true for the entire time period during which KGF effect can be noted or, alternatively, once KGF mediated thymic expansion has reached a plateau, mature single positive thymocytes may exit undisturbed. To address this later point, FITC labeling was performed 45 days after KGF/ HBSS treatment. At this time point the output to the spleen of labeled thymocytes within 16 hours was increased when compared to HBSS treated mice. This change affected both CD4^{+} and CD8^{+} T cells. However, the identical analysis of recent thymic emigrants homing to lymph nodes failed to document such a difference. These data demonstrate that the thymic output is increased in mice treated with KGF once size has increased and a steady state in thymopoiesis has been reached.

The role of KGF in GVHD associated pathology to the thymus

Thymic stroma cells constitute a typical target of GVHD mediated tissue destruction by donor derived alloantigen specific T cells. This pathology appears to limit the capacity of recipients of an allogeneic hematopoietic stem cell transplantation (HSCT) to rapidly and completely reconstitute the peripheral T cell compartment. Any preventive or therapeutic measures aimed at the structural and functional maintenance of thymic epithelial cells has, therefore, been tought to be of great benefit to the outcome of HSCT. Thus, the provision of KGF was assessed for its capacity to act as a cytoprotective or cytoreparative agent in a clinically relevant model of GVHD mediated injury to the thymus.

Treatment of transplant recipients with KGF diminishes thymic GVHD

To investigate the role of KGF in the prevention and repair of thymic GVHD, a murine haploidentical transplantation model was used that is independent of total body irradiation or other cytotoxic pre-conditioning regimens that may adversely affect thymocytes and thymic epithelial cells. Severe acute GVHD was elicited in $B6D2F_1$ mice by transfer of 25 x 10^6 unseparated splenocytes from C57BL/6 donors. In addition, the transplanted mice (B6 -> $B6D2F_1$) were treated subcutaneously from day –3 to day +3 of transplantation with either HBSS or KGF (5 mg/kg/day). HBSS treated $B6D2F_1$ recipients of 25 x10^6 syngeneic $B6D2F_1$ splenocytes served as controls.

The effect of KGF on thymic weight, cellularity and function was investigated on day 13 after transplantation. As demonstrated in *Figure 22a* and *b*, allogeneically transplanted mice injected with HBSS suffered a significant loss in thymic weight and cellularity when compared to syngeneic controls. However, treatment with KGF fully inhibited this GVHD-induced thymic injury. Thymocytes of syngeneically transplanted mice treated with KGF displayed normal thymocyte maturation comparable to $B6D2F_1$ -> $B6D2F_1$ mice treated with HBSS. Notwithstanding, thymic weights were increased by approximately 50%.

As the extent of thymic damage had been previously correlated with the presence of mature donor T cells entering the thymus (*142*), we next determined the frequencies and absolute numbers of infiltrating T cells of donor origin in these mice. Thymic single cell suspensions of Ly5.2[+] recipients were analyzed by flow cytometry for the *in situ* presence of mature Ly5.1^{+} cells. KGF-treatment diminished the relative percentage of thymus-infiltrating T-cells, and in particular that of mature CD8^{+} cells, when compared to HBSS-treated mice (*Figure 22c*). However, the absolute number of donor-derived T-cells was several-fold higher in KGF-treated hosts when compared to control transplant recipients (i.e. injected with HBSS). Thus,
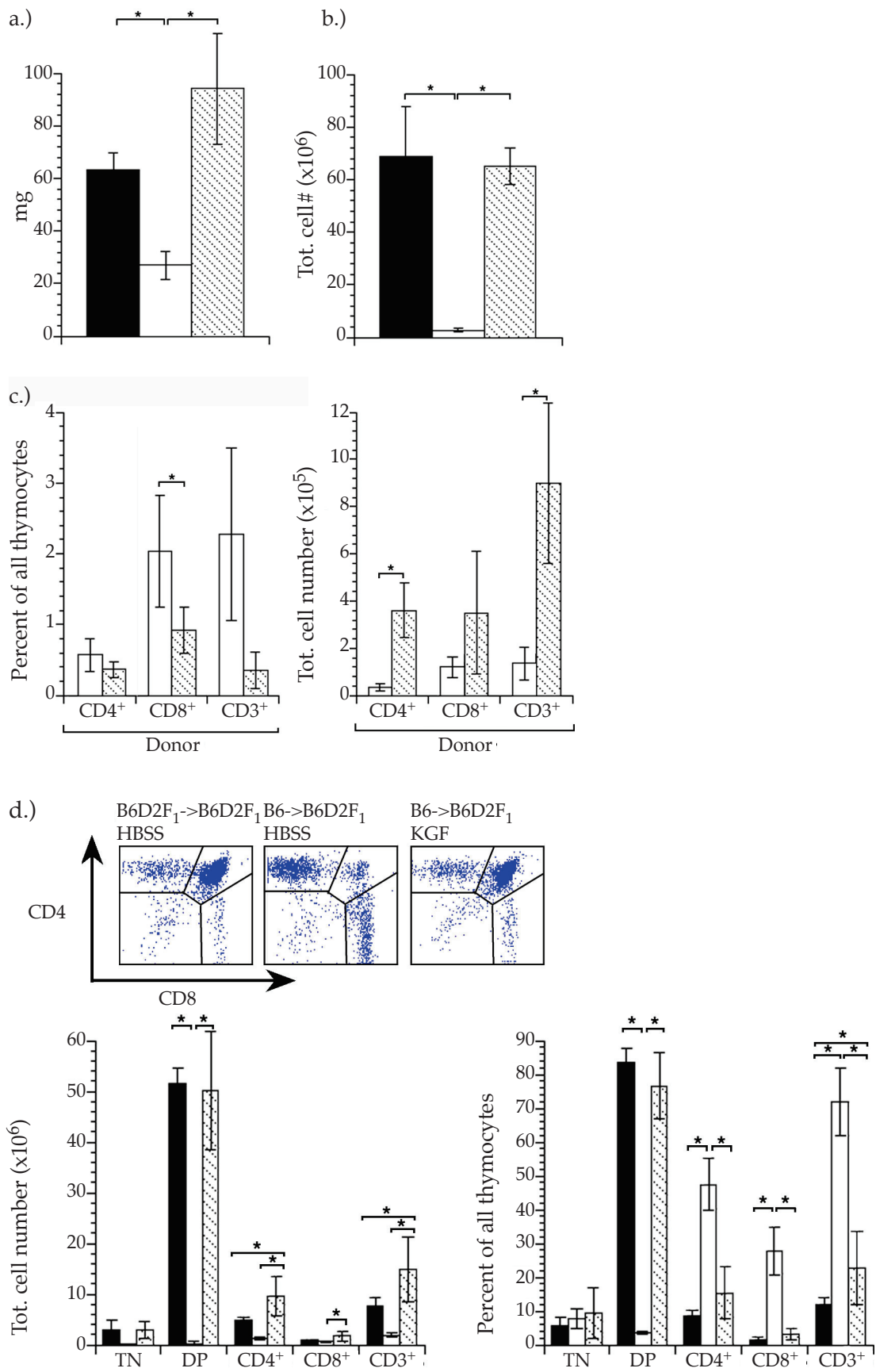
a.)

b.)

c.)

d.)

B6D2F$_1$->B6D2F$_1$ B6->B6D2F$_1$ B6->B6D2F$_1$
HBSS   HBSS   KGF

CD4

CD8

Figure 22 Treatment of mice with KGF diminishes thymic GVHD. Acute GVHD was induced by transfer of 25 x 10^6 parental B6 splenocytes to unirradiated B6D2F$_1$ mice. Recipients were analyzed on day 13 after transplantation. Mice were treated from days -3 to +3 of transplantation with KGF (i.p., 5.0 mg/kg/mouse/day KGF)◪ or HBSS □ . B6D2F$_1$ mice transplanted with syngeneic splenocytes served as non-GVHD controls and did receive neither KGF nor HBSS ■ . Panels a.) and b.): thymus weight (mg) and cellularity (total cell number x 10^{-6}) were determined. Panel c.): infiltration of donor T cells into the thymus (in percent, left, and total cell number x 10^{-5}, right). Donor-derived T cells were distinguished from host cells (CD45.2$^+$) by the expression of CD45.1. Panel d.): cell surface expression of CD3, CD4 and CD8 on thymic cells was analyzed by flow cytometry and the major thymocyte subsets were quantified (% of total cells, panel below). TN, CD3,4,8-triple negative; DP, CD4,8 double-positive. The graph represents pooled data from three independent experiments; with 10 mice analyzed for each group. Analysis by ANOVA; *p<0.05.

the protective effect of KGF on thymocyte development in presence of GVHD was not due to a decrease in thymus-infiltrating mature T cells.

Thymocytes of syngeneically and allogeneically transplanted recipients treated with either HBSS or KGF were further analyzed for expression of CD3, CD4 and CD8. Comparing the three experimental groups, significant changes in the relative number of TN thymocytes were not observed (*Figure 22d*). However, the DP population was severely diminished while single-positive mature thymocytes were greatly increased in B6 -> B6D2F$_1$ recipients treated with HBSS. These changes were typical for GVHD-mediated thymic pathology and are accounted by both enhanced apoptosis of DP thymocytes and the presence of mature donor T-cells in the thymus (*142*). In contrast, mice injected with allogeneic T cells and KGF displayed normal thymocyte maturation with the notable exception of an increased number of CD3$^+$ T cells, likely reflecting the presence of mature T cells of donor origin. Taken together, these results demonstrated that treatment with KGF prevented the GVHD-associated thymic changes in weight, cellularity and cell maturation, independently of effects on the thymic recruitment of donor-derived T cells.

KGF treatment maintains normal cell cycle progression of resident TCR$^-$CD4$^-$CD8$^-$ thymocytes despite acute GVHD

