Expression of human leukocyte antigens in diffuse large B cell lymphomas

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Chapter 1

General Introduction

Contents

1 Human Leukocyte Antigens

1.1 HLA molecules and the cellular immune response

To eradicate pathogens, the human immune system can employ several distinct defense mechanisms. The humoral immune response is primarily mediated by antibody-producing B-cells. The cellular immune response is elicited by T-cells recognizing peptides derived from pathogens in the context of Major Histocompatibility Complex (MHC) molecules, called Human Leucocyte Antigens (HLA) in humans. These highly polymorphic peptide-binding molecules can be divided in HLA class I and HLA class II molecules. HLA class I molecules mainly present small peptides of nuclear or cytoplasmic origin. Extracellularly derived peptides, degraded in endosomal compartments are preferentially bound to HLA class II molecules [1-4]. Cytotoxic T-cells (CTL) bearing the CD8 co-receptor for the T-cell receptor (TCR) complex recognize antigens associated with HLA class I molecules while $CD4^+$ T-helper (Th) cells confer reactivity towards HLA class II bound antigens [5,6]. The presentation to circulating T-cells of peptides derived from intra-cellular and extra-cellular proteins in the context of HLA enables the immune system to constantly screen for infected or transformed cells. HLA class I molecules are in principal expressed on nearly all nucleated cells [7]. The class II molecules are constitutively expressed on antigen presenting cells (APCs) such as B-cells, dendritic cells, monocytes, skin Langerhans cells and macrophages but also on activated T-cells [8]. Upon priming of naïve CD4+-T cells by non-self antigenic peptides in the context of HLA class II molecules on APCs, T- cells develop into Th1-cells or Th2- cells. Th1-cells generate CTL responses by producing the crucial cytokines IL-2, TNFß and IFN-γ. Direct interaction of co-stimulatory receptors present on CTL such as CD27, CD134 and HLA class II with the corresponding ligands on the Th-cells enhances the proliferation and survival of the CTLs during the Th1- response [9]. The presence of IL-4 during priming of naïve T-cells however, directs the immune response towards proliferation of Th2-cells producing IL-4, IL-5, IL-6, IL-13 which results in antibody production by activated B-cells and proliferation of eosinophils [10,11].

For the transposition of a local response to a more generalized immune response, prolonged surface expression of the peptide/HLA class II complex is required during migration of APCs from the initial inflammation site to draining lymph nodes [12]. TCR triggering by the long existent peptide/class II complex can occur transiently and repeatedly which can ultimately result in activation of many T-cells [13]

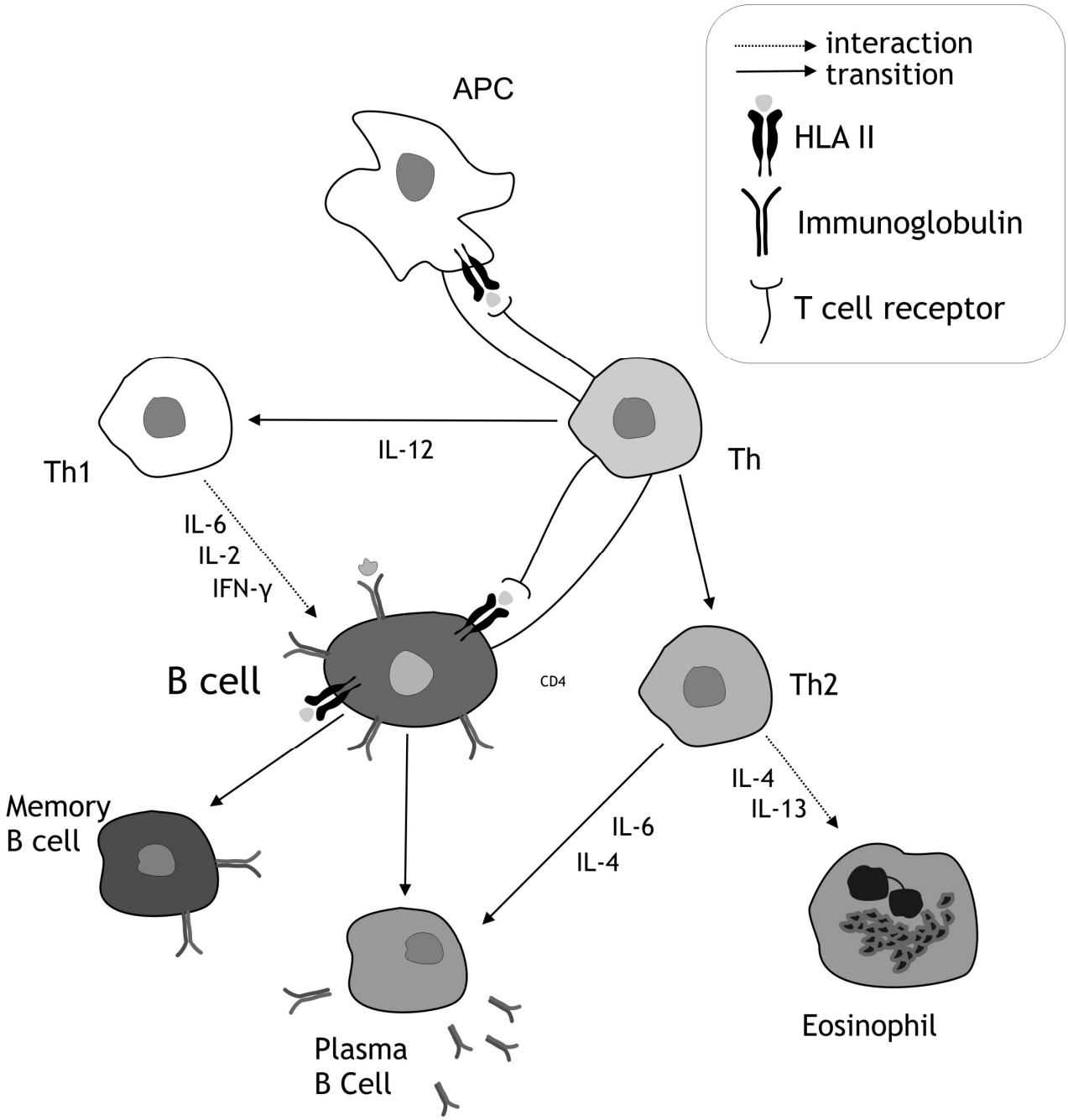

Figure 1. A schematic representation of B and T cell activation. Antigen presenting cells (APCs) including B cells, present HLA class II bound antigens to naïve $CD4^+$ T helper cells. These become activated if the APCs express the required accessory co-stimulatory molecules during interaction. Activated Th cells can either differentiate in Th1 or Th2 cells depending on the cytokines present during activation. Th1 cells produce cytokines required for a cytotoxic T cell response (IL-2, IFN-γ and TNF-ß). B cells can become activated as well during this response. The Th2 response also results in antibody production and in proliferation of eosinophils. Memory B cells express surface immunoglobulin (Ig) and plasma cells secrete Ig (antibodies).

1.2 HLA region at chromosome 6p21.3

The Human Leucocyte antigens (HLA), control immune responses to pathogens, graft acceptance or rejection and tumor surveillance [14]. The HLA molecules are encoded for by genes that are located in the 4 Mb large HLA region at chromosome 6p21.3 [15]. This very gene-rich segment contains at least 240 gene loci (128 predicted to be expressed) [16] including a large number of polymorphic multicopy genes of which more than 40% is involved in the immune response. The region is thought to have evolved from repeated gene duplication, insertion and inversion events during evolution [17,18]. The genes for the heavy chain of the classical HLA class I molecules HLA-A, -B and –C are located at the telomeric end of the HLA-region. In addition, the non-classical genes HLA-E,-F,-G and –H, class I related genes MICA and MICB are also mapped to this 1 Mb large class I region. The central class III region (2 Mb) has the highest density of genes including complement encoding genes, heat shock proteins, adrenal steroid hormone genes, TNF α and ß and the lymphotoxin genes.

The centromeric end of the HLA region contains amongst others, the genes for both the α and ß polypeptide chains of the HLA class II molecules HLA-DR, -DQ and -DP. Other genes involved in the immune response, mapped to this region are HLA-DO, HLA-DM, the transporter-associated with antigen processing genes (TAP1 and 2) and the large multifunctional immunoproteasome genes (LMP2 and 7) [19]. Both HLA class I and class II molecules are highly polymorphic based upon small nucleotide differences in the sequences of the coding regions. So far, several hundreds of alleles have been described and new alleles are continuously being reported (www.ebi.ac.uk/imtg/hla).

The combination of certain class I, class II and class III genes on one chromosome is designated a haplotype. Each individual inherits one of the haplotypes of each parent and the combination of those two inherited haplotypes is called the HLA genotype. All HLA class I and II genes of both chromosomes are expressed and this is called the individuals HLA phenotype. Interestingly, the DNA content of the different haplotypes, is not constant. The DQ and DP subregion size is equal in all haplotypes, but the HLA-DR region is not. The HLA-DRB genes have been assigned to nine different loci comprising four expressed loci (DRB1, DRB3, DRB4, DRB5) and five pseudogenes (DRB2, DRB6-9) [20-22]. Five different haplotype groups have been described according to the allelic size polymorphism of the DRB1 gene and the number and nature of the DRB loci expressed (see Figure 2). The DR3, DR11-14 haplotypes contain the minimal amount of DNA in the DR-DQ region with DR2 having approximately 30 kb and the DR4 haplotype approximately 110 kb more DNA in the DR subregion [23]. In addition, also the DR7 and 9 were also found to contain ~ 110 kb extra

DNA [24]. Currently different haplotypes are being sequenced but it appears that the extra amount of DNA does not contain functional genes and is in fact junk DNA. The difference in DNA content between the haplotypes may however be an important factor in the meiotic recombination process as it was previously shown in mice MHC that recombination frequencies differed between haplotypes and that high sequence homology was required at the recombination hotspot [25,26].

