Cloning and characterization of the human CD200 promoter region

by

Zhiqi Chen

A thesis submitted in conformity with the requirements for
the degree of Master of Science,
Graduate Department of the Institute of Medical Sciences, University of Toronto

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Finally, I am eternally grateful to my mother Jiashu, for her emotional support and enormous love.
Cloning and characterization of the human CD200 promoter region

By Zhiqi Chen

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Graduate Department of the Institute of Medical Sciences, University of Toronto

Abstract:

We previously reported that expression of CD200 was increased in animals rendered specifically hyporesponsive to organ and tissue allografts. Using monoclonal antibodies to this molecule, and a soluble immunoadhesin prepared from it, evidence was accumulated supporting the hypothesis that CD200 provided immunoregulatory signals to the immune system, in opposition to those delivered by the so-called costimulatory molecules, CD40, CD80/CD86 (amongst others).

We hypothesized that systematic application of regimens to augment immunoregulation by CD200 would depend upon our understanding potential differences in the transcriptional and translational regulation of expression of these molecules. The work described in this thesis outlines a characterization of the human promoter region for
CD200. During these studies an additional exon was defined. The promoter described 5' of this new exon 1 was mapped and its activity was confirmed. Detailed mapping studies confirm a number of potentially important cis-acting regulatory elements in the promoter region.
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<td>Ag:</td>
<td>Antigen</td>
</tr>
<tr>
<td>APC:</td>
<td>Antigen presenting cell</td>
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<tr>
<td>B7h:</td>
<td>B7 homologues</td>
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<tr>
<td>CD:</td>
<td>Cluster determinant of antigens used to characterize a cell surface</td>
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<tr>
<td>CD200 (OX-2):</td>
<td>A cell membrane glycoprotein which belongs to immunoglobulin supergene family</td>
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<tr>
<td>CD200R</td>
<td>CD200 receptor</td>
</tr>
<tr>
<td>CD200*</td>
<td>CD200 truncated molecule</td>
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<tr>
<td>CTL:</td>
<td>Cytotoxic T lymphocyte</td>
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<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte antigen-4</td>
</tr>
<tr>
<td>DC:</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>GAS:</td>
<td>Gamma-interferon activation site</td>
</tr>
<tr>
<td>ICOS:</td>
<td>Inducible costimulator</td>
</tr>
<tr>
<td>Ig:</td>
<td>Immunoglobulin</td>
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<tr>
<td>ILs:</td>
<td>Interleukins</td>
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<tr>
<td>IFN-γ:</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IRF:</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>Jak:</td>
<td>Janus tyrosine kinase</td>
</tr>
<tr>
<td>LIGHT:</td>
<td>Homologues to lymphotoxins, inducible expression, competes with herpes simplex virus glycoprotein D for herpes virus entry mediator</td>
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LMP: Low molecular mass polypeptide
mAb: Monoclonal antibody
MCP-1: Monocyte chemoattractant protein-I
MHC: Major histocompatibility complex
PCR: Polymerase chain reaction
pv: Portal venous
5'-RACE: Rapid amplification of 5' cDNA ends
RPA: Ribonuclease protection assay
STAT: Signal transducer and activator of transcription
TAP: Transporter Associated with antigen processing
Th: T helper cell
TCR: T cell receptor
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CHAPTER 1

General introduction to research on CD200
1.1 Introduction:

Organ transplantation represents a key therapeutic modality for end-organ failure, but control of rejection of the transplanted organs or tissues remains a limitation. The immunosuppressive drugs that are already in clinical use lack immunological specificity. These drugs suppress not only the immune response directed against the foreign (histocompatibility) antigens of the donor, but also other host-protective immune responses that the transplant recipients may need to make after transplantation (e.g. to pathogens). Recipients treated with these drugs are therefore susceptible to opportunistic infections and malignancy. In addition, the long-term administration of these “toxic” agents may lead to the development of other non-immunological side effects such as cardiovascular, neurological or dermatological complications. One way to overcome at least some of these problems would be to induce specific immunological unresponsiveness or tolerance to the transplanted organs or tissues. The major cells responsible for antigen-specific graft rejection are T cells, both CD4+ cells (recognizing antigen in association with Class II Major Histocompatibility Molecules (MHC), and CD8+ cells (recognizing antigen in association with Class I MHC).

1.2 Mechanisms of tolerance induction:

T cell tolerance is operationally defined as a situation in which a cell or cell population fails to develop an expected immune response following the interaction of antigen with antigen receptors on those lymphocytes. At least three basic mechanisms have been implicated in immunological tolerance, namely: deletion, anergy and suppression.

Central deletion of self-reactive clones of cytolytic (CD8+) and/or helper (CD4+) T lymphocytes occurs as a normal and essential stage of differentiation/maturation of cells in the
thymus(1). Some self proteins never reside in the thymus, and thus developing specific clones of T cells specific for these antigens may not be (centrally) deleted. It is not surprising then that a number of groups have been able to characterize mechanisms whereby tolerance to these (and other) molecules could be induced in the periphery. As one example of such mechanisms, it has been observed that the intravenous administration of foreign alloantigen can lead to the apoptosis of mature T cells in the periphery of TCR-transgenic mice(2, 3). While some of this non-responsiveness represents peripheral deletional events, evidence was also reported for a functional inactivation, or anergy, of potentially responsive cells as another mechanism of tolerance induction in these animals(4, 5).

It is now accepted dogma that at least two signals are required for effective activation of T cells(5). The first signal is provided by binding of T cell receptor to MHC-foreign antigen complexes on antigen presenting cells (APCs). The second signal is delivered by the interaction of costimulatory molecules on T cells (such as CD28, CTLA4 or CD40L) with their ligands on APCs (CD80, CD86 or CD40)(6, 7). A number of groups have documented that the delivery of the first signal in the absence of the second signal leads to T cell anergy. In this case antigen-specific T cells persist(8), but in a condition in which they have lost their ability to respond to antigen challenge, even when this now takes place in the context of adequate costimulatory signals(7).