Two weeks after transplantation, the absolute cellularities within each of the four phenotypically distinct TN thymocyte subpopulations were significantly decreased in mice injected with saline (*Figure 23, upper panel*). In contrast, treatment with KGF preserved cell numbers for each of the TN subpopulations. Thus, KGF prevented the loss of the most immature pro-T and pre-
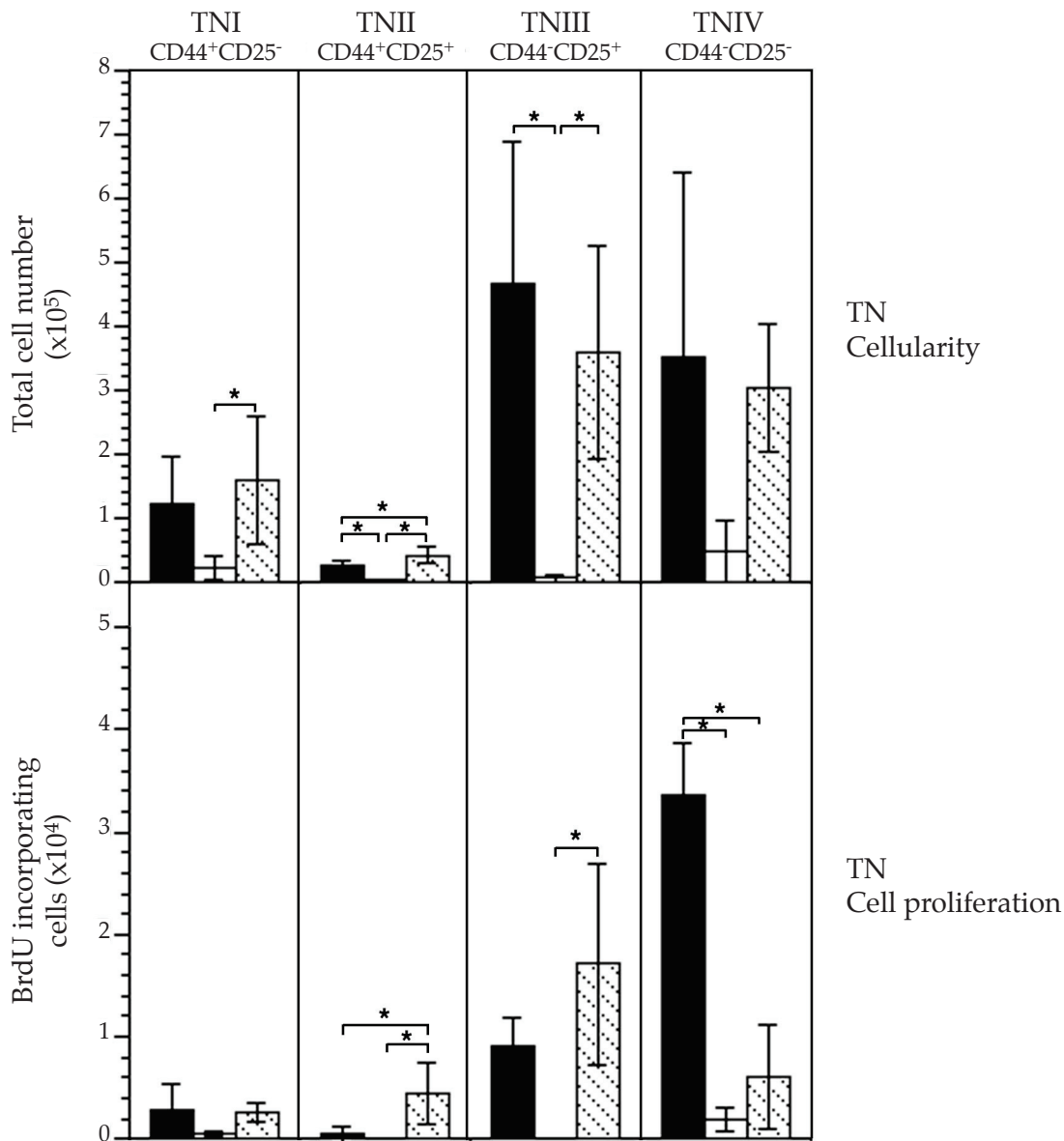
Figure 23 KGF treatment maintains normal cell cycle progression of the most immature thymocyte subsets. Acute GvHD was induced in unirradiated $B6D2F_1$ mice as in *Figure 22*. CD3,4,8-triple negative (TN) thymocytes were analyzed 13 days after transplantation for surface expression of CD44 and CD25 and for BrdU incorporation. Upper panels: TN cellularity (total TN cell number x 10^{-5}) in mice treated with KGF ⬚ or with HBSS ☐ was ascertained and compared to untreated syngeneically transplanted $B6D2F_1$ mice ■. Lower panels: the absolute cell numbers (x 10^{-4}) of BrdU$^+$ cells among TN thymocytes were determined. Each graph represents pooled data from two independent experiments, with 7 mice analyzed for each group. Analysis by ANOVA; $^*p<0.05$.
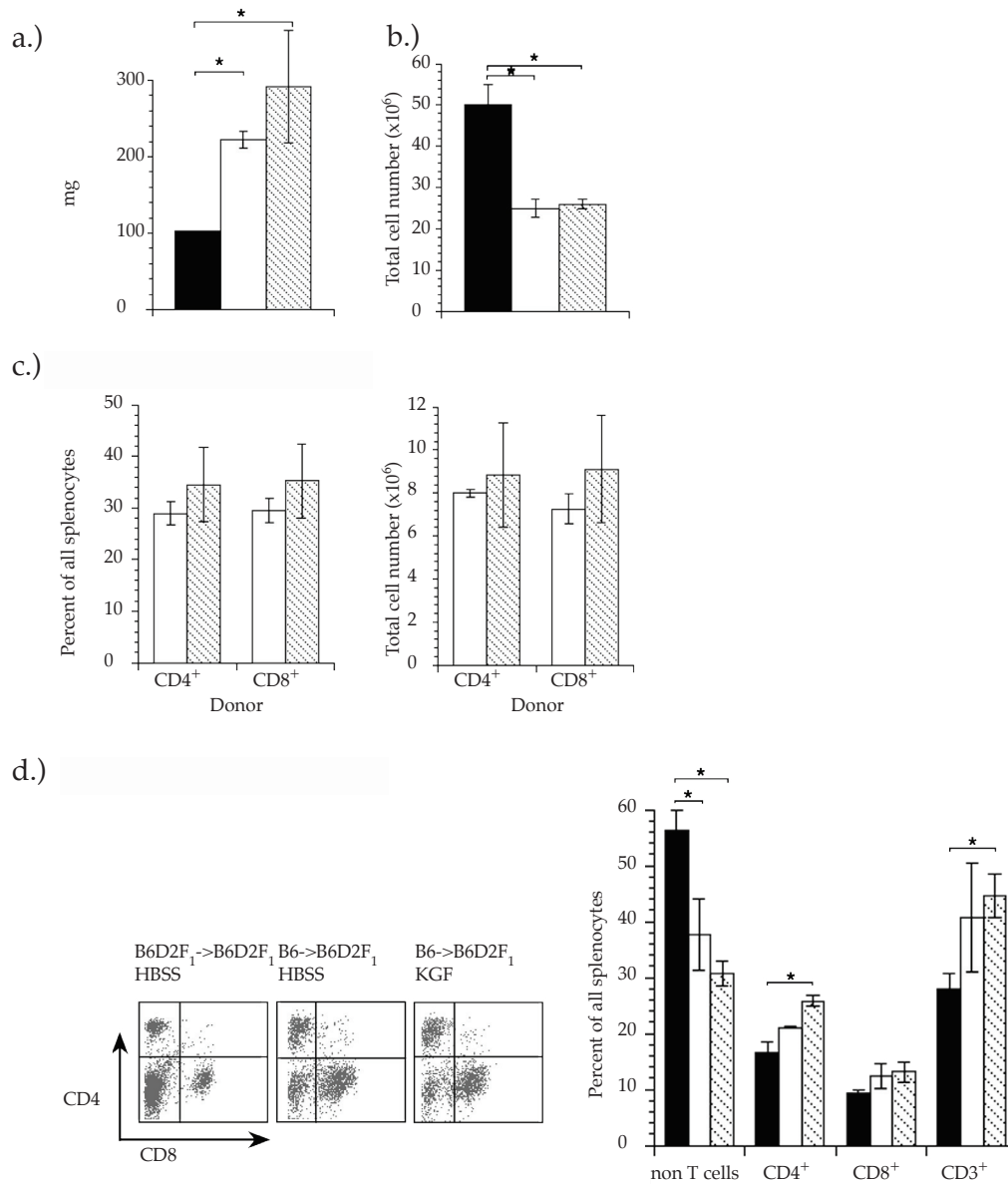
Figure 24 KGF treatment does not affect the severity of splenic GVHD. Acute GVHD was induced in unirradiated B6D2F$_1$ mice as in *Figure 22* and recipients were analyzed on day 13 after transplantation. Panels a.) and b.): spleen weights (mg) and cellularities (cells x 10^{-6}) in transplant recipients treated with KGF ▨ or HBSS ▢. Untreated syngeneically transplanted B6D2F$_1$ mice served as non-GVHD controls ■ Panel c.): infiltration of donor T cells into the spleen (in percent, left panel, and total cell number x 10^{-6}, right panel) was determined. Donor-derived T cells were distinguished from host cells (CD45.2$^+$) by their expression of CD45.1. Panel d.): the surface expression of CD4 and CD8 on splenocytes was analyzed by flow cytometry and quantified (% of total splenocytes). The graphs represent pooled data from three independent experiments; with 10 mice analyzed for each group. Analysis by ANOVA; *p<0.05.

T cells in the presence of alloreactive T cells.

As the loss of TN cells during GVHD is the consequence of impaired cellular proliferation, we investigated whether KGF averted this functional deficiency. BrdU incorporation was used to determine cell cycle progression (*155*), which normally occurs at all phenotypic TN stages and in particular within stages II and IV. In B6 -> B6D2F$_1$ recipients injected with HBSS, viable

a.)

B6D2F$_1$ -> B6D2F$_1$, HBSS   B6 -> B6D2F$_1$, HBSS   B6 -> B6D2F$_1$, KGF

b.)

B6D2F$_1$ -> B6D2F$_1$, HBSS   B6 -> B6D2F$_1$, HBSS   B6 -> B6D2F$_1$, KGF

Major cortical epithelial cells Blue

Minor cortical epithelial cells White

Major medullary epithelial cells White

Minor medullary epithelial cells White
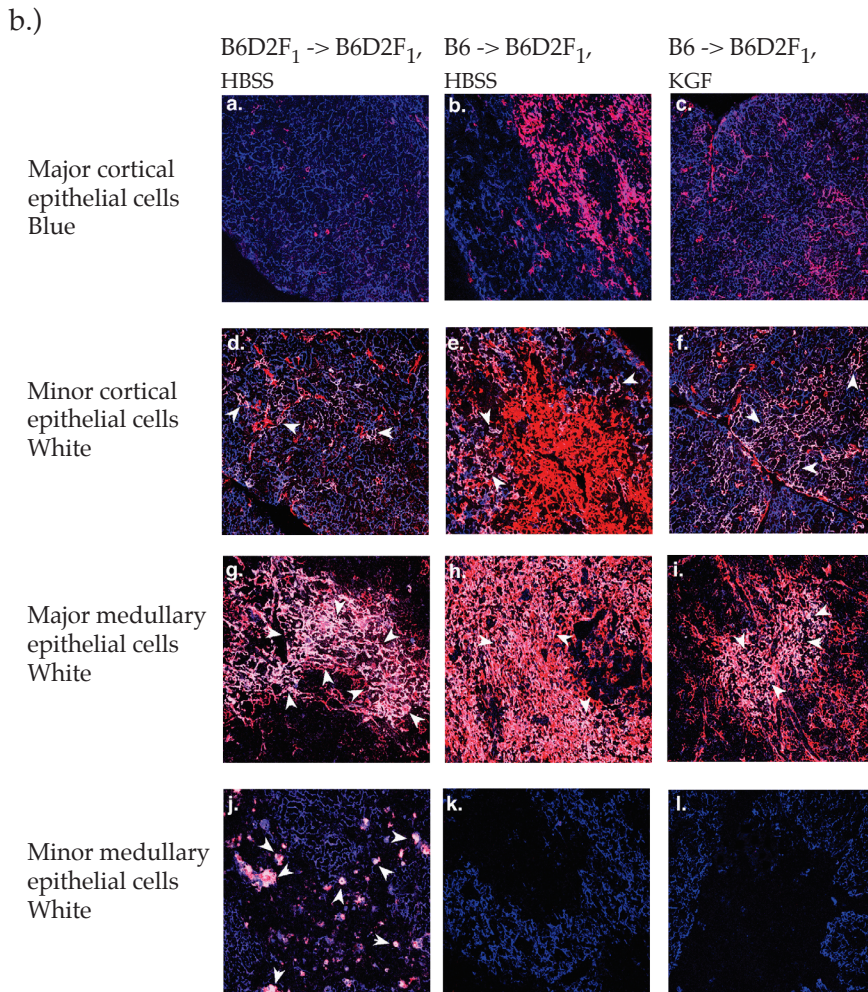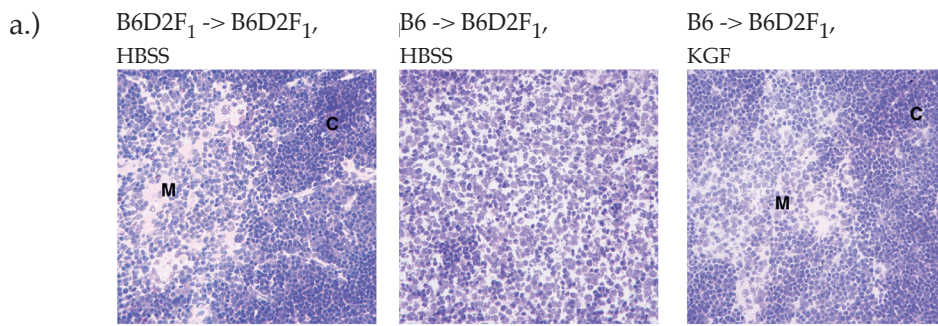
Figure 25 KGF treatment preserves the thymic microenvironment during acute GVHD. GVHD was induced in KGF- or HBSS-treated B6D2F$_1$ mice, as described in *Figure 22* and mice were analyzed on day 13 after transplantation. Panel a.): frozen thymic sections (6 *μ*m) from the three transplant groups were analyzed for histopathology (H+E stain; magnification x 200). Panel b): immunohistology of four distinct subpopulations of thymic epithelial cells in allogeneically and syngeneically transplanted mice. Thymic epithelial cells were identified using a panel of antibodies according to *Table 1*. Two- and three-color immunoflourescent analyses, respectively, were performed using a confocal microscope (Carl-Zeiss). Major cortical cells appear blue (a-c), whereas minor cortical TEC (d-f), major medullary TEC (g-i) and minor medullary TEC (j-l) appear white (i.e. the combination of blue and red fluorescence, altered by computer assisted management, see *Table 1*). The arrows denote individual positive cells. Magnification x 200. The data substantiate that KGF treatment preserves in the presence of GvHD the normal structure of cortical and major medullary TECs but not of the subpopulation of minor medullary TECs.