Also in family studies the sites of recombination differed between the class II haplotypes possibly due to the molecular organization of the DR region [27]. The enormous degree of polymorphism of the HLA genes is thought to have been maintained during evolution on the premise that individuals heterozygous at the different HLA loci are able to present a wider range of antigenic peptides than homozygotes, resulting in a more productive immune response to the vast array of pathogens [28]. Recombination in the HLA region (crossing over) between two non-sister chromatids occurs during meiosis and results in shuffling of haplotypes thereby contributing to the diversity which might be beneficial to a population against a recently introduced pathogen (for review see [29]). On the other hand, conservation of certain haplotypes may be beneficial in mediating an immune response against relatively old, common pathogens. For instance, *cis* association of specific DQA1 and DQB1 alleles ensures a functional DQ heterodimer [30] and the maintenance of specific DR-DQ combinations seem to protect against auto-immune disease [31]. Recombination does not seem to occur at random and by the use of many micro-satellite markers located in the HLA class II region, the cross-over sites in families with recombinant chromosomes were determined resulting in the definition of three "recombination hotspots" (see Figure 2) [32].

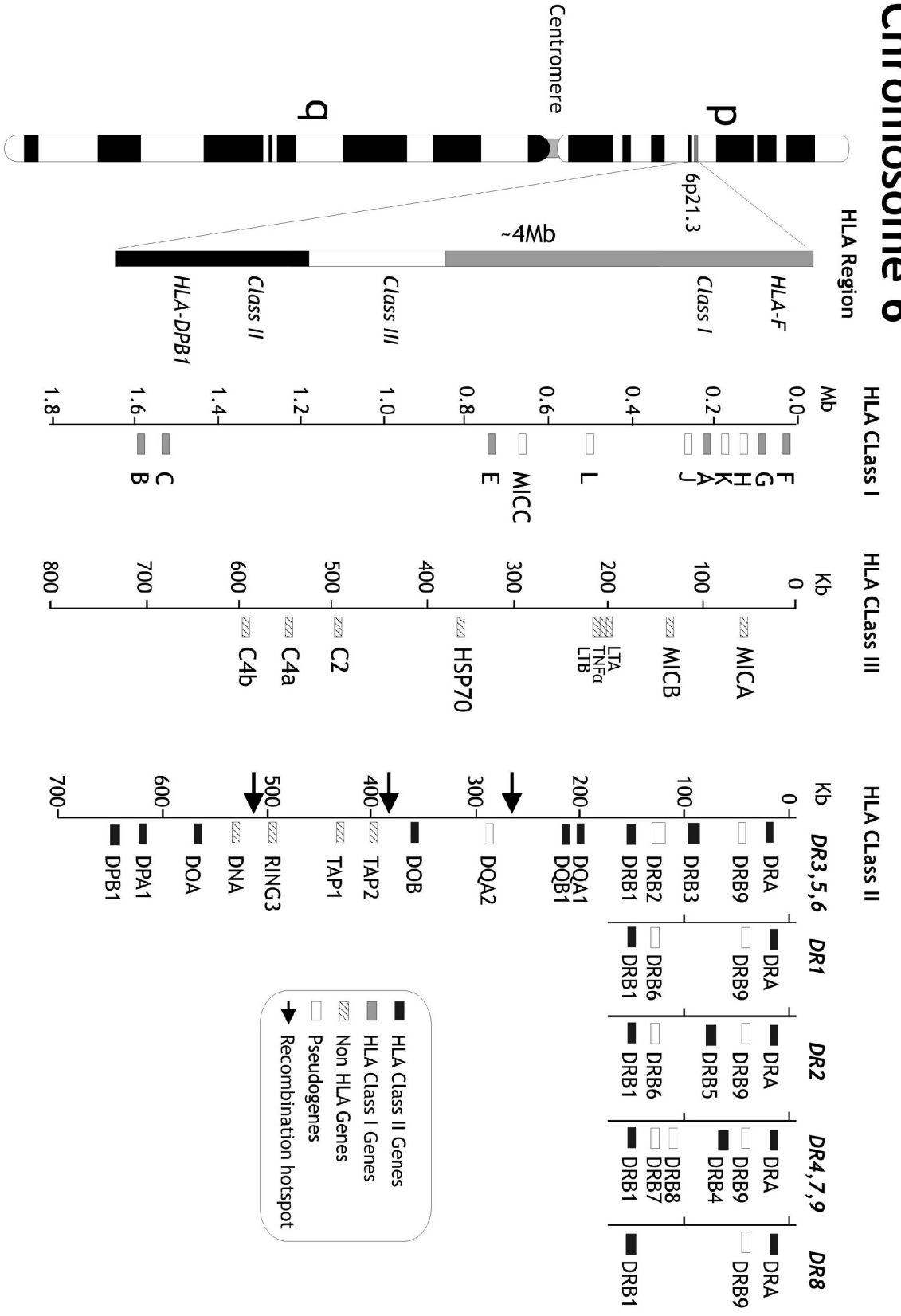

Figure 2. Schematic representation of the HLA-region located at chromosome 6p21.3. This 4 Mb large region contains over 240 genes and pseudogenes with the HLA class I genes being located at the telomeric part and the class II genes at the centromeric part of the region. In between, in the class III region, many genes reside that are involved in the immune response. The "recombination hot spots" within the class II region are indicated with an arrow. The DNA content of the HLA-DR sub-region is not constant in the different haplotypes. Five different haplotypes groups have been described according to the allelic size polymorphism of the DRB1 gene and the number and nature of the DRB loci expressed.

1.3 Structure and assembly of HLA class I molecules

The HLA class I molecules are transmembrane glycoproteins that assemble as heterodimers consisting of a highly polymorphic 43-kD heavy α-chain encoded by the HLA genes at chromosome 6p21.3 and an invariant 12-kD ß2-microglobulin ($ß_2M$) light chain encoded by a gene at chromosome 15q21 [33,34]. The α-chain consists of two-peptide binding domains (α1 and α2), one immunoglobulin-like domain (α3), the transmembrane region and the cytoplasmic tale. The extracellular helical parts of the α1 and α2 domains form the rim of a groove in which the antigenic peptide is bound. Peptide binding is restricted by the size and shape of the groove which is determined by the allotype encoded by the HLA genes. The size of the peptides is usually limited to 9 amino acids [35,36]. In the floor of the groove, the ß-pleated sheet formed by the α domains contains allele specific pockets, by which the peptide binding specificity of the particular HLA class I molecule is determined [37-39]. The HLA class I molecules will first become stable after association with $ß_2M$ [40] and antigenic peptide [41]. The assembly of newly synthesized class I and $ß_2M$ chains with antigenic peptides is called antigen processing [42]. Peptides are mostly derived from degradation of endogenous proteins from the nucleus or the cytosol by the proteasome which is encoded by the LMP genes in the HLA class II region. The transporter associated with antigen processing (TAP), which is composed of two subunits (TAP1 and TAP2) that have different biochemical and functional properties subsequently transports the 8-10 amino acids long peptides form the cytosol into the endoplasmatic reticulum [43]. In the endoplasmatic reticulum, the newly synthesized class I heavy and $ß_2M$ chains are loaded with the peptides emerging through the TAP channel resulting in the formation of a stable HLA class I complex. Multiple chaperone molecules including calnexin, calreticulin and tapasin are involved in this assembly process [44]. The class I complexes are subsequently transported through the Golgi complex to the cell surface where presentation of peptides to circulating $CD8^+$ T-cells takes place.

Constitutively all nucleated cells express HLA class I molecules but expression levels vary. The regulation of expression is modulated by cytokines, hormones and viruses. At the transcription level, cytokines are probably the most relevant immunomodulatory substances as they interact with promoter elements [45]. For instance, INF-γ and TNF-α synergistically upregulate class I expression [46] while IL-10 suppresses class I expression [47]. Most viruses stimulate production of IFN-γ/ß and consequently upregulate class I expression. However, some other viruses such as Adenovirus, Human Immunodeficiency virus and Cytomegalovirus may interfere with the MHC class I assembly pathway, thereby downregulating class I expression and facilitating evasion from the immune attack [48,49].

1.4 Structure and assembly of HLA class II molecules

The structure of the HLA class II molecules DR, DQ and DP is very similar to the class I molecules but both the 34 kD α-chain as well as the 29 kD ß-chain are encoded for by genes within the class II region. Both chains have four domains: the peptide binding domain (α1 and ß1), the immunoglobulin-like domain (α2 and ß2), the transmembrane region and the cytoplasmic tale [50]. The peptide binding groove is formed by two parallel α-helices of the α1 and ß1 chain and is more open compared to class I molecules, allowing for binding of longer peptides, 12-25 amino acids [51]. The peptides bind with a core region of similar length, with the non-bound flanking region extending from the ends of an open groove [52]. Typically, five main pockets whose size, hydrophobicity and charge vary in an allele-specific manner accommodate the so-called HLA-anchor residues. The peptide positions are to varying extents exposed to solvent and thus potential T-cell receptor (TCR) contacts [53-55]. The ß2 chain is associated with T-cell receptor (TCR) binding [56]. Unlike for class I, peptide processing and loading of extracellular proteins is TAP- and LMP-independent [57]. Extracellular proteins are endocytosed and merge with intracytoplasmic lysosomes containing proteolytic enzymes where after the proteins are degraded into peptides [58]. Within the ER, the class II molecules associate with invariant chain (Ii), a specific class II chaperone protein [59,60]. CLIP (class II-associated invariant chain peptides}, a set of nested peptides derived from Ii, occupies the class II binding groove thereby precluding the premature loading of newly formed class II molecules by immunogenic peptides [61]. The endosomes carrying the peptides then merge with the membranous vesicles that enclose the complexes of HLA class II molecules and Ii. The HLA class II binding groove is subsequently liberated through dissociation of CLIP by a protein highly homologous to HLA class I and II molecules, called DM (encoded for by a gene located in the HLA class II region: *HLA-DM*) thereby enhancing the binding of specific peptides [62,63]. After peptide binding, the HLA class II/peptide complex is transported to the cell surface where it is expressed to circulating $CD4^+$ T-cells [64].