Suppression is yet another mechanism responsible for tolerance induction. Evidence for this mechanism generally depends upon the demonstration that inhibition of an expected immune response from a population of normal responder lymphocytes is produced by addition of a pool of cells from tolerant individuals(9-11) Suppressor T cells comprise a class of lymphocytes which are believed to be distinct from helper and cytolytic T lymphocytes(12). In the context of
the transplantation studies currently discussed, our laboratory was one of the first to show that
tolerance could be adoptively transferred to naive mice with cells from animals bearing long-
term surviving allografts(13-15).

1.3 The alteration of cytokine production by T cells in tolerance and graft rejection:

Based on the cytokines they produce, helper T lymphocytes have been subdivided into two
different populations: type-1 cytokine-producing cells (Th1) and type-2 cytokine-producing cells
(Th2)(16). Th1 cells produce interleukin-2 (IL-2) and interferon-γ (IFN-γ), both of which are in
general increased after transplantation in individuals in graft rejection occurs(17-19). In contrast,
those cytokines produced by Th2 cells, such as IL-4, IL-10 and IL-13, have been associated with
the development of hyporesponsiveness in such models(20, 21).

Previous work from our laboratory has shown that survival of both non- vascularized (skin)
and vascularized (kidney and small intestine) allografts was increased in animals receiving
antigen-specific pretransplant immunization via the portal vein (pv)(14, 22). It was also shown
that this increased graft survival was associated with increased type-2 cytokine production (IL-4,
IL-10 and IL-13), and simultaneously decreased type-1 cytokine production (IL-2, IFN-γ and IL-
12). Confirmation that these changes in cytokine production were of functional relevance came
from studies in which we used anti-IL-10 monoclonal antibody (mAb) alone, or in combination
with rIL-12, to show this would reverse the graft prolongation in these pv immunized
animals(20). In contrast, graft survival could be extended with the use of rIL-13, in association
with anti-IL-12(21).

However, in some cases no correlation between cytokine production and graft outcome was
seen. As an example, the use of anti-IFN-γ mAb in grafted animals was found neither to inhibit
graft rejection nor block or facilitate the induction tolerance(23, 24). Data such as these suggested that other events were also crucially involved in the regulation of graft survival following transplantation.

1.4 The role of γδ T cells in regulation of graft rejection:

While the majority of T cells in the thymus, peripheral lymph nodes and spleen express TCRs comprising αβ heterodimers, the majority of T cells in the skin and the epithelial lining of gut, lung and reproductive organs are γδ TCR-expressing cells(25). γδ T cells are believed to play a primary role in the defense against pathogens and it has been observed that this population of T cells is associated with chronic inflammatory diseases such as multiple sclerosis(26) and arthritis(27). More recently, γδ T cells have been implicated as important “players” in allograft tolerance induction(28). Evidence supporting such a role include studies showing increased graft survival could be adoptively transferred by γδ T cells obtained from animals receiving antigen-specific pretransplant immunization via the portal vein (pv), and that increased graft survival in such animals could be abolished by anti-γδ TCR mAb(29, 30). Interestingly, it was also shown that γδ T cells producing IL-10 and TGF-β, cytokines previously implicated in tolerance in these models, were induced by pv immunization(31). Despite these interesting observations, indefinite survival of organ or tissue allografts was never achieved by adoptive transfer of γδ TCR+ cells alone.

1.5 Documentation of a role for other molecules in regulating graft rejection:

Much of the work described above implied that regulation of cytokine production and antigen presentation was important in tolerance induction in allografted animals. A crucial role in antigen presentation, as already discussed, has been ascribed to costimulatory molecules and
their counter-receptors. Lack of costimulatory signals (signal 2) has been shown to lead to T cell anergy(5, 7). Primary importance in T cell activation has been attached to costimulatory pathways involving CD28 and CD80/86, CTLA4 and CD80/86, and CD40L and CD40 interactions(32-35) In addition, more recently a variety of other costimulatory pathways have been found to be capable of regulating T cell immunity, including LIGHT with LIGHT(36), 4-1BB with 4-1BBL(37), ICOS with B7h(38, 39) and OX40 with OX40L(40) (in all cases the counter-receptor expressed on APC is the last named in the pair). However, in all these individual cases studied to date, blockade of any one of these interactions by mAb injection has not proven to be a reproducible means to promote graft survival universally in all systems tested(41). Even using combinations of costimulation blockade has not proven effective. Our laboratory speculated that there were other molecules which played important immunoregulatory roles, which had yet to be discovered.

As one approach to exploring evidence for such (hypothetical) molecules, we asked whether there was evidence for differential expression of novel genes in pv immunized animals, which might in turn be associated with the prolongation of graft survival in such situations. Using a subtractive hybridization approach, we identified several molecules which were preferentially expressed in mice receiving allogeneic skin or renal grafts along with pretransplant donor-specific pv immunization(42). One of these molecules had been previously described by Barclay and colleagues at Oxford, and given the designation MRC OX-2(43, 44). Its function had remained an enigma. The new nomenclature for this molecule (followed below) is CD200.

1.6 Functional studies with CD200:

CD200 was first defined by a mAb developed by immunizing mice with glycoproteins purified from rat thymocytes(44). The molecule was found to be a type I membrane glycoprotein,
a member of the so-called immunoglobulin supergene family. It possesses a hydrophobic leader peptide, an extracellular Ig V-like and C-like domain, a transmembrane and a cytoplasmic domain. Immunohistological analysis confirmed that CD200 is present on thymocytes, dendritic cells, activated T cells, resting B cells, neurons, endothelium and epithelial cells in mouse, rat and human(43, 44). Interestingly, subsequent genetic mapping has suggested that human CD200 gene shares the same chromosome location (chromosome 3) as the genes encoding CD80 and CD86(45).