TN cells of stages II, III and IV were severely affected by GVHD in their proliferative capacity (*Figure 23, lower panel*). However, in KGF treated mice with GVHD, the TN subpopulations II and III proliferated at normal (TNIII) or even increased (TNII) levels. For TNIV cells, the GVHD-induced inhibition of cell cycle progression was only partially reversed by KGF.

KGF treatment fails to modulate splenic GVHD

Since the spleen serves as a typical target of acute GVHD, we next assessed the extent of splenic damage in B6 -> B6D2F$_1$ mice treated either with KGF or HBSS. KGF influenced neither weights nor absolute splenic cellularities in allogeneically transplanted recipients when compared to mice injected with HBSS (*Figure 24a and b*). Moreover, treatment with KGF had no effect on the degree of donor T-cell infiltration (*Figure 24c*). Phenotypic analysis by flow cytometry of splenocytes revealed for both groups of recipients a loss in non-T cells (e.g. B lymphocytes) in lieu of CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ T cells (*Figure 24d*). Thus, *in vivo* administration of KGF did not affect the severity of splenic GVHD.

KGF treatment essentially preserves the thymic microenvironment despite GVHD

The loss of normal thymic morphology is a typical feature of GVHD and has recently been linked to the alloimmune response directed against thymic stromal components. Since normal T-cell development was preserved by KGF despite acute GVHD (as judged by splenic alterations) and since KGF receptor expression was detected exclusively on thymic epithelial cells in all anatomical compartments of the thymus, the cellular stromal composition and architecture of the thymic microenvironment was investigated in B6 -> B6D2F$_1$ recipients. *Figure 25a* shows that the clear separation between cortex and medulla was lost in acute GVHD in mice treated with HBSS but was clearly maintained in recipients injected with KGF. As already detailed in the first section of this work immunohistology using UAE-1 lectin, MTS-10 and antibodies to cytokeratin (CK) 5 and CK18 distinguished thymic epithelial cells into four distinct subpopulations: the major cortical (CK18$^+$CK5$^-$UEA-1$^-$MTS10$^-$), the minor cortical (CK18$^+$CK5$^+$), the major medullary (CK5$^+$MTS10$^+$) and the minor medullary epithelial cells (CK18$^+$UEA-1$^+$) (*28*)(see *Table 1*). In HBSS treated B6 -> B6D2F$_1$ mice the major cortical epithelial cells were severely diminished in cell number in response to acute GVHD, thus contributing to a smaller cortex (*Figure 25b, panel b*). In contrast, treatment with KGF preserved the cellularity of this subpopulation to a degree indistinguishable to that of syngeneic transplantation controls (*Figure 25b, panels a and c*). The frequencies of minor cortical and major medullary epithelial cells also remained unchanged in mice injected with KGF when compared to syngeneic transplantation controls, whereas recipients injected with HBSS sustained a substantial loss of both of these subpopulations (*Figure 25b, panels d-i*). Only minor medullary epithelial cells

appeared to be unaffected by KGF treatment because both treatment groups of B6 -> B6D2F$_1$ recipients were shown to display an almost complete loss of this subpopulation (*panels j-l*). However, this subpopulation of epithelial cells did express the KGFR in naive thymi.

KGF affects thymic epithelial cell function
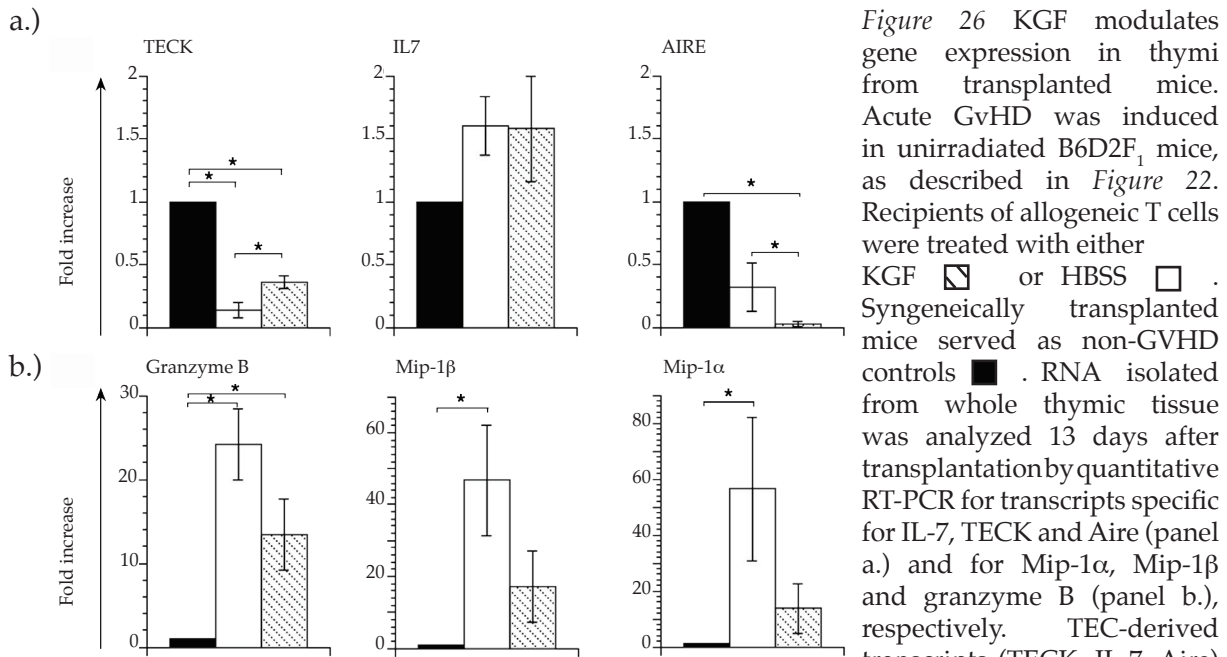
Figure 26 KGF modulates gene expression in thymi from transplanted mice. Acute GvHD was induced in unirradiated B6D2F$_1$ mice, as described in *Figure 22*. Recipients of allogeneic T cells were treated with either KGF ▨ or HBSS ▢. Syngeneically transplanted mice served as non-GVHD controls ■. RNA isolated from whole thymic tissue was analyzed 13 days after transplantation by quantitative RT-PCR for transcripts specific for IL-7, TECK and Aire (panel a.) and for Mip-1α, Mip-1β and granzyme B (panel b.), respectively. TEC-derived transcripts (TECK, IL-7, Aire) were normalized for EVA expression whereas hematopoietic cell-specific transcripts (granzyme B, Mip-1a and b) were normalized for GAPDH expression. Each graph represents pooled data from one experiment; with three mice analyzed for each group. Analysis by ANOVA; $^*p<0.05$.

The effect of KGF on thymic epithelial cells may include alterations in gene expression that result in enhanced biological functions, thus maintaining normal thymocyte maturation despite the presence of allogeneic T cells. In normal thymic development, epithelial cells produce necessary chemotactic and activating stimuli to lymphoid precursors, (e.g. thymic epithelial chemokine; TECK (*156*) and macrophage inflammatory protein-1; Mip-1 (*157*)). Moreover, these cells provide crucial survival and proliferation signals for TN cells, (e.g. IL-7) (*158*), and unique molecules necessary for repertoire selection of DP thymocytes (e.g. autoimmune regulator; Aire) (*159-161*). To investigate whether KGF influence the expression of these molecules during acute GVHD, quantitative RT-PCR analysis was performed on thymic tissues. Thymic epithelial cell specific transcripts were normalized to the expression of the epithelial V-like antigen, EVA (*157*). Expression of the chemokine TECK was significantly diminished in epithelial cells during GVHD but was partially restored by KGF treatment (*Figure 26a*). In contrast, IL-7 expression was increased among thymic epithelial cells during GVHD albeit not statistically significant. KGF treatment had no further effect. Analysis for the expression of the transcription factor Aire, which is usually expressed in minor medullary

epithelial cells (*160*), revealed a decrease of specific mRNA following GVHD. KGF treatment did not restore Aire expression, which is in agreement with a lack of minor medullary epithelial cells in KGF-treated mice with GVHD (*Figure 26a*).

Since infiltrating T cells mediate a strong inflammatory response possibly affecting thymic epithelial cell, in the non-irradiation model of GVHD used here, was investigated whether KGF altered the thymic microenvironment and resulted in decreased inflammation. The mRNA species for granzyme B, Mip-1α and Mip-1β were therefore measured by quantitative PCR and normalized to GAPDH expression. Transcripts for granzyme B, a cellular activation marker of cytotoxic T cells, were greatly increased in thymic tissue affected by GVHD (*Figure 26b*). Treatment with KGF reduced the number of granzyme B-specific transcripts by two-fold, despite the fact that the absolute number of donor T cells was increased in these mice when compared to saline treated animals with GVHD. The expression of macrophage inflammatory proteins-1a and 1b, i.e. important mediators of inflammation (*162*) was upregulated during acute thymic GVHD (*Figure 26b*). Treatment with KGF reduced specific transcripts of these chemokines by 3- to 4-fold; despite the increased number of infiltrating donor T cells in the thymus.