DO, also encoded for by a gene in the HLA-class II region (*HLA-DO*), is protein expressed in a subset of APCs, mainly B cells, further modulates the peptide loading process [65,66]. The B-cell receptor (BCR) binds antigen and transports it to endosomal compartments where DO physically associates with HLA-DM and inhibits it function. As a consequence, DO restrains presentation of exogenous antigens delivered through nonreceptor-mediated mechanisms (for review see [67]). Only high-affinity antigen qualifies for expression in complex with HLA class II on the B-cell surface leading to T-cell dependent proliferation and secretion of antibodies [68].

HLA class II molecules are constitutively expressed on cells that serve as APCs for $CD4^+$ T-cells but may also be induced on other cells by IFN-γ (for review see [69]). Class II expression is also modulated by other cytokines such as IL-4, IL-10, IFN-α/ß, TNF-α and also glucocorticoids. The expression patterns are tightly regulated by a complex interaction between *cis*-acting DNA elements and trans-acting nuclear regulatory proteins [26]. The *cis* elements, important for both constitutive and inducible class II expression are located in the promoter region and are thought to be conserved among the various class II genes [70] (see also below Ch. 1.6). Usually DR, DQ and DP are concomitantly expressed but some B-cells solely DQ and others DR. The expression levels of the different class II molecules varies with HLA-DR being highly expressed followed by DP and DQ. In most individuals, each chromosome expresses two DRB genes and their different ß-polypeptide chains assemble as heterodimers with the α-chain (DRA) product. The number of alleles and the level of expression differs between the DRB genes with only the DRB1 gene, present in all DR haplotypes, being highly expressed and very polymorphic [71]. Interestingly, due to sequence variation in the promoter region, the transcription activities of the DRB1 promotor in the various haplotype groups differed, with the DR52 group (DR3, DR11-14) showing the highest transcriptional activity followed by DR1, DR51 (DR15, DR16) and DR8. The DR53 group (DR4, DR7, DR9) showed just 25-50% of the activity of the DR52 group [72].

Figure 3. Three dimensional crystal structure of one α ß heterodimer of HLA-DR1. The α chain is the lighter shade, the ß chain is the darker shade. The bound peptide is indicated by the arrow.

SEROLOGY DEFINED				DNA DEFINED			
DR	**SPLIT**	**DR**	**DQ**	**DRB1***	**DRB>1***	**DQA1**	**DQB1**
DR1			DQ5	1		0101	0501
DR1		DR51	DQ5	1	5*0101	0101	0501
DR2	DR15	DR51	DQ6	15	5*0101/0102	0102	0602
	DR16	DR51	DQ5	16	5*02	0102	0502
DR3	DR17	DR52	DQ2	3	3*0101/0202	0501	0201
	DR18	DR52	DQ4	0302/0303	3*0101	0401	0402
DR4		DR53	DQ7	4	4*0101/0103	0302	0301
		DR53	DQ8	4	4*0101/0104	03	0302
		DR53	DQ4	405	4*0101	0302	0401
		DR53	DQ2	4	4*0101/0103	0303	0202
DR5	DR11	DR52	DQ7	11	3*0202	0501	0301
	DR11	DR52	DQ5	1101	3*0202/0301	0102	0502
	DR12	DR52	DQ7	12	3*01/02/03	0501	0301
DR6	DR13	DR52	DQ6	1301	3*0101/0202	0103	0603
	DR13	DR52	DQ6	1302	3*0301	0102	0604/0609
	DR13	DR52	DQ7	1303/1305	3*0101	0501	0301
	DR14	DR52	DQ5	1401/1407	3*0202	0104	0503
	DR14	DR52	DQ7	1402	3*0101	0501	0301
	DR14	DR52	DQ7	1406	3*0202	0501	0301
DR7		DR53	DQ2	701	4*0101	0201	0202
			DQ9	701	4*0103	0201	0203
DR8			DQ4	8		0401	0402
			DQ7	803		0601	0301
			DQ6	803		0103	0601
DR9		DR53	DQ9	901	4*0101/0103	03	0303
		DR53	DQ2	901	4*0101/0103	03	0202
DR10			DQ5	1001		0104	0501

Table 1. Most common HLA-DR and HLA-DQ haplotypes defined by serology and by DNA typing

1.5 Cancer and immune surveillance

Cytotoxic T-cells

Cytotoxic $CD8^+$ T-cells play a central role in the host defense against tumors. CTL recognize tumor associated antigens (TAA) expressed in the context of HLA class I molecules. For CTL priming, presentation of tumor-derived peptides by professional APCs to $CD4^+$Th cells is crucial [73]. Only a few HLA-peptide complexes are required for triggering a CTL response [74] but efficient tumor cell lysis depends on both the amount of available peptide and the levels of HLA-expression [75]. Tumor-specific CTLs have been identified in various tumor types and have allowed the identification of TAA and their HLA restriction elements [76]. Several categories of TAA have been reported including antigens preferentially expressed in tumors and germ cells (i.e. MAGE, BAGE and GAGE families) [77,78] , differentiation antigens (i.e. melanocyte/melanoma specific Melan-A) [79], antigens derived from mutated genes [80-82] and viral antigens [83]. Also B cell lymphomas present highly specific TAA, including their own idiotypes (Id) [84-86], antigenic determinants localised to the variable (V) regions of immunoglobulin (Ig) [87-89].

Natural Killer cells

Natural Killer (NK) cells also play a role in tumor immunity as they not only kill virus-infected cells by anti-body dependent cellular cytotoxicity but also transformed cells with downregulated HLA [90]. Their cytolytic activity is regulated by recognition of HLA-A, -B and –C molecules and HLA-E on the potential target cell by respectively inhibitory immunoglobulin-like receptors (KIR) and CD94 on the NK cells. CD94 ‘gauges‘ the overall HLA class I expression on target cells and if no inhibitory signal from a HLA class I specific receptor is received, killing of the target will occur. Karre and Ljundggren proposed the concept of NK cells recognizing ‘missing self’ [91]. KIRs are expressed by overlapping subsets of NK cells and each individual NK cell expresses at least one receptor for one of the self HLA class I alleles enabling them to detect even single allele losses on a given target cell (for review see [92]). Therefore, in theory the majority of tumors would be expected to be susceptible to NK cell attack as they often lack one or more HLA class I molecules on their surface. However, NK cells are not activated probably due to the absence of required ligands for the cytotoxicity receptors [93] and the presence of the non-classical class I molecules HLA-E and –G on the target cells serving as a decoy target for the KIRs on the NK cells [94] Soluble HLA-G also inhibited NK-mediated cell lysis of HLA class I negative cells [95] .

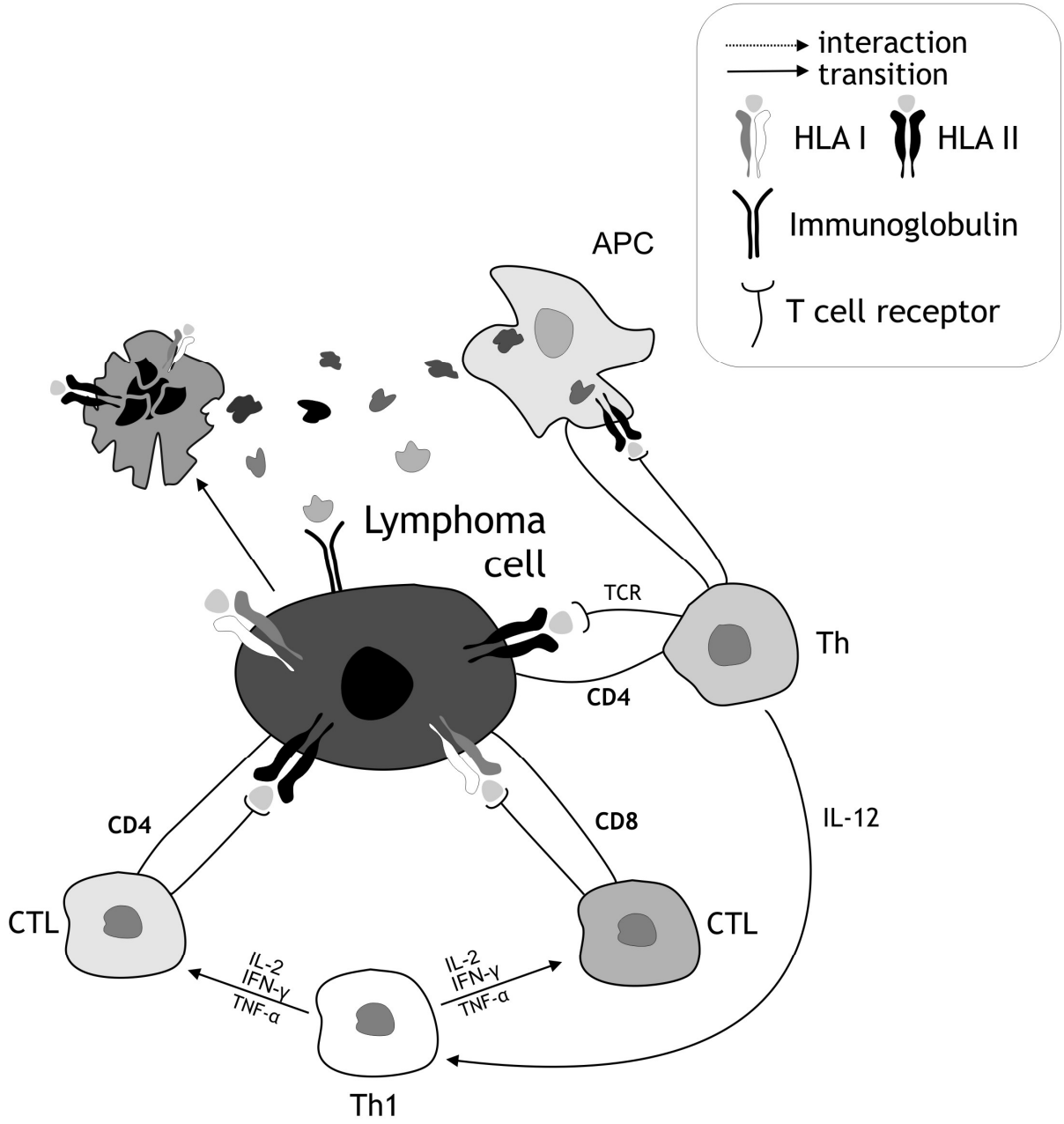

Figure 4. Schematic representation of a cytotoxic T cell response against tumours. Early during lymphoma development, a malignant B cell itself may provoke a cytotoxic T cell response by expressing tumour associated antigens, including its own idiotype, in the context of HLA class I and II. Other APCs in the vicinity may as well, by presenting peptides derived from apoptotic lymphoma cells. A lymphoma that during tumour development loses HLA class I and II expression may escape from "immune surveillance" as it is no longer susceptible to cytotoxic T cell attack.