Despite the large body of literature describing the expression pattern of CD200, as indicated above, no definite function for this molecule has yet been shown. With the evidence from subtractive hybridization studies in our laboratory which showed a clear association between increased expression of CD200 and increased graft survival, and also with the polarization of cytokine production to type-2 cytokines (increased production of IL-4 and IL-10), we hypothesized that CD200 may be a molecule capable of delivering a key immunoregulatory signal to antigen-activated T cells-for schematic of regulation of T cell activation by costimulatory molecules and CD200 see Figure 1.

As supportive evidence for this hypothesis we were able to document that an anti CD200 monoclonal antibody could abolish graft prolongation and reverse the altered cytokine profile seen in pv immunized animals, both rats (with intestinal transplants(46)) and mice (with skin or renal allografts)(47). Interestingly CD200 has a high homology with CD80 and CD80, yet Borriello and colleagues reported that it failed to bind either CD28 or CTLA4(48). They concluded therefore that it was not likely to be an additional (antagonist) ligand in the CD28:CD80/CD86 or CTLA4: CD80/CD86 costimulating pathways. Further support for this conclusion came from data showing that the immunoadhesins, CD28-Ig and CTLA4-Ig, both
stained CHO cells transfected with adenovirus vectors to express CD80 or CD86, but neither reagent stained CHO cells transfected with adenovirus vectors to express CD200(48).

At this time our laboratory speculated that CD200 was a qualitatively different kind of molecule that the extended family of costimulator molecules previously described, and that it was capable of delivering an immunoregulatory (suppressive) signal to activated T cells. We postulated that this tolerizing (negative) signal delivered to responder lymphocytes led to altered polarization in cytokine production, and the prolongation of graft survival. Interestingly, the molecular structure of CD200 showed that the intracellular domain lacked its own signaling motifs, or sites for "docking" of adapter molecules which might co-opt other signaling molecules. Accordingly, tolerance delivered by CD200 was in turn proposed to depend upon engagement of a receptor (CD200R) on a target cell(47).

A soluble immunoadhesin was prepared in which the extracellular domains of CD200 were linked genetically to a murine IgG2a Fc region, the molecule then being expressed in a Baculovirus expression system(49). We found that this soluble form of CD200 (CD200:Fc) was indeed immunosuppressive both in vitro and in vivo, as we had predicted. Both graft prolongation, inhibition of induction of alloreactive CTL in culture, and polarization to type-2 cytokine production, occurred in the presence of CD200:Fc. Moreover, and further supporting much of our earlier data on the auxiliary role of γδTCR⁺ cells in pv induced tolerance (the initial model system in which increased expression of CD200 had been observed) we found that immunosuppressive γδTCR⁺ cells were dependent on CD200 expression for their continued persistence and suppressive ability(50).
Emphasizing a general role for CD200 interactions in delivery of immunoregulatory signals, in collaborative experiments with a group at McMaster University, we showed a crucial functional role for CD200 in regulation of fetal loss in mice(51). These studies help explain the early observations of Barclay and colleagues documenting expression of CD200 in placental tissue. Blocking CD200 expression (with mAb) increased fetal loss, while we found that infusion of CD200:Fc protected from fetal loss(51).

1.7. A key role for CD200R in delivery of immunoregulatory signals:

In 1997 a report from Preston et al. suggested that there existed a counter-receptor for CD200 on macrophages(52). However, when our laboratory used a FITC-conjugated CD200Fc to detect CD200R+ cells, we found evidence both for an F4/80+ macrophage cell population with binding activity, and for binding by activated T cells. In particular, some 15% of αβTCR+ cells bound CD200Fc, while >85% of γδTCR+ cells bound this molecule(53). A more recent molecular analysis by Wright et al from Barclay’s laboratory documented a cDNA sequence for a macrophage-derived CD200R, and again confirmed, using a new mAb to CD200R, expression of this molecule on cells of the myeloid lineage(54). We have cloned and sequenced an identical cDNA both from macrophages and from γδTCR+ hybridoma lines which we described many years ago (Kai and Gorczynski-unpublished).

The conundrum concerning the failure of Barclay’s group to detect CD200R on other cells may soon be resolved. In association with Dr.Marsden’s laboratory we have recently found evidence for significant genetic heterogeneity in CD200R, with at least three independent genes encoding a family of CD200R molecules present in the germ line. The cellular expression of independent members of this family is under active investigation.
Interesting data confirming the physiological significance of CD200:CD200R interactions comes from recent studies by Hoek et al, from DNAX (in association with the Barclay group)(55). These workers constructed a mouse with a homologous deletion for CD200 (a CD200 knockout mouse), and showed, again as we had predicted earlier, that this animal showed increased susceptibility to autoimmune disorders (experimental allergic encephalomyelitis, EAE, an animal model for multiple sclerosis, and collagen-induced arthritis). Interestingly, however, the most marked histopathological abnormalities seen involved inflammatory changes in cells of myeloid lineage. Unfortunately no reports of tissue/cell staining with anti-CD200R were given in these studies.