KGF reduces CD80 expression in thymic GVHD

CD80 (B7.1) is a critical co-stimulatory molecule for thymocyte development and mature T cell function (*163, 164*). Therefore the CD80 expression in thymic tissue sections of syngeneically and allogeneically transplanted mice was determined. The comparison of the staining revealed a pattern compatible with CD80 expression on thymic epithelial cells (data not shown)). We found that CD80 expression was upregulated in the thymus in the presence of acute GVHD (*Figure 27*). Treatment with KGF diminished this expression in both the cortical and medullary compartment to a degree comparable of syngeneic transplant recipients despite an increase in the
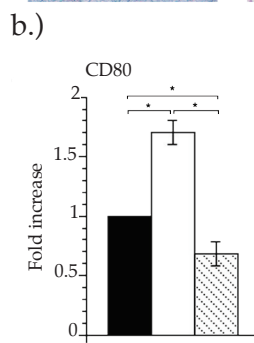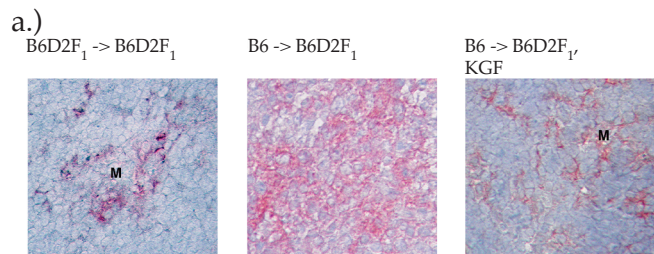
Figure 27 KGF reduces CD80 expression on thymic stromal cells in recipients with GVHD. Acute GVHD was induced in KGF-treated or HBSS-treated B6D2F$_1$ mice, as described in *Figure* 22. Panel a.) Thirteen days after transplantation, frozen thymic sections (6 *μm*) from the three transplant groups were stained with anti-CD80 moAb and developed with AEC. Slides were then counterstained with hemalaun. Magnification x 200. Panel b.)RNA isolated from whole thymic tissue was analyzed 13 days after transplantation by end-point RT-PCR for transcripts specific for CD80. Transcripts were normalized for EVA expression. The graph represents pooled data from one experiment; with three mice analyzed for each group. Analysis by ANOVA; *p<0.05.

absolute number of mature donor-derived T cells. These immunohistologic findings (*Figure 27a*) were further corroborated by quantitative PCR analysis (*Figure 27b*). Thus KGF treatment reduces the cell surface expression of the costimulatory molecule CD80 on stromal cells (and possibly other cells, including activated T cells) and may thus hamper their allogeneicity.

Donor T cell activation in thymic tissue exposed to KGF

Thymic epithelial cells are typically targeted by donor T cells in the course of GVHD; changes in the architecture and composition of these stromal cells become apparent between day 9 and 13 after donor T cells transfer. While the thymocyte cellularity remained normal in recipients treated with KGF when compared to animals injected with HBSS, mice of both treatment groups displayed first a decrease and by day 13 a complete loss in the population of minor medullary epithelial cells as a consequence of GVHD (*Figure 25b*). The understand of this feature detailed studied between day 9 and 11 were carried out(*Figure 28*): donor T cells infiltration was studied on histological sections and in *Figure 28b* it is clear that the infiltrating donor T cells preferentially circulate near to the minor medullary epithelial cell subpopulation. Although the complete loss of this population at day 13, likely to be directly mediated by allogeneic T cells; histological attempts, to detail the precise molecular mechanisms by which these particular epithelial cells are destroyed, remained unsuccessful (data not shown). For
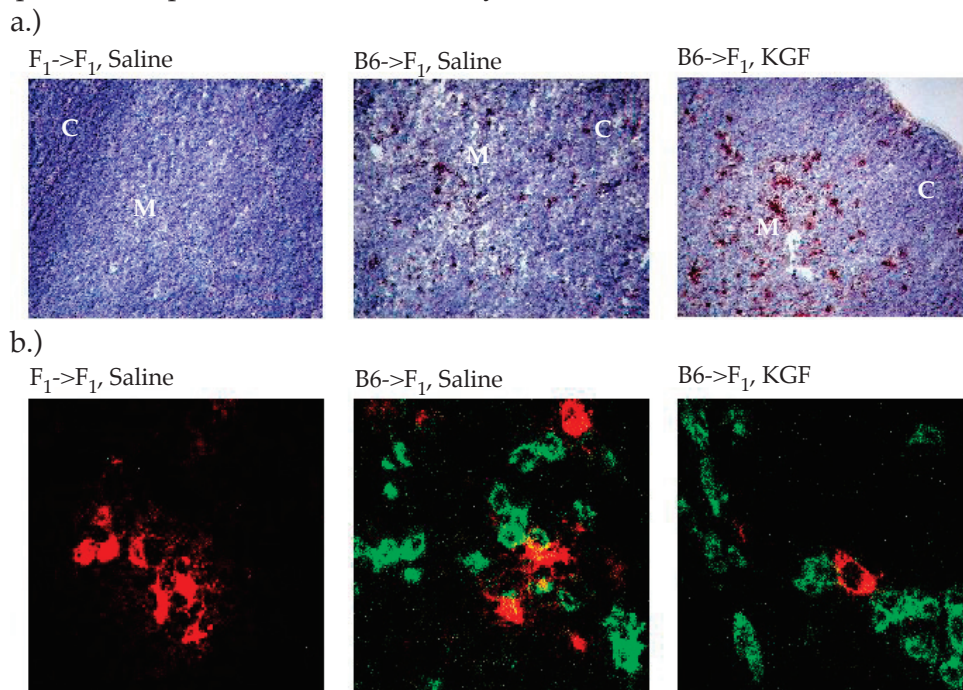
Figure 28 Donor lymphoid cells infiltrating the thymus are mostly located in the medullary compartment. GVHD was induced as described in *Figure 22.* Analysis was performed at day 9 post transplantation. a.) Immunohistological staining for CD45.1$^+$ cells in the thymus (in red). Donor lymphoid cells are located mostly in the medulla. b.) Fluorescent detection for UEA-1 lectin (minor medullary TEC) in red and CD45.1$^+$ cells in green.
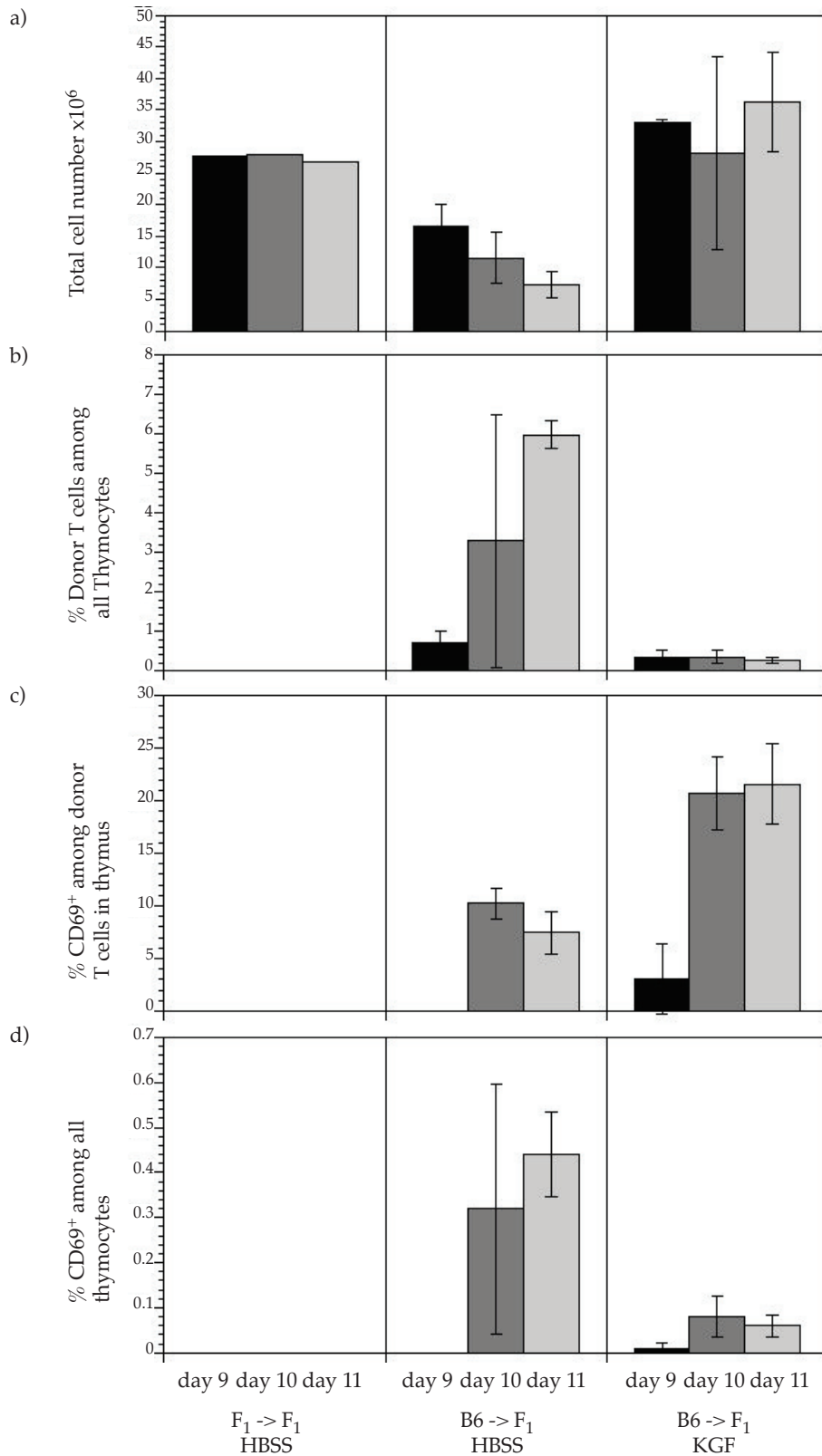
Figure 29 Early thymic infiltration of T cells is not modulated by KGF. GVHD and KGF treatment were performed as described in *Figure 22*. a.) Total thymic cellularity at d9, d10 and d11 after transplantation. b.) Relative cells number of donor T cell in the thymus. c.) Relative cell numbers of activated CD69$^+$ among donor T cells. d.) Relative cell numbers of activated CD69$^+$ among all thymocytes. F_1->F_1: n=1 per time point; B6 ->F_1 -/+ KGF: n=3 per time point

example, all attempts to stain tissue section for the presence of the activated form of caspase-3 using phospho-specific antibodies could not confirm perforin-mediated apoptosis in this specific thymic epithelial cell subpopulation.

To further elucidate the pathomechanisms operating in this loss of minor medullary epithelial cells, thymus-infiltrating donor T cells were analyzed for their anatomical location and state of activation in the recipient. On day 9 after transfer, these cells had accumulates in the thymic medulla (*Figure 28a*). In the further course of the disease (*Figure 29*), thymic cellularity decreased over the next 2 days progressively in the allogeneic HBSS treated mice. In contrast, recipients injected with KGF did not display a change in total thymocytes number (*Figure 29a*). Moreover, these changes correlated with the relative number of donor T cells infiltrating the thymus (*Figure 29b*). Although the relative percentage of an activated phenotype (i.e. $CD69^+$) among the thymic infiltrating donor T cells detected in the thymus of KGF treated mice was higher than in the HBSS treated group (*Figure 29c*), these cells represent only a small minority among all thymic cells in this treatment group (*Figure 29d*). Thus, KGF does not prevent in absolute terms the influx of donor T cells into the thymus and does not alter their activation when compared to mice pretreated with HBSS and injected with allogeneic T cells.