1.6 Loss of HLA class I and II expression in tumours

Loss of HLA class I expression has been observed at frequencies of 30-70% in melanoma, various solid tumor types including lung, breast, colon, laryngeal and cervical cancer as well as in haematological malignancies [96-104]. Despite the high frequencies of HLA loss reported, the incidence is presumably under-rated as most studies used monoclonal antibodies (mAb) directed against monomorphic or locus-specific class I determinants and very few allele-specific mAbs. It is important to elucidate the underlying mechanisms of HLA loss in tumors in order to understand the nature of these aberrations in the course of carcinogenesis. In addition, for immunotherapy, the distinction between structural and transcriptional defects is crucial as the latter may be overcome by treatment with modulatory cytokines such as IFN-γ and TNF-α [105].

Five phenotypes of altered HLA class I expression have previously been described [106]

Complete loss of HLA class I expression

Complete loss of HLA class I expression, usually defined by absence of W6/32 staining (monomorphic anti-class I mAb) was at first described in melanoma [107-110] but later also in breast [111], colon [112], prostate, bladder and laryngeal cancer [113]. Several mechanisms explaining complete loss of HLA class I expression have been described. In several different tumor types, deleterious mutations of the $ß_2M$ gene were reported as an important mechanism underlying loss of class I expression, although the frequencies were low [114-117]. However, in a series of laryngeal carcinomas, loss of heterozygosity at chromosome 15 was found in approximately 30% of the cases but no mutations of the remaining $ß_2M$ gene [118,119] were detected. Thus, if the $ß_2M$ gene was to be a real tumor suppressor gene, one would expect genetic aberrations of both alleles. As both genes are normally expressed, loss of one gene-copy through a mutation or genomic loss will therefore not result in complete absence but possibly in reduction of synthesis of $ß_2M$ molecules and concomitant decrease of class I expression [120]. A second mechanism that contributes to HLA class I expression involves defects the antigen processing pathway. Mutation of TAP1 was reported in a melanoma cell line [121] while in three small cell lung carcinoma cell lines downregulation of both TAP and LMP genes resulted in loss of class I expression [122]. However, additional mechanisms subverting TAP-dependent peptide loading of HLA class I molecules have been described [123-125].

Hypermethylation of the promoter regions of the HLA-A,-B and –C genes is a third mechanism leading to complete loss of class I expression, which is however reversible. In squamous cell carcinomas of the esophagus, 45% of the samples exhibited methylation of one or more of the three HLA loci while 21% exhibited methylation of all three loci. The

HLA-B gene locus was most frequently involved (38%) [126]. After demethylating treatment of a cell line obtained from a melanoma patient not responding to T-cell mediated immunotherapy, peptide specific cytotoxic T-cell clones were recovered [127].

HLA haplotype loss

Loss of heterozygosity (LOH) through chromosome loss, mitotic recombination or hemizygous deletions will result in loss of one haplotype [128-130]. Using sets of polymorphic micro-satellite markers spanning 6p21.3, LOH of the HLA region was observed in cervical [131], colon [132], laryngeal [133,134], breast [135] and lung carcinoma [136] as well as melanoma [137] and diffuse large B-cell lymphoma [138].

Locus-specific HLA downregulation

Locus-specific MAbs are required to study this type of HLA loss. Many HLA-A and HLA-B specific MAbs are available but just one recognizes the HLA-C locus [139]. Loss of HLA-B expression in human melanoma cell lines was found to be mediated by a regulatory element, 20 bp region downstream of the transcription-initiation site [140] and not by *c-myc* overexpression as previously thought [141]. In colorectal cancer cell lines, under-representation of locus-specific DNA-binding proteins correlated with loss of HLA-B expression [142]. Moreover, in a lymphoma cell line, locus-specific de novo methylation caused down-regulation of MHC class I [143].

Allele specific loss

Of three melanoma cell lines the mechanisms underlying expression loss of a single HLA-A allele were reported, including a deletion of two exons and an intronic mutation leading to alternative splicing [144-146]. In cervical cancer some cases showed loss of a single HLA-A or -B allele due to single base pair substitutions resulting in stop codons or small intragenic deletions [147,148].

Composite loss of class I expression

Carcinogenesis is a multi-step process and independent events may result in a combination of different aberrations, for example haplotype loss in combination with loss of an additional allele [149,150].

Loss of HLA class II expression

HLA class II expression in tumors has not been studied as extensively, as most normal epithelial cells do not constitutively express these molecules. In some types of carcinoma (i.e. breast, stomach, larynx and colon) up-regulation of HLA-DR was associated with a better prognosis [94,97,96,95}. In addition, in cervical carcinoma HLA class II up-regulation was seen in the primary tumor whilst no class II expression was seen in the metastasis, suggesting that subsequent downregulation is associated with tumor progression [151,152]. Also in B-cell lymphomas, several authors reported a correlation between loss of HLA class II

expression and worse prognosis [153-155]. In contrast, in melanomas expression of HLA class II molecules is widely accepted as a marker of disease progression [156].

So far, few reports have been published about mechanisms involved in loss of class II expression in transformed cell lines and tumors. DeMars et al. described homozygous deletions of the HLA-DR and HLA-DQ region in Epstein-Barr (EBV) virus-transformed irradiated human B-lymphoblastoid cell lines selected for absence of class II expression [157]. Loss of DR expression resulted from an intragenic deletion of the HLA-DRB1 gene in an irradiated human lymphoblastoid cell line [158] while in another lymphoblastoid cell line a missense mutation in DRA resulted in conformational changes of the expressed HLA-DR molecules [159].

Knowledge of transcriptional defects of HLA class II expression comes from studies in the Bare Lymphocyte Syndrome (BLS). Defects in constitutive and IFN-γ induced transcription of HLA class II genes is the hallmark of this disease, a rare primary immunodeficiency disorder with a poor prognosis, characterized by a complete lack of cellular and humoral immune responses to foreign antigens. Usually during the first year of life recurrent bronchopulmonary infections and chronic diarrhea occur. The patients die at very young age (mean 4 years) mostly as a result of an overwhelming viral infection. Bone marrow transplantation can be curative in quite a number of patients (for review see [160]).

Mutations in any of the four regulatory *trans*-acting genes specific and essential for class II expression, RFX5, RFXAP, RFXANK and CIITA (class II transactivator} lead to the same clinical presentation [161]. The first three are subunits of RFX, a trimeric complex that binds to all class II promoters, including DM, DO, invariant chain and even the class I genes and $ß_2M$ [162]. CIITA is a non-DNA-binding co-activator that functions as the master control factor by association with the promoter-bound RFX complex [163].

Several authors reported defects in CIITA expression in cell lines derived from different carcinomas. In small cell lung carcinoma (SCLC) no HLA-DR was expressed in contrast to non-SCLC due to non-inducible lack of CIITA [164]. In some gastro-intestinal carcinoma cell lines, methylation of one of the promoters of CIITA explained the absence of HLA-DR after IFN-γ treatment [165]. In neuroblastoma cell lines similar defects in CIITA were reported, but although IFN-γ induced transcription of CIITA was restored after treatment with DNA demethylating agents, HLA class II expression was not [166]. Negative regulators of CIITA transcription, typically overexpressed in SCLC and neuroblastoma including L-myc and N-myc, probably played a role in this process [167].

1.7 Implications of HLA downregulation for anti-tumour immunotherapy

An important mechanism of tumour escape is maintenance of immunological tolerance which might occur if tumour-specific antigens are presented at very low levels either by tumour cells or via cross-priming by professional APCs and if additional pro-inflammatory stimuli are lacking [168,169]. The T-cells in these individuals are probably not tolerant but still susceptible for priming as they behave as naïve lymphocytes that have never encountered antigen [170]. Through tremendous effort over the last few decades, many experimental models of immunotherapy have been established. Despite the promising results in animal models, in humans the results of immunotherapy for cancer have in general been disappointing, although fairly high clinical response rates have been reported in some clinical trials [171,172]. A commonly used method is T-cell mediated immunotherapy, an elegant highly specific strategy that has been proven to be effective in several experimental models and clinical trials. Adoptively transferred EBV-specific $CD8^+$ CTLs were successfully used to prevent EBV-related lymphoma in bone marrow transplantation patients in one study [173]. Autologous expansion of CTLs directed at tumour-specific antigens is another possibility to generate tumour-specific T-cell clones. In the last few years, activated professional APCs (dendritic cells) presenting tumour-specific antigens are used to generate specific CTL clones (for review see [174,175]). Numerous tumour-associated proteins or peptides have been used as vaccines, including idiotype Ig in non-Hodgkin's lymphoma (NHL) patients (for review see [175]). Effective T-cell immunity heavily depends on tumour-specific $CD4^+$ T-cells both in the induction and the effector phases [176]. Adoptive transfer of $CD4^+$ IFN-γ producing Th1 cells resulted in activation of dendritic cells and expansion of tumour-specific $CD8^+$ CTLs. Apart from this, Th1 cells can recruit and activate tumouricidal macrophages by the cytokines they produce [177]. Tumour-derived peptide presentation by tumour cells is HLA restricted and a prerequisite for efficient T-cell based immunotherapy. The expression of HLA class I will therefore be under strong selective pressure and selective loss of the relevant TAA presenting HLA allele has been described in a melanoma cell line derived from a metastasis [178]. Since many tumours show loss of one or more HLA class I alleles (see Ch. 1.5), $CD8^+$ T-cell based immunotherapy might be of limited value.