1.8 Transcriptional regulation of gene expression in APC in development of immunity:

As is evident from the discussion above, in a minimalist model, T cell activation can be considered to be regulated by the integration of signals resulting from TCR and costimulator molecule engagement. It should be realized that this ignores (at least temporarily), for ease of discussion, the potential role for exogenous cytokines in cell activation, beyond their role in regulating expression of costimulator molecules(56, 57)

For further simplification, from the perspective of the ligands expressed on APC, the “key” players in regulating stimulation are considered to be members of the CD80/CD86 family, CD40 and now CD200. Characterizing the regulation of gene transcription, and the promoter, enhancer and silencer regions in genes which are responsive to cis/trans activating factors, can often suggest novel mechanisms for regulating biological function. For the CD200 molecule, such information would provide valuable data on mechanisms involved in the endogenous and cytokine-activated differential expression of costimulator molecules and CD200.
The endogenous levels of expression of many of these molecules is controlled by cytokines in the milieu (58, 59). Culturing dendritic cells (DC) in the presence of different cytokines is known to alter their costimulatory function (60). IL-12 and IFNγ increases T cell activation (for IL-2 production) while DCs cultured with IL-10 and IL-4 have decreased stimulatory activity (for IL-2 production) (60, 61). Cytokines and interferons are known to stimulate proliferation, differentiation, and survival signals, as well as specialized functions in host resistance to pathogens. Although they activate multiple signaling pathways that can together mediate these functions, one of the primary pathways by which they produce their pleitropic effects is the so-called Jak-STAT pathway, involving transcriptional activation of gene expression.

The Jak-STAT pathway is triggered by both cytokines and interferons, and rapidly allows the transduction of an extracellular signal into the nucleus. The pathway uses a novel mechanism in which cytosolic latent transcription factors, known as signal transducers and activators of transcription (STATs), are tyrosine phosphorylated by Janus family tyrosine kinases (Jaks), allowing STAT protein dimerization and nuclear translocation. STATs then modulate the expression of multiple target genes. The basic biology of this system, including the range and potential functions of known Jaks and STATs, is discussed in detail in a recent review (62).

The importance of the STAT family of transcription factors to transplant biology is further emphasized in a recent study by Zhou et al (63). It has been previously documented that STAT 4(-/-) mice have impaired type-1 T cell differentiation, whereas STAT 6(-/-) mice fail to generate type-2 responses. Zhou studied the role of type 1 and type 2 T cell differentiation in acute cardiac allograft rejection and in the induction of tolerance in wild-type, STAT 4(-/-), and
STAT 6(-/-) recipients. Although all recipients rejected the grafts with a similar tempo, in situ cytokine gene expression in the allografts confirmed differences in these mice. Thus, as expected, decreased levels of IFNγ were seen in STAT 4(-/-) recipients and no detectable IL-4 and IL-5 was seen in STAT 6(-/-) mice.

This group then went on to examine the effect of blockade of the CD28:CD80 costimulatory pathway on cardiac graft survival. Increased survival for > 100 days was seen in all CTLA4-Ig-treated wild-type and STAT 4(-/-) mice, but ~15% of CTLA4-Ig-treated STAT 6(-/-) mice rejected their grafts between 20 and 100 days. Moreover, some 60% of STAT 6(-/-) mice eventually rejected their grafts by ~100 days. Splenocytes harvested on day 145 posttransplant from CTLA4-Ig-treated rejecting STAT 6(-/-) recipients adoptively transferred accelerated rejection (secondary to transfer of preimmune cells) to SCID recipients, unlike the “tolerance” transferred by splenocytes from CTLA4-Ig-treated wild-type or nonrejecting STAT 6(-/-) mice. The authors concluded that long-term prolongation of cardiac allograft survival by CTLA4-Ig in this model was STAT 1-independent but, at least in part, STAT 6-dependent, and that the mechanism involved reflected not simply the balance of type 1 and type 2 cytokines, but the “tolerance” induced by CD28:CD80 blockade.

There is evidence that tolerance to/rejection of allografts is not simply a reflection of the relative levels of STAT 1 and STAT 6. Thus, for example, activation of STAT5 proteins (STAT5a and STAT5b) has been described as one of the earliest signaling events mediated by the IL-2 family of (type-1) cytokines, allowing the rapid delivery of signals from the membrane to the nucleus. Among all of the STAT family proteins, STAT5a and STAT5b are the two most closely related molecules, and along with other transcription factors and co-factors, they are
known to regulate the expression of different target genes in a cytokine-specific fashion. While the activities of STAT5a and STAT5b are activated, like STATs 1, 4 and 6, by cytokines, they, and a number of other STAT proteins, are negatively controlled by CIS/SOCS/SSI family proteins(64). Thus the outcome of STAT5 activation in regulating expression of target genes varies, depending not only upon the complexity of the promoter region of the target genes, but on the other signaling pathways that are activated by each cytokine as well. Lin et al(64) provide a detailed review of the molecular mechanisms of activation of STAT 5 by IL-2 family cytokines, using a variety of biochemical approaches as well as gene-targeting analyses.

There are a number of more specific studies assessing gene regulation of expression of different components of the antigen processing machinery in APC themselves. As one example, ChatterjeeKishore et al examined expression of two molecules known to be involved in processing antigen for presentation to CD4\(^+\) T cells, low molecular mass polypeptide (LMP) 2 (involved in processing proteins into immunogenic moieties) and transporter associated with antigen processing (TAP) 1, responsible for transport of the immunogenic peptide complex in its association with MHC(65). Both genes are encoded by closely linked genes within the MHC class II subregion. While the two genes are known to share a bi-directional promoter, LMP2 and TAP1 have differential cellular expression. TAP1 is expressed constitutively, while LMP2 expression requires induction by IFN\(\gamma\) in most cells (see also Fruh et al(66)). The regulatory elements within the LMP2/TAP1 promoter and the transcription factors that bind these elements have been defined by other groups, and include an interferon responsive consensus sequence-2 and a GAS (gamma-interferon activation site) region. The mechanism(s) by which these transactivators regulate differential TAP1 and LMP2 gene transcription was addressed by
analyzing melanoma cell lines with distinct phenotypes of LMP2 and TAP1 expression. Their data suggested that binding of either interferon regulatory factor 1 (IRF1) or STAT1 to the overlapping interferon consensus sequence-2/GAS was sufficient for regulating transcription of the TAP1 gene, but that binding of both factors was required for LMP2 gene transcription. Thus restoration of LMP2 gene transcription was shown following transfection of wild type STAT1α or interferon regulatory factor 1 cDNA into cells which lacked these transcription factors. This greater flexibility in the regulation of the TAP1 gene (vs LMP2) was postulated by these workers to reflect a (more) important role for this molecule in maintaining immune surveillance.