KGF increase long-term survival of mice with acute GVHD

To determine the effect of KGF on survival following the transfer of allogeneic T cells, mice were treated with either KGF (5 mg/kg/day) or HBSS for 6 consecutive days and transplanted on the third day with 25×10^6 splenocytes. KGF treatment resulted in the long-term survival of the 80% of the transplanted mice. In contrast, all mice that had received HBSS did succumb to GVHD (*Figure 30a*). Moreover, there were no differences in total body weight measurement during the observation period of 49 days when comparing KGF treated mice receiving allogeneic T cells with mice engrafted with
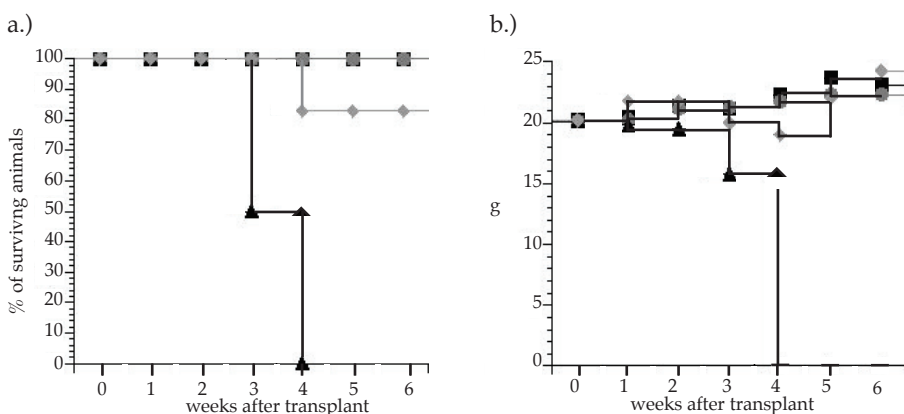
Figure 30 KGF treatment improves the survival and body weight of mice transfused with allogeneic T cells. Mice were transplanted and treated as described in *Figure 22.* a) Survival curve (n=6 per group). b) Weight curve (n=6 per group). F_1 ->F_1, HBSS:■; F_1 ->F_1, KGF:◯; B6 ->F_1, HBSS:▲ ; B6->F_1, KGF:◇.
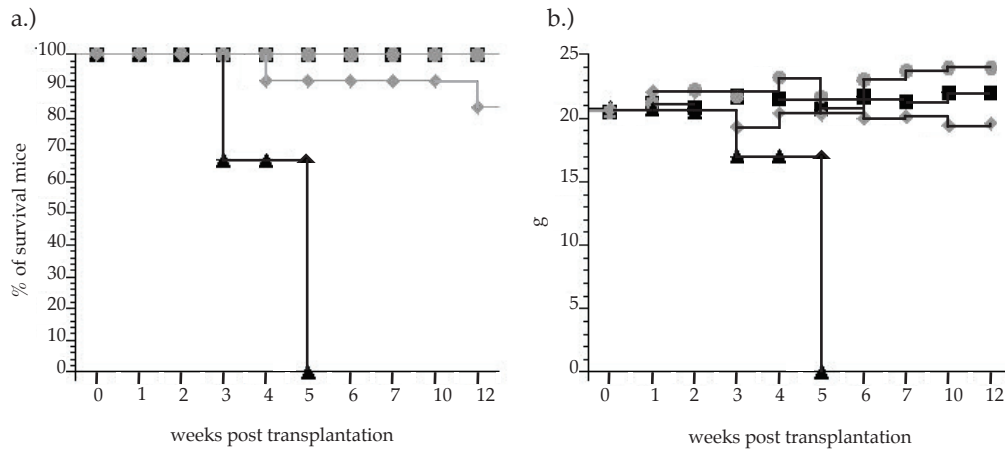
Figure 31 Addition of KGF 3 weeks after T cell engraftment does not alter the clinical course. Mice were ransplanted and treated as described in *Figure 22*. a) Survival curve . b) Weight curve (n=6 per group). $F_1 ->F_1$, HBSS:■ (n=6 per group); $F_1 ->F_1$, KGF:◯(n=6 per group); B6 ->F_1, HBSS:▲(n=6 per group); B6->F_1, KGF:◇(n=12 per group).
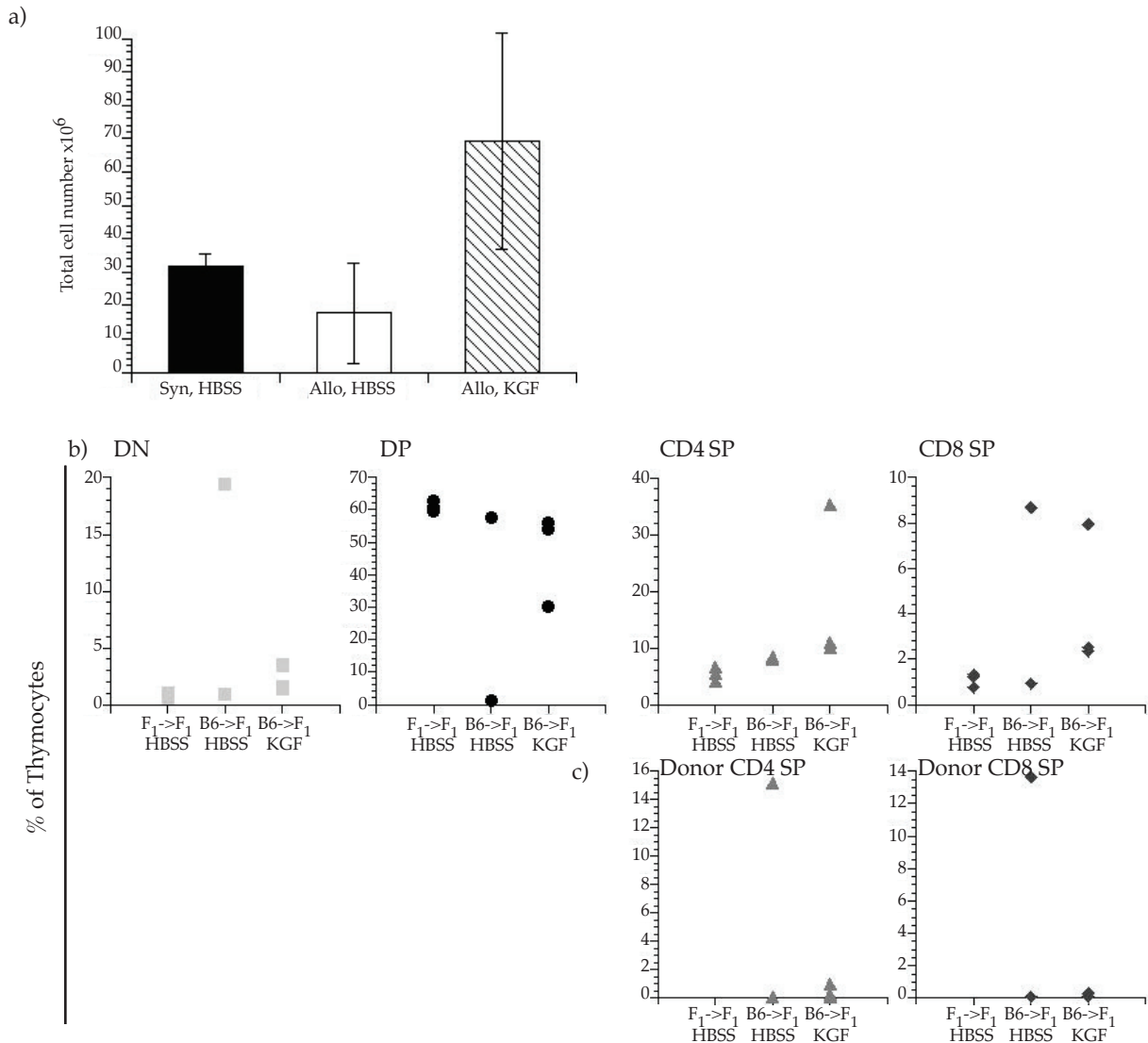
Figure 32 Analysis of the thymus at week 4 post transplantation. a) Thymic cellularity. b) Thymic phenotype according to DN, DP, CD4 SP and CD8 SP. c) Donor derived T cells resident in the thymus; CD4 SP and CD8 SP. Groups: F_1->F_1, HBSS, n=3; B6->F_1, HBSS, n=2; B6->F_1, KGF, n=3.

syngeneic T cells (*Figure 30b*). Neither survival nor changes in body weight were affected when additional doses of KGF were provided to engrafted mice on day 21, 22 and 23 after transplantation (*Figure 31*). Thus, the additional treatment of transplanted mice with KGF three weeks after the transfer of allogeneic T cells did not convey a beneficial effect even when animals were observed for a total of 12 weeks.

To asses the thymic damage in mice 4 weeks after infusion of allogeneic splenocytes (25×10^6), thymic cellularity was determined in the KGF and HBSS treated groups. As demonstrated in *Figure 32a*, thymic cellularity was increased in KGF treated mice when compared to the respective control group. The different values for cellularity and phenotype of maturing
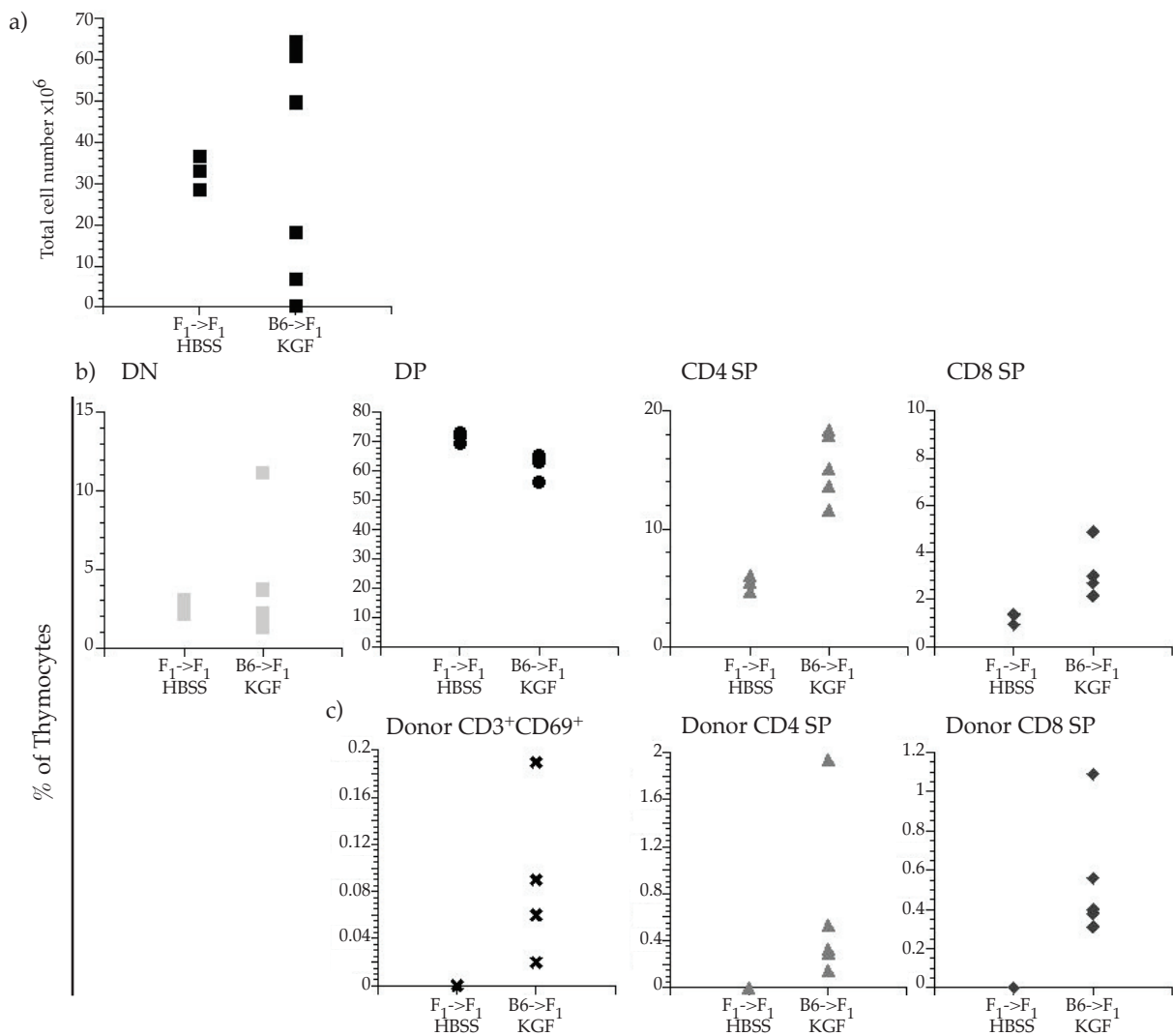
Figure 33 Analysis of the thymus at week 12 post transplantation. a) Thymic cellularity. b) Thymic phenotype according to DN, DP, CD4 SP and CD8 SP. c) Donor derived T cells resident in the thymus; CD3+CD69+ active donor T cell, CD4 SP and CD8 SP. Groups: F_1->F_1, HBSS, n=3; B6->F_1, KGF, n=6.