HLA class II molecules as a target for immunotherapy has gained interest in recent years. Several methods have been developed to induce HLA class II expression in tumours originating from somatic cells that do not constitutively express these molecules. A tumour-specific $CD4^+$ T-cell response can be elicited if tumour cells present peptides derived from transformed intracellular proteins in the context of HLA class II. However, tumour cells can escape by up-regulating Ii/CD74 expression which blocks binding of

endogenous peptides to newly synthesized class II molecules [179] as observed in colon carcinoma [180]. Dr. Ostrand-Rosenberg and colleagues developed potent vaccines consisting of tumour cells transfected with MHC class II genes and showed tumour rejection in mouse models [181]. The engineered tumour cells directly presented endogenous peptides to specific $CD4^+$ T-cells provided they did not express Ii/CD74 and induced even a stronger immune response than the host APCs [182]. Moreover, several reports also described direct killing of tumour cells by cytolytic CD4+ T-cells [183-185]. However, due to the heterogeneity of HLA, transfection of autologous HLA class II alleles is clinically not feasible and therefore alternative approaches have been developed, inducing intra-tumoural class II expression with CIITA or IFN-γ and suppressing Ii expression by antisense methods [186].

The use of monoclonal antibodies as anti-cancer treatment has been extensively studied since Kohler and Milstein described the method of mAb production [187]. Most B-cell lymphomas constitutively express HLA class II molecules and are potential targets for class II directed antibody-mediated immunotherapy. Engagement of HLA class II molecules can result in proliferation, activation of apoptosis depending on the status of differentiation and activation of malignant B-cells or the presence of co-stimulatory signals. Crosslinking of HLA-DR but not HLA-DQ or –DP induced programmed cell death in activated B-cells [188]. Humanized mAbs directed against a variant of the ß-chain of HLA-DR (Hu1D10) predominantly present on malignant B-cells [189] and mouse mAb Lym-1 directed against an epitope on the ß-chain of HLA-DR10 [190], have in the last few years been used in clinical trials with rather disappointing results (for review see [191]).

Neoplasms that show defects in HLA class II expression during tumourigenesis will of course no longer be sensitive to any of the above described immunotherapeutic regimens unless class II expression is still inducible.

1.8 HLA association with disease

For many years, the HLA genotype has been considered to be the most important genetic marker of susceptibility to many diseases. Rather than with individual genes, the strongest disease associations exist with combinations of alleles at multiple loci. The different combinations of alleles were called ancestral haplotypes (AH) as they showed a high degree of conservation and were probably derived from a common remote ancestor. The 8.1 AH (HLA-A1, C7, B8, C4AQ0, C4B1, DR3, DQ2) is carried by most Caucasians with HLA-B8 and associated with many autoimmune diseases such as myasthenia gravis, diabetes mellitus, systemic lupus erythematodes and with impaired survival after human immunodeficiency virus infection (for review see [192]). The carriers of this haplotype tend to produce low IL-2 and IFN-γ responses and normal levels of IL-4 which may suggest a bias

towards a Th2-type immune response. Peptide binding studies defined DRB1*0301 as an unique molecule amongst the other class II molecules as it required a specific amino acid at anchor point P4 [193]. Associations with certain HLA alleles have not only been reported for autoimmune diseases but also for different types of cancer including haematological malignancies. Associations of Hodgkin disease with the HLA class I region have been reported since the 1970s [194,195] while more recently, positive associations with the class II alleles DRB1*1501 (DR2) and DQB1*0602 were reported [196,197]. Cutaneous T-cell and enteropathy-associated T-cell lymphoma showed a positive association with certain DR and DQ alleles [198,199] and homozygosity for HLA-DRB4*01 was found to be a risk factor for childhood acute lymphoblastic leukemia in boys [200]. Few studies have addressed the HLA haplotypes in B-cell lymphomas. In Thai NHL patients HLA-DRB1*15 was increased and HLA-DRB1*0803 was decreased compared to healthy controls [201]. The only study in caucasoid DLBCL patients showed a association between adverse outcome and null HLA-DRB1*15 [202] although the distribution of the HLA-DR alleles was not different from the controls .

2 Diffuse large B-cell lymphomas

2.1 Normal B cell development

The primary effectors of the adaptive immune responses are B and T lymphocytes. The response of mature B-cells to T-cell dependent antigens requires both signaling through the B-cell receptor (BCR) and presentation of antigen to Th-cells [203]. Three features are essential for this type of response: great diversity to deal with all possible antigens, discrimination of self from non-self and long-lasting immunologic memory.

Precursor B-cells

The pluripotent precursors of the B lymphocytes are found in the bone marrow and the thymus, the primary lymphoid organs. B-cell development progresses through a number of defined sequential stages [204,205]. The precursor cells, committed to the B lineage can be identified by their expression of differentiation antigens on the cell surface and in the cytoplasm. The V(D)J gene rearrangement process, a hallmark of B lymphocytes, has not taken place yet in the pro-B-cells and therefore Immunoglobulin Heavy (IgH) or Immunoglobulin Light (IgL) chains are not expressed at this stage. However, the required cytoplasmic proteins for the rearrangement process and subsequent assembly of the B-cell antigen receptor (BCR) are already present. During early stages of differentiation the precursors express CD34 and TdT (terminal desoxyribonucleotidyl transferase). In pro-B-cells Ig gene rearrangements are a tightly regulated process starting at the Heavy chain locus with at first random combinatorial joining of D (diversity) and J (joining) gene

segments and then with V (variable) genes [206,207]. For V(D)J recombination, expression of recombinase activating genes (RAG1 and RAG2) is required [208,209] and two sequential rearrangements of the IgH locus are necessary to produce a functional gene. The IgH becomes membrane bound after association with two "surrogate" light chains. The surrogate receptor subsequently associates with Igα and Igß, required for surface expression and signaling [210,211]. The surrogate Ig complex in the early pre-B-cells initiates further differentiation into late pre-B-cells in which rearrangement of the light chain occurs. Subsequently, surface Ig is produced in the very small population of precursor B-cells that successfully completed the rearrangements of both IgH and IgL loci. Cells with incomplete or out of frame rearrangements are not allowed to mature and are subject to apoptosis resulting in a reduction of B-cell precursors (for review see [212]). TdT inserts N-segments between the V, D and J region during recombination and it switches off after this process has been completed. The recombination process generates an enormous antibody diversity as each surviving B-cell has its own unique combination of the different V-,D- and J-genes of the IgH and the IgL chain. This diversity is further increased by random nucleotide introduction in the joint regions during recombination.

The specificity of the expressed BCR determines whether the newly formed B-cells will survive or will be eliminated. The clonal selection model originally proposed the deletion of self-reactive B-cells. However, later several authors reported small sets of self-reactive B-cells that had undergone receptor editing: the autoreactive receptors were deleted and entirely new receptors had developed through V(D)J recombination upon interaction with auto-antigen [213-216].

Peripheral mature naïve B-cells

Mature naïve $IgM^{+}IgD^{+}$ B-cells that survived the selection process migrate out of the bone marrow to lymph nodes, the spleen or mucosa associated lymphoid tissues (MALT) where they form primary B-cell follicles in association with follicular dendritic cells (FDC). B-cells at this stage live for only a few days unless they encounter cognate antigen and receive T-cell help. This leads to the formation of germinal centers (GC) in secondary lymphoid organs [217]. Activation of the B-cells initially occurs outside the follicles in the T-cell rich zones in association with interdigitating cells and T-cell help.

Germinal center B cells

A small number of B-blasts colonize each follicle after which they undergo massive clonal expansion within the dark zone of the GC. At the same time a hypermutation mechanism directed at the immunoglobulin variable genes is activated, leading to a dramatic increase in affinity towards antigen binding [218]. The proliferating blasts give rise to centrocytes that express hypermutated sIg and are able to interact with follicular dendritic cells that hold

antigen. Fas/APO-1 (CD95), a member of TNF receptor family transduces apoptotic cell death and is expressed on GC B-cells but not on mantle cells while Bcl-2, a potent apoptosis inhibitor shows an inverse expression pattern. Self-reactive B-cells formed during the GC reaction are eliminated to prevent autoimmunity [219]. In fact, most centrocytes undergo spontaneous apoptosis and just a few centrocytes survive by positive signaling via the BCR and CD40 expressed at their surface [220-222].

Post-germinal center B-cells

B cell that are positively selected, migrate to the light zone of the germinal center and receive growth and differentiation signals promoting isotype switching, generation of long-lived memory cells or terminal differentiation towards the plasma cell stage. Isotype switching permits a more diverse immunologic response by altering the effector function of the antibodies.

IL-10 and IL-21 direct GC B cell differentiation toward the generation of plasma cells, while the absence of IL-10 stimulation leads to the generation of memory B cells [223,224].

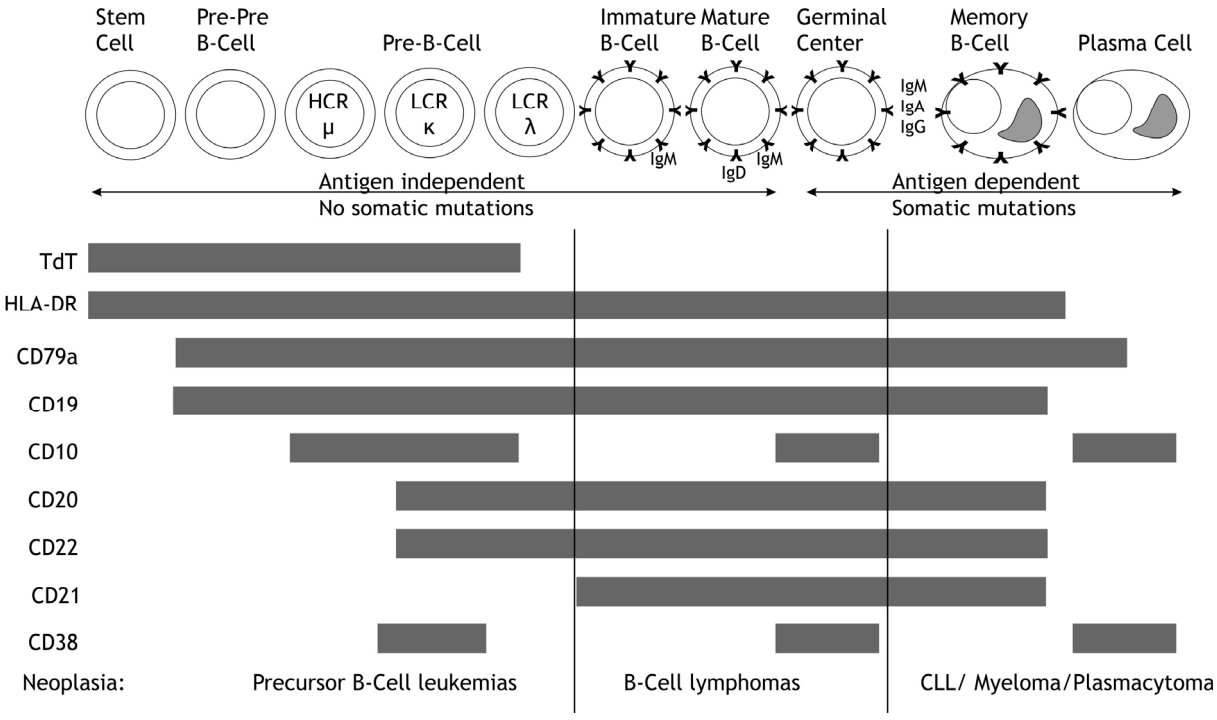

Figure 5. B cell development, the expression of markers during each stage and the corresponding leukemias and lymphomas.