In a similar vein, a number of studies have examined regulation of gene expression of CD80/CD86 by cytokines. Studies examining the chromatin configuration of the human CD80 gene in intact nuclei from various cell types identified a tissue-specific deoxyribonuclease I hypersensitive site 3kb upstream of the transcription start site as a cell type-specific enhancer region(67). This 183-bp region was responsive to both lipopolysaccharide and dibutyryl cAMP, both of which have been previously described as regulating CD80 expression. Further deleterional and site-directed mutagenesis revealed the presence of multiple critical cis elements within this region, one of which was a nuclear factor NFκB consensus sequence. Members of the NFκB family of transcription factors have already been implicated in signal transduction pathways relevant to CD80 expression. The NFκB family of transcription factors is itself composed of NFκB1 (p50/p105), NFκB2 (p52/p100), RelA (p65), c-Rel, and RelB, which, following homo-or heterodimerization bind to κB motifs (see(68) for review). The dimers are retained in the cytoplasm by the inhibitory protein IκB, which is phosphorylated and degraded following
activation, thus releasing the NFκB complexes which translocate to the nucleus. Detailed immunological studies have been performed on the role of NFκB regulation of T cell activation, where it seems that p65/p50 and p65/c-Rel heterodimers act as positive regulators of T cell activation and cytokine expression(69), while p50/p50 homodimers may be negative regulators (since the p50 molecule lacks the transactivation domain present in p65 and c-Rel)(68).

More recently, Li et al (70) reported a series of studies designed to understand the mechanisms by which the expression of the human CD86 gene was transcriptionally regulated by IFNγ, using an approach analogous to that used in this thesis, namely by cloning and characterizing the 5'-flanking region of the human CD86 gene. A series of luciferase reporter gene constructs were prepared and used for transfection of cells, in their case from the monocytic line U937 and Raji B cell line. Basal expression was detected in Raji cells, which themselves show high constitutive expression of the CD86 molecule, but not in U937 cells, which show low expression of CD86 in non-activated state. However, induction of CD86 expression followed stimulation of U937 cells with IFNγ, and depended on the presence of two functional GAS elements. Additional gel mobility shift assays showed that the two GAS elements specifically bound an IFNγ-induced transcriptional complex. This DNA-protein complex was itself further “supershifted” by antibody to Stat1α but not by antibodies to Stat 2, Stat 3 and Sp1, indicating that the GAS elements interacted uniquely with Stat1α. As further documentation of their functional significance, point mutations in the GAS elements prevented the formation of the DNA-protein complex and significantly reduced the responsiveness of the reporter gene to IFNγ. This group concluded that two functional GAS elements within the human CD86 promoter
played critical roles in the induction of CD86 gene expression by binding to IFNγ-induced Stat1α.

Focusing attention on the site of expression of co-receptors for APC expressed costimulatory molecules, Parra et al(71) investigated transcriptional regulation of human CD40 Ligand expression. Again using luciferase constructs they documented existence of a functional CD28 responsive element some 170 nucleotides upstream of the start codon, and that this site bound a transcription factor complex containing JunD, c-Fos, p50, p65, and c-Rel, but not c-Jun. Other transcription factors have been identified in regulation of gene expression in APC following inflammation. Induction of the monocyte chemoattractant protein-1 gene (MCP-1) by the inflammatory cytokine tumor necrosis factor (TNFα) occurs through the coordinated assembly of an NFkB-dependent distal regulatory region and a proximal region which binds Spl(72). ICAM expression on APC is also upregulated following stimulation by TNFα and/or IFNγ. As a result, although ICAM-1 is expressed constitutively on the cell surface, its expression is further increased following inflammation, primarily through activation of gene expression. The architecture of the ICAM-1 promoter itself is quite complex, containing a large number of binding sites for inducible transcription factors, the most important of which is NFkB. This acts in concert with other transcription factors, prominent amongst which is AP1, via specific protein-protein interactions, producing an assembly of distinct transcription complexes on the ICAM-1 promoter. The AP-1 transcription factor too is a complex between members of the Fos (cFos, FosB, Fra-1 and Fra-2) and Jun (c-Jun, JunB and JunD) families of proteins(73). AP-1 transcriptional activity is thus regulated at the level of both fos and jun gene transcription, and by post transcriptional modifications of the corresponding proteins. The AP-1 transcription complex
has been described to regulate expression of a number of cytokine genes(73), as well as being implicated in control of cell cycle progression(74) and expression of costimulatory molecules (see above).

1.9 Promoter analysis of CD200 gene expression in APC:

The work described earlier documents an important role for CD200 in immunoregulation per se. Our laboratory thus became interested in understanding in more detail the molecular events which might control gene expression of CD200, under constitutive and inducible conditions. Characterization of these events would form the prelude to addressing the issue of the control of CD200 expression relative to that of known costimulator molecules (such as CD80, CD86 and CD40). It was felt that detailed analysis of the promoter region of the CD200 gene was an essential starting point. Since a longer-term goal was to study regulation of expression of human CD200, we decided to restrict our analysis to the human CD200 gene.