thymocytes (*Figure 32b*) were, however, distributed over a large range reflecting a variable response to KGF. Moreover, donor derived T cells could still be detected in thymic tissue at low frequency despite normal to increased thymocytes numbers demonstrated in *Figure 32c*. The comparative analysis at 12 weeks after transplantation revealed two separate groups of recipient mice in the allogeneic transplanted and KGF treated group as discriminated by total thymic cellularity (*Figure 33a*). Almost all the mice treated with KGF had a regular distribution of the DP thymocytes but shows an increased relative cell number of SP thymocytes (*Figure*
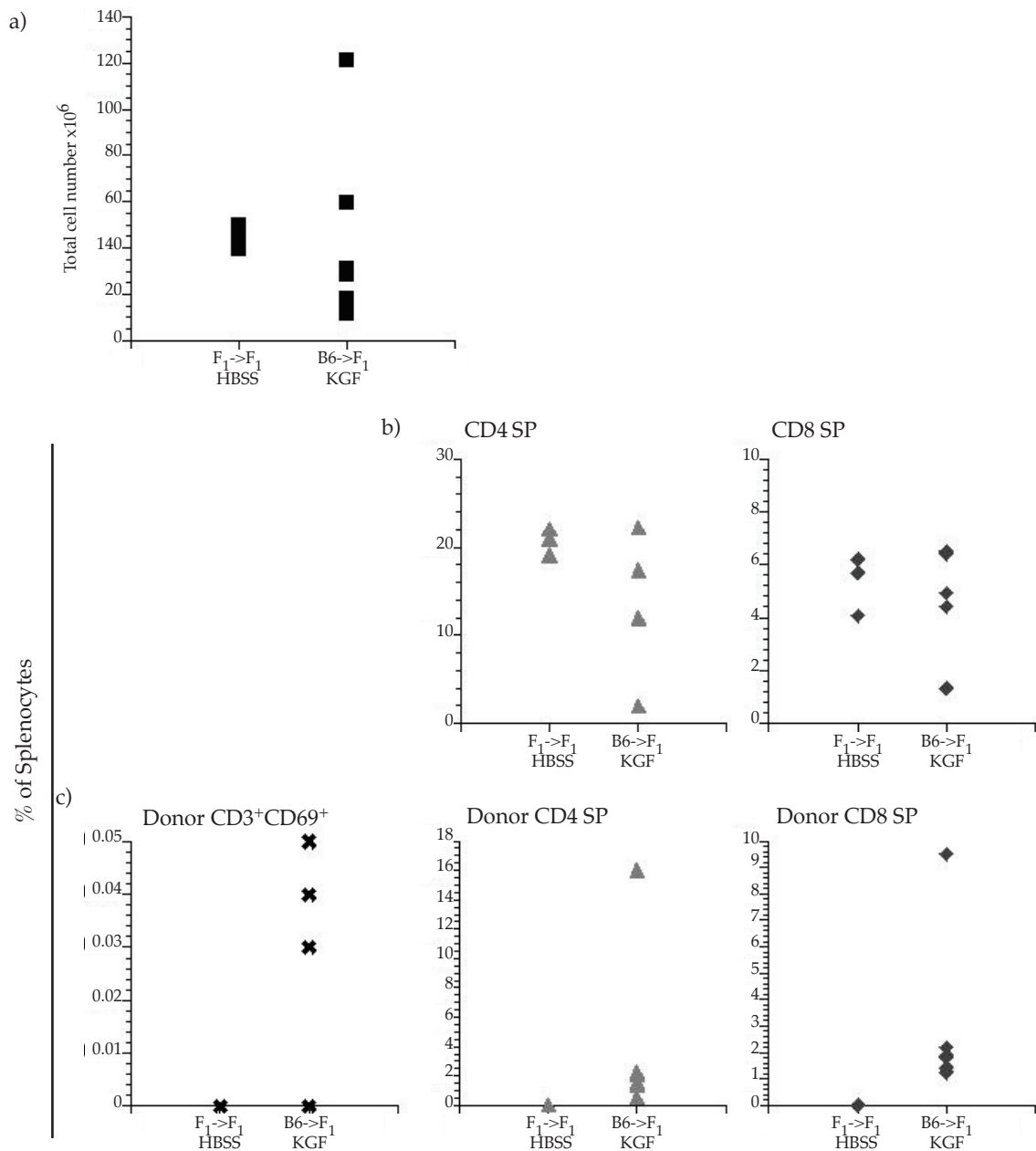
Figure 34 Analysis of the spleen at week 12 post transplantation. a) Spleen cellularity. b) Spleen phenotype according to CD3- CD4 SP and -CD8 SP. c) Donor derived T cells resident in the thymus; $CD3^+CD69^+$ active donor T cell, CD3- CD4 SP and -CD8 SP. Groups: F_1->F_1, HBSS, n=3; B6->F_1, KGF, n=6.

33b). Analysis of donor T cells present in the thymus of these mice revealed heterogeneous values for CD4 SP and CD8 SP (*Figure 33c*) and high values, but heterogeneous, for the early activation marker CD69.

The analysis of the spleen at 12 weeks after transplantation demonstrated similar findings (*Figure 34*). With the notable exception of two mice, the total cellularity was decreased in all recipients of allogeneic transplanted and KGF treated mice (*Figure 34a*). This loss was paralleled by a persistence of donor T cells whereby a higher number of these alloreactive cells corresponded also to an increased frequency of activated donor T cells (*Figure 34c*). The host T cells consisted of CD4 and CD8 subpopulation at an almost normal ratio (*Figure 34b*).

The persistence of donor T cells for at least 12 weeks after engraftment and the decrease in total thymic and splenic cellularity in some of the transplanted mice rescued with KGF are reminiscent of chronic GVHD. This disorder is highlighted by the production of antibodies against nuclear components and single-strand (ss) DNA. To asses whether the prolonged survival of these KGF treated recipients of allogeneic splenocytes resulted in a state of chronic GVHD, the sera of mice were tested for the presence of auto antibodies. For this purpose, kidney and liver tissue sections from Rag-/- mice were incubated with serum collected 12 weeks post transplantation from syngeneically transplanted mice, HBSS treated or allogeneic transplanted mice treated with KGF. As demonstrated in *Figure 35* serum derived the latter group of mice contained anti-nuclear auto-antibodies, while serum from the control group
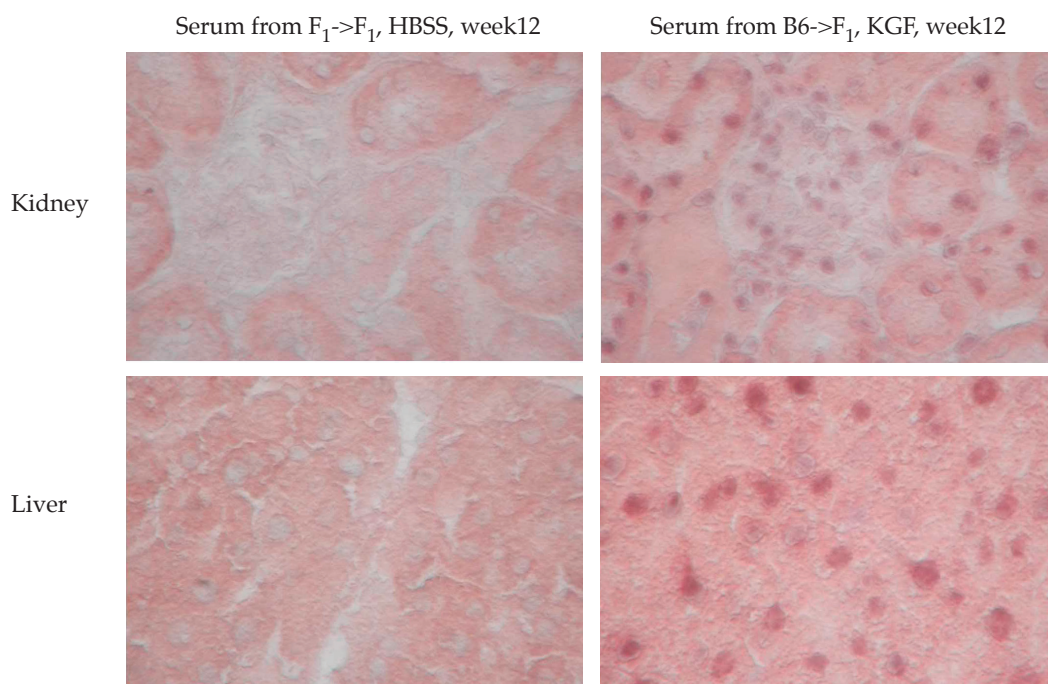
Figure 35 Anti nuclear auto-antibodies are present in the serum of allogeneic transplanted mice treated with KGF.

70

was deprived of auto-antibodies. Taken together, the extended survival secondary to KGF treatment appears to be paralleled with the detection of anti-nuclear antibodies, a characteristic feature of chronic GVHD.

Discussion

The thymus is the primary lymphoid organ for the generation and selection of T cells (*1, 2*). The loss of thymic function, either as a consequence of physiological senescence or the result of disease- and/or treatment-related pathology, affects the individual's capacity to maintain a broad T cell antigen receptor repertoire (*165*). In consequence, the ability to mount an efficient adaptive immune response may become restricted. Novel cell therapeutic and pharmacological strategies that have been designed to maintain regular thymic function despite the presence of adverse influences (e.g. radiation, chemotherapy, or GVHD) have, therefore, attracted an increased interest for their clinical application (*166, 167*). In this context, the therapeutic use of KGF holds a significant potential and great promise. The work presented here reports on the preclinical results supporting the use of KGF in the treatment of immune suppression following hematopoietic stem cell transplantation.

In vivo treatment of naive mice with a short course of pharmacological doses of KGF results in a 2- to 3-fold increase in thymocyte cellularity as early as two weeks after initiation of treatment. This effect lasts for at least 12 weeks and affects both lymphoid and epithelial cells. As the specific receptor for KGF, FgfR2IIIb is exclusively restricted in its expression to the thymic epithelial cells, changes in the number of thymocytes have to occur through an indirect mechanism, i.e. through the physiological mechanisms of lympho-stromal cross-talk. It is perceived as likely that the increase in thymic epithelial cells provides a larger number of micro-environmental niches that are able to support thymopoiesis. Independent of this contention, it is striking that not all thymic epithelial cells express FgfR2IIIb when analyzed by immunohistochemistry. While the putative precursor population of MTS24^{+} thymic epithelial cells and the UEA-1^{+} minor medullary epithelial cells stain uniformly positive for FgfR2IIIb, only few of the major cortical and major medullary epithelial cells stain positive for this specific receptor. Despite this restricted FgfR2IIIb expression pattern, KGF treatment results interestingly in a proportionally correct expansion of the thymic epithelial compartment. This may either be brought about by the expansion and maturation of subpopulation-specific precursor cells FgfR2IIIb positive or, alternatively, the exposure of KGF stimulates the secretion of other growth and differentiation factors which in turn triggers now all thymic epithelial cells regardless of whether they express the KGF-specific receptor. Whether the FgfR2IIIb positive thymic epithelial identify a specific cell type with distinct functional characteristics is not yet known. Experiments have therefore been initiated that seek to determine whether KGF displays in addition to its potent mitogenic effect also a potential for thymic epithelial cell differentiation. It is, however, conceivable

that FgfR2IIIb positive cells can undergo an asymmetric division whereby the daughter cells are phenotypically and functionally divergent in comparison to the FgfR2IIIb positive precursor cells. In this context, it was also noted that the thymic FgfR2IIIb expression density and pattern as analyzed by immunohistology did not change following KGF stimulation, a finding confirmed by quantitative PCR (data not shown). Finally, other stromal cell types such as fibroblasts, dendritic cells, and macrophages do not express FgfR2IIIb and their expansion following the exposure to KGF is therefore the consequence of successful signaling in thymic epithelial cells.