Memory B-cells

Memory B-cells show little or no expression of IgD and strong expression of IgM, and in case of class switching IgG or IgA, as well as somatic mutations within V genes without signs of ongoing mutations. They represent Bcl-2 expressing [225] long-lived high affinity resting cells that preferentially home to marginal zones and that upon antigen exposure rapidly proliferate and differentiate, generating vast amounts of Ig secreting plasma cells. The GC is no longer involved in these secondary responses.

Plasma cells

Long-lived plasma cells migrate out of the GC and predominantly populate the bone marrow and organs directly exposed to foreign antigen, i.e. the gastrointestinal tract and the lung. Plasma cells maintain the humoral immunity by augmenting the production of Ig destined for secretion while expression of surface Ig is downregulated.

2.2 Role of MHC class II expression in B-cell development

The response of mature B-cells to T-cell dependent antigens requires both signaling through the BCR and the processing and presentation of antigen to T-helper cells [203,226]. In contrast, antigen binding to the BCR on immature B-cells results in negative selection, though in vitro experiments showed rescue of immature B-cells by interaction with T-helper cells, a process which was dependent on class II mediated antigen presentation [227,228]. Gene knockout experiments in mice revealed that expression of class II molecules early during B-cell development is essential for generation of mature B-cells. In the absence of invariant chain (Ii), the rate of export of newly synthesized MHC class II molecules from the endoplasmatic reticulum to the cell membrane was reduced, resulting in a marked reduction of class II expression at the surface [229,230]. The class II molecules that did reach the cell surface behaved as though they were empty or contained weakly bound peptides. The B-cells in the Ii-deficient mice were blocked in their maturation and expressed $CD23^{low}$, IgM^{high} and IgD^{low} [231].

2.3 B-cell non-Hodgkin lymphomas

Worldwide, the vast majority of lymphoid neoplasms are derived from B-lymphocytes and most B-cell neoplasms mirror the features of the different B-cell differentiation stages (see Figure 5) [232]. Lymphoblastic leukemia/lymphomas resemble precursor B-cells, while 50% of the chronic lymphocytic leukemia cases show some features of mature naïve B-cells and 50% characteristics of memory B-cells. Mantle cell lymphomas resemble naïve B cells, follicular lymphomas represent neoplastic GC B-cells and plasmacytoma/myeloma resemble normal plasma cells. However, diffuse large B-cell lymphoma (DLBCL)

representing 40% of the B-cell neoplasms is an exception to the rule. These lymphomas display genetic and immunophenotypic features of B-cells but an exact allocation to one of the B-cell populations has been impossible so far.

2.4 Diffuse large B-cell lymphoma, clinical and pathological aspects

Morphology and protein expression

In the current WHO classification, DLBCL are lumped to a single category as it proved impossible to reliably distinguish subtypes based on morphology alone [233]. DLBCL typically replaces the normal architecture of the tissue involved in a diffuse manner and is composed of large transformed lymphoid cells with vesicular nuclei (>2 x size of small lymphocytes), prominent nucleoli and basophilic cytoplasm. According to the WHO classification, four morphological variants can be discerned: centroblastic, immunoblastic, T-cell/histiocytic rich and anaplastic [234].

The tumour cells usually express CD19, CD20, CD22 and CD79a which are all pan-B markers, but may lack one of these. The GC markers CD10 and Bcl-6 are expressed in approximately 20-40% and 70-95% of the cases. MUM1, a member of the interferon regulatory factor family, is regarded as a post-GC marker and is positive in 50-75% of the cases although co-expression with Bcl-6 is seen in 50-57% of the cases [235,236]. Proteins involved in apoptosis and cell cycle regulation such as p53 and Bcl-2 are positive in respectively 20-30% and 30-50% of the cases. Surface and/or cytoplasmic immunoglobulin (IgM>IgG>IgA) is expressed in approximately 75% of the tumours. The proliferation fraction, as detected by Ki-67 is usually higher than 40% and in some cases may even be higher than 90%.

Molecular features

Despite their heterogeneity, all DLBCL display somatic hypermutations of Ig genes, a feature of the GC blasts or post-GC B-cells [237].

With the recent development of gene-expression micro-arrays, a whole new set of diagnostic tools has emerged, enabling the exploration of the molecular nature of these lymphomas. Mathematical algorithms are used in large datasets to define gene expression signatures, a set of genes characteristically expressed in a certain cell type or during a particular biological response or differentiation step [238]. According to their gene expression profile a large subset of untreated 'de novo' DLBCL patients was divided in three categories: a GC B- like expression profile showing ongoing somatic hypermutation, an activated B-like profile or a "third" group. Of the activated B-like group it was suggested that these cells were at the transition between GC B-cells and plasma cells [239,240]. The GC-like group showed a significant better response to chemotherapy than the activated B-like

subset. However, both groups were not homogeneous as within each group both clinical and molecular heterogeneity was evident. Recently, another distinct gene expression profile was seen in a group of primary mediastinal DLBCL which is however nowadays regarded as a different entity[241].

Clinical features

DLBCL show much heterogeneity with respect to presentation, response to treatment and survival and therefore most certainly comprises several different sub-entities, including a rather substantial group of transformed indolent follicular or MALT lymphomas (reviewed in [242]). Primary extra-nodal presentation occurs in approximately 35-40% of the cases of which 30% in the gastro-intestinal tract [243,244]. Other extra-nodal sites include skin, lung, thyroid, bone, central nervous system (CNS) and the testes.

Prognostic factors

First published in 1993, the International Prognostic Index (IPI) proved a very useful index of clinical features associated with survival for aggressive NHL, identifying patients with low, intermediate and high risk [245]. However, the IPI does not recognize subentities and a considerable proportion of patients classified as "low" or 'low-intermediate" risk eventually died of disease prompting many authors to conduct studies to find other biological prognostic markers.

In two large cohorts of DLBCL, significant differences in survival were reported between extra-nodal and nodal disease at various clinical stages [246,247]. Several authors reported Bcl-2 expression as an adverse prognostic factor [248-251], which was however not confirmed by others [252]. The same accounts for p53 mutations [253,254]. In multivariate analysis, rearrangement of 3q27 and the absence of a GC phenotype were associated with a poor prognosis [255]. Moreover, FOXP1 (Forkhead box-P1), a winged-helix transcription factor differentially expressed in resting and activated B cells, was almost exclusively confined to patients in the activated B-cell like subset and associated with particularly poor prognosis [256]. The better prognosis for GC B-like compared to the activated B-like DLBCL, as determined by gene expression profiling was confirmed by immunohistochemical studies using GC B-cell (CD10 and Bcl-6) [257] and activation (MUM1/IRF4 and CD138) markers [258].

Therapy

DLBCL are intermediate to high grade lymphomas, but approximately 40% of the patients can be cured by multi-agent chemotherapy which is generally CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) [259]. Recently, multivariate analysis of a multi-center randomized trial showed a significant improvement in response to treatment and event-free survival in elderly DLBCL patients who were treated with R-CHOP, CHOP in combination with rituximab, a chimeric mAb directed against surface CD20, in comparison

to treatment with CHOP alone [260]. Moreover, R-CHOP showed a significant beneficial effect in case of Bcl-2 expression of the tumours [261].

2.5 Genetic defects in DLBCL

Cytogenetic studies have revealed different types of chromosomal abnormalities in over 90% of the DLBCL including gains and losses of several chromosomes [262]. Amongst these, reciprocal translocations involving the activation of various proto-oncogenes are the most common. Erroneous repair of double strand breaks generated during the rearrangement and class switching processes is supposedly the most important mechanism responsible for the translocations [263]. Besides, lymphomas are prone to mutations as a result of the somatic hypermutation machinery, active during normal B-cell differentiation and activation.

Bcl-2

The characteristic translocation of follicular lymphomas t(14;18) (q32;q21) is also present in approximately 20% of the DLBCL [264]. In this translocation the *BCL-2* proto-oncogene (18q21) is juxtaposed to an IgH gene (14q32) [265-267] causing sustained high levels of Bcl-2, an apoptosis inhibitor [268]. However, significantly more DLBCL (40-50%) without detectable t(14;18) show high expression of Bcl-2 which might be due to gene amplification as detected by comparative genomic hybridization [269].

Bcl-6

Reciprocal translocations between the 3q27 region harboring the BCL-6 gene and various alternative partner chromosomes, including the sites of IgH, Igκ, Igλ and CIITA are present in 20-35% of DLBCL [171,172, 187}. Bcl-6, selectively expressed in the GC, encodes a zinc-finger DNA binding protein required for GC formation during B-cell development. Very recently, Bcl-6 was identified as a suppressor of p53 transcription. Absence of p53, a tumour suppressor gene modulating damage-induced apoptotic responses, allows germinal-centre B cells during the GC reaction to tolerate the physiological DNA breaks required for immunoglobulin class switch recombination and somatic hypermutation [270]. From gene expression studies, Bcl-6 also appeared as a repressor of Blimp-1 [271], a repressive transcription factor sufficient for driving B-cells towards plasma cell differentiation [272] and of p27kip1, an inhibitor of the cycle-dependent kinases that blocks the cell cycle when highly expressed [273]. In addition, Bcl-6 is thought to modulate the T-cell mediated immune response as Bcl-6 deficient mice developed a Th2-mediated hyperimmune response [274]. Somatic mutations of the Bcl-6 promotor region are, independent of translocations, present in up to 75% of DLBCL [275].