The genomic organization of human CD200 had been reported many years ago by Barclay’s group(45), and of mouse CD200 more recently by Borriello et al(75). However, comparison of the genomic sequences, and of our reported cDNA sequences for human and mouse CD200(42), suggested that there might be additional coding sequences for the human CD200 gene upstream of the defined “first exon” in the reported genomic human CD200 gene. Accordingly to begin these studies it was necessary first to isolate and sequence genomic DNA containing sequences 5’ upstream of the previously reported first exon, to define the full genomic organization of CD200. Thereafter sequences 5’ upstream of this “true 5’ end” of the genomic DNA were assessed to identify the promoter, using various well-characterized techniques (5’RACE, primer-extension(76) and RNase protection(77). Regions in the putative promoter were analysed for the existence of sequences characterizing binding sites for known transcriptional modifiers.
(enhancer/silencer activity). Finally, we confirmed promoter activity by examining transcription of a reporter gene (luciferase) in CD200 promoter-reporter constructs, both in cells normally expressing CD200 (dendritic cells, endothelial cells) and in normally non-expressing cells (Hela). These results form the basis of the work described in the thesis below.

1.10 Summary and Hypothesis:

We have reported evidence that increased expression of a novel gene, CD200, capable of delivering immunoregulatory signals, is a key feature of processes leading to increased transplant graft survival. We predict that characterization of the promoter region of the CD200 gene will identify both potential enhancer-like elements and silencer-like elements, which may contribute to regulation of human CD200 expression. Moreover, comparison of the CD200 promoter with described promoter regions for CD80, CD86 and CD40, may shed light on how cells (animals) can differentially regulate expression of molecules which deliver a T cell activating signal (leading to graft rejection) from those signals (e.g. from CD200) which decrease rejection.
CHAPTER 2

Cloning and Characterization of the Human CD200 5'-Flanking Region
2.1 Abstract

CD200 has been characterized as a type I membrane glycoprotein which is expressed preferentially on a number of cell types uniquely relevant to the inflammatory and immune cascade, amongst which are dendritic cells, endothelial cells and activated T cells. Our laboratory has shown that increased expression of CD200 is associated with increased graft survival following antigen-specific pre-transplant immunization via the portal vein, and that treatment with an anti CD200 antibody abolishes graft prolongation. In addition, an immunoadhesin incorporating the CD200 extracellular domain prolongs both allo- and xenograft survival. We propose that understanding the molecular basis of constitutive and inducible expression of human CD200 will be important for therapy designed to regulate immune injury. We have cloned and characterized the 5'-flanking region of the human CD200 gene, including an exon1/intron1 boundary region that was previously unidentified. Start sites for transcription initiation are defined. In the 5'-flanking region of the gene a number of sites potentially relevant for regulation of CD200 mRNA expression have been identified. The complete genomic organization of the human CD200 gene is provided, along with characterization of promoter activity in the cloned 5' flanking region.

Keywords: CD200; promoter; transcriptional start site; dendritic cell; mRNA splicing
2.2 Introduction:

CD200 antigen is a type I membrane glycoprotein with a hydrophobic leader peptide, an extracellular Ig V-like and C-like domain, a transmembrane and a cytoplasmic domain. CD200 is expressed on dendritic cells, thymocytes, activated T cells, resting B cells, neurons, endothelium and epithelial cells in mouse, rat and human (43, 78). Recent studies from our laboratory, as well as that of Barclay et al, have provided novel insight into the function of this molecule. For instance, we documented increased expression of CD200 in association with increased graft survival following donor specific immunization via the portal vein (pv), a protocol which is also associated with polarization of cytokine production to type-2 cytokines (increased production of IL-4, IL-10 and TGF-β) (19, 22, 49, 79, 80). Preston et al. suggested that a counter-receptor for CD200 may exist on macrophages (52) and more recently Wright et al reported cloning of such a molecule, with documentation for its immunoregulatory role in autoimmune disease models (54). We also reported that interaction of CD200 with its receptor in macrophages enhanced suppression of graft rejection (53). Although CD200 has high homology with CD80 and CD86 at the nucleotide and amino acid level, it binds neither CD28 nor CTLA4 and therefore is likely not an additional ligand in the CD80/CD86: CD28 or CD80/CD86: CTLA4 costimulatory pathway. This hypothesis is supported by data showing that CD28-Ig and CTLA4 Ig reagents stained CHO cells transduced to express CD80/CD86 but not cells transduced to express CD200 (48). We showed that bone marrow-derived dendritic cells expressing CD200 could inhibit the stimulation of type 1 cytokine production by CD80 and CD86-positive dendritic cells(81). We speculated that CD200 may be a co-regulator molecule distinct from costimulator molecules such as CD80 and CD86, and that CD200 delivers a
tolerizing (negative) signal to responder lymphocytes to prolong graft survival (49, 80).

To date the rat, human and murine CD200 genes have been cloned and their cDNA sequences characterized (42, 43, 75, 78). Interestingly, comparison of the published sequence of the leader exon of human CD200 with that of the rat or murine homologues indicated that the reported human leader exon lacked the first four amino acids at the N-terminal sequence. McCaughan et al. suggested that a further unidentified 5’ exon may exist in the human gene (78). An EST of a neuronal precursor cDNA clone (accession number: AA100426) has the same cDNA sequence as that of the reported human CD200 gene, with an additional 36 nucleotides 5’ upstream of the leader exon as predicted.

We report below the cloning of the 5’-flanking region of the human CD200 gene, with identification of the first exon; the genomic organization of the 5’ region; the transcriptional start site; and documentation of functional promoter activity.