Experiments were carried out to elucidate the molecular mechanisms by which KGF enhances thymic epithelial cell proliferation and thymocyte development. Little is presently known regarding target genes of KGF signaling, be it in the normal physiological steady-state condition of organ maintenance or be it following cell injury after specific insults (*168-170*). Coinciding with the relatively late discovery of FgfR2IIIb, a precise knowledge of the signaling pathways engaged by KGF ligand binding to is receptor has not sufficiently been detailed yet. To identify KGF target genes in adult thymic epithelial cells, naive mice were treated with either KGF (15 mg/kg/day) or HBSS. Epithelial cells were then isolated 24 hours after injection and subjected to cDNA generation and quantitative RT-PCR analysis. In a first step, transcripts for IL-7, the bone morphogenic proteins (BMP) 2 and 4 and particular members of the Wnt family were quantified since these factors have previously been identified to play a role in epithelial cell development and early thymopoiesis. IL-7 acts as an important survival factor for early thymopoiesis and its specific receptor, IL-7R is expressed on TNI cells. While speculations have been made that functional IL-7R is also expressed on thymic epithelial cells, data supporting this claim are so far not been published. The bone morphogenic proteins are part of the TGF-β superfamily of multifunctional factors and participate in developmental events as diverse as patterning of the early embryonic mesoderm, epithelial-mesenchymal interactions, apoptosis and inhibition of early thymocyte development (*171-174*) and review in (*175*). BMP2 and BMP4 may therefore act directly on several cell types present in the thymus including the lymphoid compartment. Wnt molecules constitute a family of 18 different glycoproteins, which control functions such as cell fate specification, proliferation, migration, polarity and cell death. An involvement of the Wnt signaling pathway in thymus organogenesis has been suggested by experiments demonstrating that thymic epithelial cells can receive Wnt-mediated signals and that the transcription factor FoxN1 is upregulated in response to Wnt4 and Wnt5b but not Wnt1 (*176*). Moreover, blocking Wnt activity by the provision of soluble Wnt receptors, solFz result also in a partial maturational block of the progression of TN to DP thymocytes. Since lymphoid cells normally express on their cell surface several of the Wnt-specific receptors, changes in Wnt availability can affect their growth and differentiation in either an autocrine as a paracrine fashion (*152, 177*). The treatment of adult mice with KGF resulted in a robust upregulation of

IL-7 transcripts in thymic epithelial cells, in a modest increase in mRNA for Wnt4, Wnt5b and Wnt10b, and in a significant stimulation of BMP2 and BMP4 transcription. Biological effects of these molecules on thymic epithelial cell proliferation (and possibly differentiation) are plausible but will have to be tested formally. Not knowing the precise molecular mechanisms by which KGF stimulates changes in thymic lymphoid and stromal cellularity, changes in the effector molecules operational in thymocyte-epithelial cell crosstalk are a likely explanation for this observed phenomenon. To detail the global transcriptional changes brought about by KGF signaling to thymic epithelial cells, experiments are presently under way to analyze, by use of gene chip, the transcripts of *in vivo* stimulated and unstimulated primary thymic epithelial cells. These experiments are also designed to address the possibility that the thymic epithelial cell precursors with an MTS24^{+} phenotype may respond in a different fashion to KGF than the population of phenotypically mature cortical or medullary thymic epithelial cells.

In a separate set of experiments, thymic organ cultures depleted of lymphoid cells were also analyzed for changes in gene transcription in response to KGF. To this end the lobes harvested at E15.5 of gestation were first exposed for 6 days to 2dGuo prior to KGF stimulation. Although this treatment does not arrest thymic tissue maturation at the chronological age of isolation, epithelial cells did not acquire a mature phenotype at the time KGF was added to cultures. Several differences have been noted when comparing changes in transcripts stimulated by KGF in fetal thymic epithelial cells with those derived from adult thymic epithelial cells. An upregulation of IL-7 transcripts was not detected after KGF stimulation of embryonic thymic stromal cells, while mRNA-specific for IL-7R increased in these cells by approximately 7-fold. Although not yet independently verified, these results would certainly argue for the expression of IL-7 receptor on thymic stromal cells. The pattern of KGF-mediated upregulation of Wnt molecules was considerably different when compared with the corresponding changes observed in adult thymic epithelial cells. An explanation for this discrepancy might be reflective of the general differences observed when comparing the gene expression profiles of specific molecules between fetal and adult cells of a given tissue. Interestingly, transcripts for FoxN1 were severely decreased as a consequence of KGF signaling. This result is unexpected as the lack of FoxN1 causes an athymic phenotype known as "nude" but KGF-mediated signaling via FgfR2IIIb has been viewed as critical for thymic epithelial cell development of the organ primordium (*60*). While it appears likely that FoxN1 transcription is not inhibited in the presence of physiological KGF concentrations, three additional observations suggest that pharmacological doses of KGF decrease FoxN1 transcripts. First, low FoxN1 messages parallel the lack of an upregulation of Wnt4 and 5b in response to KGF. These specific Wnt family members have previously been demonstrated to enhance the transcription of FoxN1 (*176*). Second, preliminary experiments, in collaboration with Y. Takahama (Division of Experimental Immunology, Institute for Genome Research, The University of Tokushima,

Japan), have analyzed the repopulation of thymic lobes by early precursor cells revealed a decreased capacity for the homing of immature thymocytes to microenvironment previously exposed to KGF. Third, the absolute number of early thymic precursors (ETP (*36*)) has been found to be decreased in adult mice exposed 9 days earlier with pharmacological doses of KGF (unpublished data).

The pathways by which KGF signaling is transduced to stimulate changes in gene transcription have not yet been characterized. In analogy to the signaling pathways activated by other FGF receptors, experiments were designed to determine whether binding of KGF to its specific receptor also activates signaling through NFκB, ras, or p53. To this end, thymic fetal lobes depleted of lymphoid cells by 2dGuo treatment were exposed to KGF in the presence or absence of separate, specific inhibitors. None of the KGF-triggered transcriptional changes appeared to be controlled exclusively by only one of the second messengers. For example, BMP2, but not BMP4, transcription was abrogated when ras activity was blocked but neither Wnt10b nor IL-7R expressions were affected in the presence of NFκB, ras, or p53 inhibitors. In contrast, inhibition of FoxN1 transcription by KGF was lost when ras and p53 were inactivated. Finally, it also remains to be defined how KGF exerts its cytoprotective effect on thymic epithelial cells after injury by radiation or GVHD. Observations in experimental models focusing on epithelium of the respiratory or alimentary tract have recently begun to light on the mechanism of protection. For example, the protection exerted by KGF on lung and intestinal epithelial cells from radiation-induced damage is paralleled by reduction in apoptosis and sustained proliferation (*178-180*). Furthermore, experimental evidence suggests that stabilization of actin filaments (F-actin), important cytoskeletal structure that allow formation of stable construction in the cells and are also fundamental for cell movement, via a PKC-dependent pathway is associated with resistance to radiation (*179*). However, it remains to be established whether this association is coincidental or causal. Finally, KGF signaling may also cross-talk to establish pathways to enhance cell survival and resistance to cell damage. For example, irradiated and HSC-transplanted mice treated with a combination of p53 inhibitor and KGF revealed a robust decrease in tissue damage of pulmonary, intestinal and skin epithelium in an experimental model of acute GVHD when compared to recipients treated only with one of these pharmacological blockers. In addition, histological analyses of thymic sections of mice irradiated and HSC-transplanted treated with p53 inhibitor and KGF revealed a normal cellularity and tissue architectural organization, while animals treated with only one of these molecules demonstrated limited structural deficiencies (data produced in collaboration with Bruce Blazar, University of Minnesota Cancer Centre, Minneapolis).

The *in vivo* treatment of adult mice with KGF results in a proportional increase of all thymocyte subpopulations by 2- to 3-fold. Phenotypic analyses at the different developmental stages

and the use of the Vβ repertoire of positively selected, mature SP thymocytes did not reveal differences when compared to control mice. Thus, KGF treatment stimulates an elevated level of thymopoiesis without affecting thymocyte maturation or selection. Moreover, this KGF-effect is long-lasting as the increase in thymic size and cellularity was evident for at least 3 months. Differences in the KGF responsiveness as a function of age were, however, not observed. In preliminary experiments, mice older then 12 months were treated with KGF and responded with an increase in thymic cellularity similar to young animals. The KGF-effect is not restricted by the senescence of thymic tissue albeit age-dependent changes are somewhat limited in mice when compared to humans.

The initial effect of KGF on thymocyte production in adult mice appears to affect preferentially cells with a TN phenotype. Kinetic studies assessing changes in thymocyte subpopulations demonstrated a burst of TNI and TNII proliferation early after the provision of KGF. Since this effect is paralleled by an upregulated expression of IL-7, it is likely that the increased survival of IL-7R$^+$ cells is effected by this cytokine. Prior to stimulation of TN cells, KGF has creates a larger stromal compartment able to accommodate more developing thymocytes. While this interpretation of the data consist in an attractive model, several issues related to the observed biological functions of KGF remain to be addressed. For example, it is not known yet whether the increased transcripts expression of BMP2 and BMP4 specific affect the TN -> DP -> transition in treated adult mice. Similarly, changes in Wnt expression have the theoretical potential to either enhance or block early thymocyte development. It will be the task of further experiments to dissect the molecular mechanisms leading to a sustained enhancement of thymopoiesis following the short-term exposure to KGF.

Comparing the effect of KGF in adult thymic tissue with the changes in fetal thymic organ cultures, revealed several differences. For example, the accumulation of TNI thymocytes and the concomitant loss of cells with a more mature phenotype were specific for fetal tissue. Since little to no IL-7 is being produced in thymic organ cultures in response to KGF, a lack in the survival of IL-7R bearing immature thymocytes may serve as an explanation for these results. Experiments supplementing fetal thymic organ cultures with exogenous IL-7 are presently under way to test this contention directly. Interestingly, the exposure of fetal lobes to KGF was limited to 48 hours but the disruption of thymocyte development was still apparent 20 days later. Moreover, the changes observed are particularly marked by the loss of DP cells. Since there is not a complete lack of TNIV cells, a block in the differentiation of TN cells to DP cells seems to be a likely explanation. This specific developmental transition is under the control of several thymocyte-intrinsic signals including the productive rearrangement of the T cell antigen receptor β-chain and the successful signaling via the pre-T cell antigen receptor. In contrast, little is presently known regarding the requirements provided by the thymic

microenvironment to achieve this maturational progression. Although BMP2 and BMP4 have been previously demonstrated to exert a block in the TN -> DP -> transition, their functional involvement in the long-term fetal thymic organ culture experiments described here is far from clear, least for the reason that gene profiling has not been performed on fetal tissue other than 24 hours after KGF treatment.

Experiments to detail the export of thymocytes in adult mice revealed that mice treated with KGF show an increased export of early thymic emigrants, both $CD4^+$ and $CD8^+$ to the spleen within 16 hours, but only at the time were the thymus has reached the stable steady state after treatment. The discrepancies within the migration of early thymic emigrants in the spleen and in the lymph nodes, where no significant increased in this population was detected, could be explained by the homing pattern capacity that could differs between spleen and lymph nodes.