Aberrant somatic hypermutation

In the majority of DLBCL somatic mutations of proto-oncogenes such as *BCL-6*, *PIM-1*, *cMYC*, *PAX5* and *Rho/TTF* were found, sharing features with the IgV-associated somatic hypermutation mechanism. In contrast to mutations in V regions and BCL-6, however, these mutations were not detectable in normal GC B cells or in other GC-derived lymphomas. Intriguingly, the hypermutated regions of the four above mentioned genes are also susceptible to chromosomal translocations suggesting a role for hypermutation in generating DNA double-strand breaks and subsequent translocations [276].

3 DLBCL in immune privileged sites

3.1 Immune Privilege

The Th1 immune response as part of the host defense system against pathogens is crucial for the clearance of infections. However, the consequent Th1-mediated delayed type hypersensitivity (DTH) reaction can also cause extensive damage to the surrounding tissues which is nevertheless tolerated at most sites of the body. Some tissues however, have very restricted regenerative capacities and unbridled DTH would have long-term consequences for survival of the organism. At these sites (i.e. the CNS, eye, ovary and testis) therefore, immune responses are limited to prevent extensive tissue damage.

The first report about site-specific limited immune reactions appeared in 1873 when Van Dooremal described progressive growth of human tumour cells specifically in the anterior chamber (AC) of the rabbit eye [277]. Many decades later, in the 1940s, the founder of the transplantation immunology, Sir Peter Medawar, proposed the term immune privilege when he observed prolonged survival of grafts placed in the brain and the AC compared to other sites of the body [278]. His original concept of the immune system being unaware of antigenic material placed in these special sites, has significantly been altered over the years as it is now known that immune privilege is in fact actively acquired and maintained [279-281]. The strategies that immune privileged sites use to limit local immune responses to maintain their integrity are probably best studied in the AC of the eye.

Direct evidence for the existence of immune privilege came from experiments performed by Niederkorn and co-workers. They injected MHC-incompatible DBA/2 tumour cells in the AC of BALB/c mice and saw progressive intra-ocular tumour growth. Moreover these mice failed to reject DBA/2 skin grafts and failed to show DBA/2-specific DTH despite the presence of clonally expanded DBA/2 specific CTLs and antibodies in the blood and secondary lymphoid organs. This phenomenon was termed anterior chamber-associated immune deviation (ACIAD) [282,283]. However, the maintenance of immunologic privilege

strongly depended on the degree of incompatibility at H-2 loci encoding class I MHC molecules. In addition to the tolerated grafted tumour cells, some allogeneic tissues evoked very strong immune responses leading to destruction of the eye whilst other allogeneic tissues were rejected without causing structural damage [284].

ACIAD

It has now been established that ACIAD, one of the factors of immune privilege, is in fact a stereotypic Th2-directed systemic immune response to any foreign antigen present in the AC. In the absence of lymph vessels, antigens present in the AC are captured by APCs and directly transported to the venous circulation. Passage of these AC-derived antigens through the spleen is absolutely essential for induction of ACIAD. The antigen-primed macrophages migrate to the spleen where the antigenic peptides are released and captured by B-cells through the BCR. The subsequent TAP-independent peptide presentation to $CD4^+$T-cells results in IL-10 production and activation of a population of $CD8^+$ suppressor T-cells leading to suppression of the immune response [285,286].

Aqueous humour

Another important factor for immune privilege is the aqueous humour (AqH) in the AC. The AqH constitutes of several immunosuppressive factors including transforming growth factor ß (TGF- ß), vaso-active intestinal peptide, calcitonin gene-related peptide (CGRP), α-melanocyte stimulating hormone and macrophage-migration inhibitory factor (MIF) that all inhibit a Th1 immune response in case of antigen challenge [287,288].

Anatomical and molecular aspects that contribute to immune privilege are the blood – organ barrier, CD95L expression and the lack of HLA class I and II expression.

Blood-organ barrier

The blood-brain barrier constitutes of a astrocytic controlled specialized microvasculature, characterized by a continuous network of complex tight junctions, lack of fenestrations and vesicular transcytosis and expression of specific enzymes limiting exchanges of soluble substances such as hormones, growth factors, cytokines and immunoglobulins as well as trafficking of immune cells (for review see [289]). However, even under normal circumstances, small numbers of activated T-cells traffic through the brain [290]. The endothelium of the CNS is probably actively involved in this process. The blood-testis barrier does protect antigenic germ cells in the testis but is in fact in a constant state of flux as mature germ cells need to pass through to the tubule lumen (for review see [291]). Lymphocytes can also relatively easy cross the barrier [292]. In the normal rat testis, the lymphocytes are mostly CD8+ T-cells [293] and their number correlates with the number of resident macrophages which in turn is being regulated by Leydig cells [294].

CD95L

The cornea expresses functional CD95L (FasL), a membrane protein belonging to the TNF receptor superfamily, that induces apoptosis in cells expressing CD95 (Fas receptor), upon binding. Whereas CD95 is ubiquitously expressed on multiple cell types, CD95L expression is mainly restricted to activated T-cells, NK cells and tissues in immune privileged sites [295]. Instead of killing CD95 expressing T-cells, CD95L can also prevent degranulation of activated CTLs [296]. Fas-mediated apoptosis of lymphoid cells plays an important role in ACAID as prior to cell death IL-10 and TGF-ß are produced [297]. Through interaction with APCs, the IL-10 containing apoptotic cells direct the outcome of T-helper cell differentiation away from a Th1- towards aTh2-response.

HLA class I and II expression

The corneal endothelium does not express HLA class I molecules and HLA class II molecules are not present in the cornea. Human testicular germ cells also lack surface class I molecules but class II molecules are expressed on macrophages that are abundantly present in the testicular interstitium [298,299].

The endothelial cells in the CNS, on the other hand, do constitutively express class I molecules, whereas HLA class II expression can be induced by IFNγ and/or TNFα in endothelial and micro-glial cells [300].

Innate immune response

The absence of HLA class I molecules protects the corneal endothelium from recognition by CTLs but potentially renders it vulnerable to NK-cell mediated killing. NK cells together with macrophages, dendritic cells and polymorphic neutrophils are the cellular part of the innate immune system.

Unlike the adaptive immune system, the response of the innate immune cells to pathogens is very rapid and commonly results in extensive collateral tissue damage as a result of secretion of strong pro-inflammatory cytokines, chemokines, NO and activation of the complement system. NK cells and macrophages are not only involved in the induction of an aspecific inflammatory response but can also directly kill target cells.

However, as for the adaptive immune system, the AqH plays the central role in innate immune privilege with TGF-ß2 and MIF strongly inhibiting NK cell lysis, certain soluble factors inhibiting complement activation and CGRP inhibiting NO production by macrophages [301,302].

3.2 Tumour-specific immune response in immune privileged sites

Compared to other sites of the body, tumours at immune privileged sites experience growth advantage as they are thought to be protected from attack by immune cells.

Nevertheless, CTL responses directed at tumours in immune privileged sites have been described in several experimental models [303-306]. Clonal expansion of specific fully functional anti-tumour CTLs was observed after intracerebral tumour implantation in syngeneic mice. The $CD8^+$ CTLs were recruited via crosspriming by APCs, such as macrophages and activated microglia cells, resident in the tumour stroma. [307,308]. In another mouse model, vaccination with tumour-antigen induced a MHC class I-restricted CTL response resulting in protection against intracranial challenge with antigen-positive melanoma and astrocytoma tumour cells and long-term survival of 50% of the cases [309]. In a rat-model, immunization with a recombinant bacterium expressing a pseudotumour antigen induced initially immunity against subcutaneous but not intracranial tumour challenges with a gliosarcoma cell line expressing the same antigen. However, once rejection of subcutaneously grafted tumour cells occurred, rats developed systemic antitumour immunity and were completely resistant to subsequent tumour challenges both within the CNS and the periphery [310].

3.3 Testicular DLBCL

Incidence and pathology

Primary malignant lymphoma of the testis is a rare disease (for review see [311]), although the most common testicular tumour after the age of 60 years. It accounts for 1% of all NHL, 5% of all testicular and 50% of the non-germ cell testicular tumours [312,313]. In sharp contrast to germ cell testicular tumours, prevalent in young patients, testicular lymphoma is very rare in patients < 30 years [314]. There are no well established predisposing factors for testicular lymphoma, although associations with trauma, chronic orchitis and cryptorchism have been described in some reports [315,316].

The majority of testicular lymphomas are of B-cell origin and 75% of those are classified as DLBCL with the other 25% being lymphoplasmacytoid or Burkitt-(like) lymphomas.

Clinical presentation

The most common clinical presentation is a painless scrotal mass with 25-41% of the patients showing constitutional symptoms such as weight loss, fever, anorexia and night sweats [317,318] . According to the Ann Arbor classification, the majority of patients have stage I or II disease at the time of presentation [319].

Bilateral involvement is present in approximately 20% of the cases. Testicular lymphoma has a propensity to disseminate to other extranodal sites such as the skin, lung, Waldeyer's ring and most notably the central nervous system (CNS) [320].

Therapy and prognosis

The best therapeutic regimen for these lymphomas is still a matter of debate.

Orchidectomy, also providing tissue for histological examination, is the first choice of treatment, especially in localized disease, which is usually followed by either loco-regional radiotherapy, systemic chemotherapy or both. The results of radiotherapy after orchidectomy have been disappointing with often a good initial response but an overall relapse rate of about 70% (for review see [321,322]). The results for chemotherapy with or without radiotherapy were better for stage I patients with 70-87% of the patients achieving a complete clinical remission (CR). In a retrospective study of 373 patients with testicular DLBCL the relapse rate was 52% with 15% of the patients showing a relapse in the CNS [323].