2.3 Materials and Methods

2.3.1 Screening of human genomic library

A human P1 artificial chromosome (PAC) library (Genome Systems, USA) was screened using a PCR-based method with two specific primers designed to amplify a 275 bp DNA fragment within the Ig V-like domain (exon 3) of human CD200. Screening by PCR demanded the ability to amplify a minimum ~150bp amplicon, and the existing information on the only other 5’ exon known, exon 2, indicated this would not provide a sufficiently lengthy amplicon for use. The sequences of the primers used are as follows:

- Primer1 (sense primer) 5’-GCT GTA CAC AAC TGC TTC CTT -3’;
- Primer2 (antisense primer) 5’-CTG AGA TCT TCC CAA AAC CAA-3’.
PCR conditions included 25 pmol of each primer, 1.5 mM MgCl₂, 200 μM dNTPs and 1.25 U Taq polymerase (Life Technologies, Canada) in a final reaction volume of 50 μl. Initial denaturation was at 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, and final extension at 72 °C for 15 min. Two independent positive clones (named PAC 1 and PAC 2 respectively) from the PAC library were obtained. Genomic DNA was extracted from the clones by a Maxiprep method (QIAGEN, Canada).

2.3.2 Dot blot analysis

Genomic DNAs from the two clones (above) were dot blotted onto a Hybond® nylon membrane (Amersham life science, Canada) and UV cross-linked using a Stratagene 2400 lamp. The blot was hybridized with [γ-³²P]ATP end-labeled oligo 1 and oligo 2 (the same as primer 1 and 2) respectively in 6 x SSC, 5 x Denhardt’s solution, 0.5 % SDS and 0.1 mg/ml sonicated salmon sperm DNA at 55 °C overnight with rotation. The membrane was washed twice in 6 x SSC and 0.5 % SDS at 55 °C. Autoradiography was performed using Kodak X-omat AR film (Eastman Kodak, USA) at -70 °C overnight. DNA from a PBK vector (Stratagene, USA) without inserts was used as negative control. An antisense oligo (oligo 3) corresponding to the most 5’ regions of the human EST database sequence 5’-CAG ACT GCC CAT CCT TGC TC-3’ was used as a specific probe for the dot blot.

2.3.3 Southern hybridization

Based on available information of the restriction map of human CD200, 2 μg of genomic DNA from PAC 2 was digested with either Hind III or Bam HI at 37 °C overnight and separated on a 0.6 % TAE-agarose gel with 0.5 μg of EtBr/ml at 40 V for 10 hours. PAC 2 was chose (see results) because it was the clone which had evidence for DNA sequence “upstream” of the most
available 5' exon used for screening (exon 1). The gel was denatured in 0.125 M HCl, neutralized in neutralization solution, and rinsed with dH₂O. DNA was transferred from 0.6 % agarose gel onto a Hybond* nylon membrane using downward transfer at room temperature for three hours. The membrane was UV cross-linked and hybridized with [γ-³²P]ATP end-labeled oligo 3 in 6 x SSC, 5 x Denhardt's solution, 0.5 % SDS and 0.1mg/ml sonicated salmon sperm DNA at 57 °C overnight, followed by washing in 6 x SSC, 0.5 % SDS at 57 °C for 30 min, 62 °C for 15 min. Autoradiography was performed at -70 °C overnight.

2.3.4 Generation of a genomic sublibrary containing CD200

4 μg of genomic DNA from PAC 2 was digested with either Hind III or Bam HI at 37 °C overnight and separated on 0.6 % TAE-agarose gel with 0.5 μg of EtBr/ml at 40V for 10 hours. After electrophoresis, a 4 kb fragment from the Hind III digestion and a 10 kb fragment from the BamHI digestion were cut from the agarose gel and purified using a gel purification kit from QIAGEN. pBluescript II SK(-) (Stratagene, USA) was also digested with either Hind III or BamHI and treated with CIAP (Calf Intestinal Alkaline Phosphatase) at 37 °C for 45 min. Phenol and phenol/chloroform extraction were performed twice and the DNAs precipitated. The enzyme treated pBluescript II SK (-) was ligated with either the 4 kb Hind III fragment or the 10 kb BamHI fragment at a molar ratio of 1:3 using T4 DNA ligase (Life Technologies, Canada) at 14 °C overnight.

Ligation products were transformed into DH 10β E.Coli cells by electroporation using a Cell- Porator Electroporation System I (Life Technologies, Canada). 20 μl of ElectroMax DH 10β cells (Life Technologies, Canada) was transferred to the electroporation cuvette along with 1 μl of the ligation mixture precipitated by 3M NH₄Cl and 100% ethanol. Electroporation was
performed at 401V, 330μF capacitance, lowΩ, and 4kΩ (for Booster). Shocked cells were added to SOC media and shaken at 37 °C for 1 hour at 250 rpm. The cells were plated onto LB/ampicillin plates coated with 50 μl of 1M IPTG and 25 μl of X-gal (40mg/ml), incubated at 37 °C overnight, and white colored colonies picked at random. Colonies from the Hind III treated DNA plate were streaked onto another LB/ampicillin plate without IPTG and X-gal, and incubated at 37 °C overnight for subsequent manipulation.

2.3.5 Screening of the genomic sublibrary containing CD200

The 113 colonies were streaked onto 5 nitrocellulose filters (Millipore, USA) with 25 colonies on each filter. Filters were laid onto fresh LB/ampicillin plates, incubated at 37°C overnight, and then treated with denaturing solution for 1 min, neutralization solution for 5 min, and rinsing with 2x SSC. After UV cross-linking, the filters were hybridized with [γ-^{32}P]ATP end-labeled oligo 3 in 6 x SSC, 5 x Denhardt’s solution, 0.5 % SDS and 0.1mg/ml sonicated salmon sperm DNA at 57 °C overnight. The membrane was washed twice in 6 x SSC, 0.5 % SDS at 57 °C for 30 min, and then 62 °C for 15 min. Autoradiography was performed at -70 °C overnight. To confirm that the clones contained the correct size inserts, plasmid DNAs from positive clones were isolated with QIAGEN Miniprep Kit and digested with Hind III.