Despite the systemic presence of acute GVHD, KGF preserved normal thymic development as revealed by typical cellularity, frequency and cellular proliferation of the different immature and mature thymocyte subsets. The structure of the thymic microenvironment following exposure to KGF was almost normal, as assessed by the regular cellular compositions of several thymic epithelial cell compartments. The hypothesis that this effect was a direct consequence of KGF on thymic epithelial cells as thymic epithelial cells but not thymocytes or other stromal elements specifically express the KGF receptor was confirmed by analysis of the peripheral compartment, where no changes between the allogeneically transplanted and KGF treated group and the allogeneically transplanted and saline treated group were detected. Treatment with KGF also reduced CD80 expression, possibly rendering thymic epithelial cells less likely targets to allogeneic T cell recognition. Interestingly, the absolute cell number of mature donor T cells in B6 -> B6D2F$_1$ recipients treated with KGF was, however, significantly increased. Finally, KGF treatment was shown to affect the transcriptional activity of several genes described to play an important role in thymic epithelial cell function when measured under these conditions. The conclusion was therefore that thymic epithelial cells are targets of GVHD and the hypothesis that prevention or reversal of thymic epithelial cell injury by KGF has a beneficial effect on developing thymocytes by allowing a normal cross-talk to occur between thymic epithelial cells and thymocytes was confirmed. Recent investigations concerning issues of the mesenchymal-epithelial cell interactions, the morphogenesis of epithelium and the mechanisms operating in cutaneous wound repair have identified KGF as a highly specific and potent mitogen (*43, 59, 63, 64*). Thymic epithelial cells have been reported to bear cell surface markers common with epithelia of other organs, in particular the epidermal keratinocytes in the skin (*181*). Despite these phenotypic similarities it is important to recognize that the capacity to efficiently support development and selection of T cells is a

unique feature of thymic epithelial cells (*182*). The observations that FgfR2IIIb is expressed only on thymic epithelial cell cells and not on other stroma cells or thymocytes strongly imply that treatment with KGF affected thymic epithelial cells and the effects observed in this GVHD model were due to changes in thymic epithelia. Although it is not possible to completely rule out an indirect contribution of KGF acting via modulation of peripheral GVHD, the central effect of KGF on thymopoiesis is much more relevant for the above and the following reasons: clinical assessment of GVHD comparing the HBSS and KGF treatment groups did not reveal significant changes in this model (data not shown); and a role for glucocorticoids as effectors of the observed thymic changes (i.e. loss of DP cells and hypocellularity) has been ruled out in this model (*129*). Cytoprotection constitutes a promising approach to ameliorate epithelial injury inflicted by GVHD. KGF has recently been recognized as an agent for epithelial cell repair in different GVHD target organs. Administered to mice before extensive conditioning and bone marrow transplantation, KGF ameliorated both survival and GVHD related pathologies in liver, lung and skin but not in spleen, colon and ileum (*183*). Similarly, in these experiments, splenic GVHD was not alleviated. In other experimental systems, an increased survival of transplant recipients was also observed when KGF was administered prior to total body irradiation, either alone or in combination with chemotherapy (*55, 184*). Here, KGF treatment protected the gastrointestinal epithelium from radiation- and immune-mediated injury, reflecting variations in the clinical outcome depending on the choice of the experimental model used and the duration of KGF administered. Despite these differences, the central biological response to pharmacological doses of KGF was due to a potent trophic effect that may very well be specific for individual tissues. For example, the KGF effect on intestinal epithelium (*185*) included the survival of crypt stem cells (*54*), improved DNA repair (*186*) and an enhanced thickness of the entire mucosa (*55*) with an increased formation of goblet cells (*187*) and their secretory products (*188, 189*). Conversely, the decreased pulmonary damage observed after KGF treatment was secondary to enhanced epithelialization and the attenuation of immune-mediated injury (*190*). Thus, the pharmacological effects of KGF treatment are documented for tissues where KGF receptor expression has been convincingly demonstrated, e.g. intestinal epithelium, hepatocytes, skin keratinocytes, and alveolar type II cells (*185, 191, 192*).

KGF protects thymic epithelial cells from cell injury not preventing the activation of donor cytotoxic T cells. Indeed, the relative number of activated donor T cells is increased in transplant recipients treated with KGF when compared to the control population. Although these cells represented only a very small fraction of all thymic T cells in the former treated group, these donor derived mature T cells are first detected in the thymus as early as day 9 and are not activated, the activation marker CD69$^+$ on donor T cells in the thymus is acquired only at day 11 post cell transfer. A decrease in epithelial cell allogeneicity as a consequence

of KGF exposure is a plausible explanation for the observed protection of the epithelial cell compartment. The diminished cell surface expression of the costimulatory molecule CD80 on thymic epithelial cells in mice treated with KGF when compared to mice with unmitigated thymic GVHD. The engagement of this costimulatory molecule is critical for a T cell response to both nominal and alloantigens (*164*). An upregulation of CD80 expression on thymic epithelial cells is a typical feature of thymic GVHD in untreated mice. It therefore seams likely that initially allogeneic T cells are activated *in situ* by thymic epithelial cells, not only expressing MHC molecules, but that are equally competent to provide the necessary costimulatory signal for their allorecognition by donor T cells. Reduction of CD80 expression as a consequence of KGF treatment may therefore render these cells less efficient stimulators and targets of allorecognition. Finally, the expression of MHC Class II on thymic stroma cells (as measured by immunohistology) did not revealed differences between allogeneically transplanted mice treated with KGF and those injected only with HBSS. The incomplete activation of donor-derived T cells, as an indirect function of KGF, is furthermore mirrored by a decrease in the expression of transcript for granzyme B and Mip-1, usually only minimally detected in mature intrathymic T cells. Mip-1 constitutes a typical hallmark for activated T cells. Independent of these changes among the infiltrating T cells, the minor medullary epithelial cells (UEA-1$^+$) disappear by day 13 after induction of GVHD independently of the mice received KGF or HBSS. The molecular mechanism by which these epithelial cells are destroyed is presently not known.

The loss of the UEA-1$^+$ minor medullary epithelial cells in the course of GVHD seams to correlates with the downregulation of Aire, a transcription factor implied in the promiscuous gene expression in the thymus. A mutation in this transcription factor leads to the development of the autoimmune polyendocrinopathy syndrome (APS-1, a.k.a. APECED) (*160, 161, 193-196*). Whether mice with acute GVHD rescued by KGF treatment will now develop such an autoimmune disorder reminiscent of APS-1 is not yet known. However, the serum of allogeneically transplanted mice that had been treated with KGF contained anti-nuclear auto-antibodies, a characteristic serological feature of the chronic GVHD (*92-97*). Whether a state of chronic GVHD represent the price to be paid by KGF treated mice for escaping death by acute GVHD remains to be established.

Although the molecular mechanisms of KGF-mediated protection remain to be defined, the beneficial effects of KGF on thymic epithelial cells may be affected by different mechanisms. For example, KGF promotes the homeostasis of thymic epithelial cell proliferation, which in turn support regular thymopoiesis. KGF may also expedite the process of epithelial cell recovery following direct damage by cellular and humoral effector mechanisms of GVHD-mediated inflammation. In this context, it has recently been shown that KGF mediates a suppressive effect

on inflammation-induced gene expression as it prevents the interferon-stimulated trafficking of STAT1 from the cytosol to the nucleus (*197*). Furthermore, administration of KGF to animals prior to their conditioning by radio- chemotherapy had a long lasting beneficial effect on thymic function (*183*), underscoring a cytoprotective mechanism for this factor. Enhancement of mechanism of protection of thymic epithelial cell function and the modulation of thymic epithelial cell allogeneicity are not mutually exclusive.

With its defined role as a cytoprotective agent for epithelial cells, enhanced production of endogenous KGF may thus constitute an adjunct strategy for GVHD treatment following allogeneic BMT. For example, expression of KGF is subject to negative regulation such as glucocorticoids, a standard component of GVHD therapy that decreases KGF mRNA in a time- and concentration-dependent manner (*198*). In consequence, it may be of clinical benefit to administer exogenous KGF in a pharmacological dose prior to conditioning and in the presence of thymic GVHD.

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Curriculum Vitae

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Education

1991-1995	Liceo Cantonale Mendrisio (CH)
1995-1999	University of Basel (CH)
	Faculty of Philosophische Naturwissenschaften (Phil. Nat), Direction: Biology II (Biochemistry and Molecular Biology)
1998-1999	Diploma-Work in the laboratory of Pediatric Immunology, Kantonsspital Basel
	Supervision: Dr. W. Krenger
	Title: *Thymic development in acute and chronic Graft versus Host Disease (GvHD)*
1999	Diploma in Phil. Nat, Biology II , Direction: Immunology
2000-2003	PhD-Program at the University of Basel, Laboratory of Pediatric Immunology, Kantonsspital Basel
	Supervision: Prof. Dr. G.A. Holländer
	Direction: Cellbiology, Title: *On the Role of Keratinocyte Growth Factor for Thymic Epithelial Cell Development and the Protection from Graft versus Host Disease*
	Thesis supervision: Prof. Dr. Antonius Rolink
	Co-relator: Prof. Dr. Ed Palmer

Languages

Italian (mother-language), English, German and French orally and written.

Courses attended during the PhD-Program at the University of Basel (Biozentrum, University Basel)

Immunology I/II/Advanced	Prof. Dr. R. Giesler/ Prof. Dr. A. Rolink
Cell cycle and DNA repair	Dr. R. Jessberger
Cell and Molecular biology of cancer	Prof. Dr. M. Burger
Cell signaling I/II	PD Dr. K. Balmer-Hofer
Novel and emerging technique in	
Functional genomic	Prof. Dr. P. Philippsen
Transcriptional regulation	PD Dr. R.G. Clerc
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Publications

Krenger W, Rossi S, Holländer GA, *Apoptosis of thymocytes during graft-versus-host disease is independent of glucocorticoids*, Transplantation, 2000

Krenger W, Rossi S, Piali L, Holländer GA, *Thymic atrophy in murine acute graft-versus-host disease is effected by impaired cell cycle progression of host pro-T and pre-T cells*, Blood, 2000

Rossi S, Blazar BR, Farrell CL, Danilenko DM, Lacey DL, Weinberg KI, Krenger W, Holländer GA., *Keratinocyte growth factor preserves normal thymopoiesis and thymic microenvironment during experimental graft-versus-host disease*, Blood, 2002

Waldburger JM, Rossi S, Holländer GA, Rodewald HR, Reith W, Acha-Orbea H., *Promoter IV of the Class II transactivator gene is essential for positive selection of CD4$^+$ T cells*, Blood, 2003

Meetings

Wolfsberg-Immunology- Student Meeting,, March, 2001. Presentation: *Mechanisms of thymic atrophy in acute and chronic graft-versus-host disease*

Swiss Pediatric Research Meeting,, Lucern, Jun 2001. Presentation: *Keratinocytes growth factor reduces thymic injury in experimental graft-versus-host disease.* Winner of the Nestlé Research Prize for the best presentation

Thymus Meeting, Kerkrade/Rolduc, July 2001, presence

ASH (American society of Hematology), Orlando, December 2001, accepted abstract for presentation: *Keratinocyte Growth Factor Preserves Normal Thymopoiesis And The Thymic Microenvironment During Experimental Graft-Versus-Host Disease,* presented by W. Krenger

SGAI (Swiss society of Allergology and Immunology), Lugano, March 2002. Poster: *Keratinocyte Growth Factor preserves normal thymopoiesis and the thymic microenvironment during experimental Graft-versus-Host disease*

Wolfsberg-Immunology- Student Meeting, March 2002. Presentation: *Keratinocyte Growth Factor Preserves Normal Thymopoiesis And Thymic Microenvironment During Experimental Graft-Versus-Host-Disease*

Wolfsberg-Immunology- Student Meeting, March 2003. Presentation: *The effect of Keratinocyte Growth Factor on thymic epithelial cells and thymopoiesis*

ASH (American society of Hematology), San Diego, December 2003. Presentation: *Fibroblast Growth Factor-7 promotes thymic epithelial cell proliferation, enhances thymopoiesis and reduces in situ activation of thymus-infiltrating alloreactive T cells*