Molecular pathogenesis

Chromosomal aberrations or genetic defects in testicular DLBCL has not been the subject of many studies and therefore also at the molecular level very little is known about the etiology of this group of lymphomas. One case report described a deletion at chromosome 5p11 in a high-grade testicular lymphoma [324]. Bcl-2 over-expression without translocation t(14:18) was seen in all 9 cases studied [325] which might play a role in the relatively poor prognosis of these lymphomas. Also Hyland et al. did not detect a translocation t(14:18) in any of the 20 testicular DLBCL they studied and all cases were also negative for human herpesvirus 8 (HHV-8) and Epstein-Barr virus (EBV). Interestingly, sequence analysis of the VDJ region of the Ig locus did not reveal preferential VH family usage, but intraclonal IgH sequence variation was seen in all twelve cases studied implying ongoing antigenic stimulation [326].

3.4 Central nervous system DLBCL

Incidence

Primary CNS lymphomas (PCNSL) are uncommon and represent 2 % of all brain tumours and 0.7-1.7 % of all NHL. An unexplained 2-3 fold increase in incidence of PCNSL in immunocompetent individuals in the last few decades has been reported for the U.S, England and the Netherlands but not for Canada [327-330]. In contrast, in immunocompromised patients, the incidence rate is much higher. In acquired immunodeficiency disease (AIDS) patients 0.6% of the patients have PCNSL as the initial manifestation of disease and about 2% of all AIDS patients develop CNS lymphoma at some point during their life (for review see [331].

Pathology

Stereotactic biopsy is the diagnostic procedure of choice as open neurosurgical intervention is not superior and partial resection even associated with worse survival [332]. Histologically, PCNSL is a monotonous diffuse highly cellular lesion with an angiocentric growth pattern and occasional blood vessel wall invasion. Ninety percent or more of the

tumours are of B-cell origin usually of diffuse large B-cell, large-cell immunoblastic or lymphoblastic subtype expressing CD20. Varying numbers of infiltrating $CD3^+$ T-cells and small non-neoplastic B-cells are present in the tumour. In the surrounding edematous brain parenchyma, highly activated resident cell populations such as microglia and astrocytes are present. Several autopsy studies demonstrated that these tumours extensively infiltrate the brain microscopically with individual tumour cells in otherwise normal brain [333,334].The pathogenesis in immunocompetent hosts is currently still unknown as in the normal situation T-cells but not B-cells freely cross the blood-brain barrier, although small numbers of activated $CD23^+$ B-cells have been detected in normal brain parenchyma [335].

Clinical presentation

PCNSL can affect all age groups with a peak incidence in the fifth to seventh decade in the non-AIDS patients [336].The clinical symptoms are usually non-specific and include personality changes, cerebellar signs, headaches, seizures and motor dysfunction. Raised intracranial pressure affects approximately half of the patients [337,338]. In 20-40% of the patients, PCNSL is a multifocal disease at diagnosis, based on the number of contrast-enhancing lesions seen on MRI [339-341]. PCNSL disseminate to the eye in approximately 20% of the cases and in 7-8% of the patients an occult systemic lymphoma is found which is of no relevance to the prognosis [342].

Therapy and prognosis

Corticosteroids not only reduce edema but also have a rapid potent oncocytic effect with tumour cell lysis and radiographic reduction in 40% of the patients (for review see [343]). PCNSL are radiosensitive and whole-brain radiotherapy (RT) has been the standard treatment for many years resulting in median survivals of 10-18 months. The standard chemotherapy regimens for DLBCL (i.e. CHOP) are not effective for PCNSL as they do not cross the blood-brain barrier. High dose methotrexate (MTX) is the single most active agent and is used alone or in combination with other chemotherapeutic agents followed by whole-brain RT. This combined modality approach has resulted in 100% response rates and median survivals of 30-60 months. The relapse rate is about 50% and most recurrences are observed within 2 years of completing therapy. The prognosis at relapse is generally poor. Many elderly patients treated with the combined modality treatment will be affected by neurological toxicity and are at high risk of developing a progressive neurological syndrome characterized by dementia, gait ataxia and urinary dysfunction [344].

Molecular pathogenesis

Several studies provided evidence that PCNSL are derived from hypermutated GC B-cells. Mutations in the non-coding region of the Bcl-6 gene were observed in 50% of PCNSL [345]. Moreover, molecular analysis of immunoglobulin genes in PCNSL demonstrated clonally

rearranged V region genes and unusually high rates of somatic mutations and provided evidence that the tumour cells or their precursors had been selected for expression of a functional BCR [346,347]. Interestingly, both studies revealed that in nearly all cases the V4-34 gene segment was used. In the normal human adult antibody repertoire, V4-34 is overrepresented compared to other members of the VH4 family [348] and also in DLBCL the V4-34 usage was significantly higher compared to the usage in peripheral blood B cells [349]. The V4-34 segment has also frequently been detected in patients with auto-immune diseases such as cold agglutinin disease and systemic lupus erythematodes and during Epstein-Barr virus (EBV)-associated mononucleosis infectiosa [350,351]. Theoretically, an (super)antigen-driven expansion of B-cells producing antibodies encoded by the V4-34 gene may play a role in the pathogenesis of non-AIDS associated PCNSL but no candidates including viral or bacterial proteins have been identified so far. In contrast, in AIDS patients, PCNSL is nearly always related to EBV infection. The detection of EBV DNA in the cerebrospinal fluid is highly specific for PCNSL in AIDS patients and may even precede lymphoma development [352,353]. A role for other neurotropic viruses such as human herpes virus-8 in the pathogenesis of AIDS-related PCNSL has been excluded after extensive evaluation [354].

Cytogenetic aberrations in PCNSL have been described in a number of reports. Comparative genomic hybridization experiments identified a number of chromosomal imbalances with gains on chromosomes 1, 7, 9, 11, 12, 16, 17, 18 and 22. Losses involved chromosomes 18, 20 and most notably 6, with loss of 6q being significantly correlated with a worse prognosis [355,356]. Moreover, a commonly deleted region at 6q22-23, possibly harboring a putative tumour suppressor gene, was identified by high resolution mapping of chromosome 6q in a group of PCNSL [357]. Finally, inactivation of tumour suppressor genes $p16^{INK4}$, a cell cycle regulator, and $p14^{ARF}$, an upstream regulator of P53, was seen in the majority of PCNSL investigated [358-360].

4 Scope of this thesis

Diffuse large B-cell lymphomas presumably comprise several disease entities as they show marked heterogeneity with respect to presentation, response to treatment and clinical outcome. DLBCL can primarily present in lymph nodes or at many different extra-nodal sites. A minority of the extra-nodal DLBCL presents at immune privileged sites such as the CNS and the testis and patients with these lymphomas have a particularly poor prognosis. Tumours developing in the CNS or testis are thought to have a growth advantage as immune surveillance at these particular sites is hampered by a blood-organ barrier,

absence of HLA expression and the local production of suppressing cytokines. However, immune privileged sites are not completely devoid of immune cells as already under physiological circumstances small numbers of T-cells are present. Neoplastic B-cells express like their normal counterparts, the BCR and HLA class I and II molecules on their surface and can elicit a CTL response by presenting antigenic peptides including their own idiotype to Th cells. B-cell lymphomas with loss of HLA expression were previously reported to be associated with a worse prognosis compared to HLA positive lymphomas. In this thesis we describe HLA aberrations and the responsible mechanisms in testicular and CNS DLBCL and compare this to nodal DLBCL and DLBCL of other extranodal sites. Moreover we investigated whether certain HLA-DR and –DQ alleles were preferentially deleted in testicular lymphomas.

We studied HLA class I and II expression in DLBCL primarily derived from lymph nodes, the testis and the CNS using specific antibodies on paraffin-embedded formalin-fixed tissue sections (**Chapter 2**). Loss of HLA class I and especially class II expression was very frequent in testicular and CNS but not in nodal lymphomas. Using loss of heterozygosity analysis and fluorescent in situ hybridization (FISH) on interphase nuclei and DNA fibers we detected and mapped small homozygous deletions of HLA-DR and HLA-DQ in the class II region, and hemizygous deletions involving large parts of the HLA region at chromosome 6p21.3.

Loss of heterozygosity at the HLA region was in the majority of cases due to hemizygous deletions and not to mitotic recombination as we determined by using an extensive set of microsatellite markers and FISH probes specific for this region (**Chapter 3**). A new method applying interphase FISH on nuclei isolated from paraffin-embedded formalin-fixed tissue was also described in this chapter.

In **Chapter 4** we confirm in a large series of nodal and extra-nodal DLBCL our previous report that loss of HLA class I and II expression is very common in DLBCL derived from immune privileged sites but not in DLBCL derived from the stomach, skin or lymph nodes. Moreover, we show that high numbers of activated CTLs are present in testicular and CNS DLBCL compared to the other groups of lymphomas suggesting that the former are more immunogenic.

On the basis of the remarkable differences between testicular and nodal DLBCL concerning HLA class II molecules as determined by immunohistochemistry and FISH, we hypothesized that the two groups of lymphomas would show a different distribution pattern of HLA-DR and –DQ alleles. We therefore DNA-typed 50 testicular and 48 nodal DLBCL and compared the frequencies with a large cohort of healthy dutch controls (**Chapter 5**). Both groups of lymphomas showed a significant positive association with *HLA-DRB1*15*. Moreover, the

testicular lymphomas showed a positive association with *HLA-DRB1*12* and the nodal lymphomas a negative association with HLA-DRB1*07. Using FISH, we subsequently investigated in 40 testicular lymphomas whether certain DR and DQ alleles were preferentially lost or retained through genomic deletions. Remarkably, in only one tumour of 7.HLA-DRB1*03 positive patients a homozygous deletion was found. In tumours of HLA-DRB1*12 positive patients, the opposite was seen, with only one of 8 cases not being involved in either a homozygous or a hemizygous deletion. However, none of the different HLA- DR or HLA-DQ alleles showed absolute loss or retention.

In the final chapter **(Chapter 6)** HLA-DR is proposed to function as a tumour suppressor gene in DLBCL and the significance of loss of HLA-DR expression in the development of DLBCL of the testis and the CNS is discussed.

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