2.3.6 Sequence analysis of the clones bearing CD200

0.5μg of plasmid DNA from each of 4 randomly selected positive clones was used for sequencing analysis with the ABI PRISM™dRhodamine Terminator Cycle Sequencing Reading Reaction Kit (Model 377, PE Applied Biosystems, USA). M13 and T 3 primers were used for 5' to 3' and 3' to 5' sequencing, respectively. Conditions for sequencing were as follows: 96 °C for 3 min and 25 cycles at 96 °C for 20 sec, 50 °C for 10 sec and 60 °C for 4 min. Four new
sequencing primers (sense 5'-TCC TTG GTT TTT AAC TGG ATG-3' and 5'-AAC ACA GAG CTA GAC TCC GT-3'; antisense 5'-ATT CTC ACC AGC ATT GGG TGT-3' and 5'-ACT GAT CTT TGT GAT GTA AGT-3') were designed based on the outcome of sequencing to complete the sequencing. Extension products were purified by ethanol/MgCl₂ precipitation. Samples were subjected to electrophoresis on the ABI PRISM 310. The sequences were analyzed using DNAsis for windows, sequence analysis software (Hitachi Software Engineering America Ltd, USA). Another new antisense primer (5'-GTT CCA GAG TTC TTG GCT CTT-3') was designed based on the outcome of sequencing at the 5' end, and used for primer walking to sequence the DNA from PAC2 under the same sequencing reaction conditions, except that 1 µl of DMSO was also included in the reaction.

2.3.7 Rapid amplification of 5' cDNA ends (5'-RACE)

A Smart Race cDNA Amplification System (Clontech, USA) was used to amplify the 5' cDNA ends of the CD200 gene. Total RNA isolated from activated human peripheral lymphocytes was used as the template. First-strand cDNA synthesis was primed using a 5'-CDS primer 5'-T₂₅ N₁N- 3' (N₁ = A,G, or C; N = A,C,G, or T) and performed using Superscript II reverse transcriptase (Life Technologies, Canada) at 42 °C for 1.5 hours. After the reverse transcriptase reached the end of the mRNA, it added several dC residues. The Smart II oligo (5'-AAG CAG TGG TAA CGC AGA GTA CGC GGG - 3'), whose terminal stretch of dG residues annealed to the tail of the cDNA, served as an extended template for RT. Primary PCR was performed using as sense primer a Universal Primer Mix (UPM, long 5'-CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA ACA ACG CAG AGT- 3' and short 5'-CTA ATA CGA CTC ACT ATA GGG C-3') combined with an antisense Gene Specific Primer (GSP, located
within the CD200 V-like region 5'-GGT TCT CGC TGA AGG TGA CCA TGT TTT C-3'). Conditions were: 30 cycles of 5 sec at 94 °C, 10 sec at 60 °C, and 3 min at 72 °C on a PE GeneAmp System 2400 cycler (PE Applied Biosystems, USA). Amplified PCR products were diluted 1:100 and subjected to a second round of amplification (nested PCR) using both Nested Universal Primer (NUP, 5'-AAG CAG GGT AAC AAC GCA GAG T-3') and Nested Gene Specific Primer (NGSP, 5'-AGG TGA CCA TGT TTT CTG GGC TTA CAG C-3') under the same PCR condition as for primary PCR. The 5'-RACE fragments were gel-purified using the NucleoTrap Gel Extractin Kit (Clontech, USA) and ligated to PCR2.1-TOPO vector (Invitrogen, USA) at room temperature for 5 min. 2 μl of the above ligation product was mixed with 20 μl of One Shot Top10 competent cells (Invitrogen, USA) and incubated on ice for 30 min, followed by heat shock at 42 °C for 30 sec. Shocked cells were added to SOC media and shaken at 37 °C for 1 hour at 250 rpm. The cells were plated onto LB/ampicillin plates coated with 25 μl of X-gal (40mg/ml) and incubated at 37 °C overnight. Twenty seven white colonies were randomly picked and sequenced using the ABI PRISM™ dRhodamine Terminator Cycle Sequencing Reading Reaction Kit (Model 377, PE Applied Biosystems, USA). The GSP previously used for 5'-RACE was used as a primer for the sequencing reaction.

2.3.8 Primer extension analysis

Primer extension was performed as described previously(76, 77). A 26-mer antisense primer located in exon 1 (5'-CAG CCT CTC CAT CCT TGC TCC TGG AG-3') was end labeled using [γ-32P]ATP (sp. Act. 6000 Ci/mmol) and T4 polynucleotide kinase (Life Technologies, Canada) to a specific activity of 1 x 10^9 cpm/μg; 1 x 10^4 cpm of the primer was coprecipitated with 0.5 μg of poly (A)^+ RNA from activated human peripheral lymphocytes or
30 µg of yeast RNA. The samples were then incubated for 17 hours in 40 mM PIPES (pH 6.4), 1 mM EDTA (pH 8.0), 0.4 M NaCl, and 80% deionized formamide at 30°C. Reverse transcription was performed at 37°C for 1 hour followed by 48°C for 2 hours in 50 mM Tris-HCl pH 7.6, 60 mM KCl, 10 mM MgCl₂, 1 mM of each dNTP, 1 mM DTT, 50 µg/ml actinomycin D with 40 U of RNasin, and 200 U of Superscript II (Life Technologies, Canada). RNase A (Boehringer Mannheim) was used to degrade RNA templates. Extended products were extracted with phenol/chloroform, precipitated with ethanol and separated on 6% polyacrylamide/8 M urea gel. A sequencing reaction, conducted with CD200 genomic DNA and the same primer as used for primer extension, was used as the size marker.

2.3.9 Ribonuclease protection assay

Two PCR primers were designed to amplify the DNA fragment (-100 to +12) using genomic DNA as a template. The primers were as follows: 5'-GGT GCG GCA GGG CAC AGG TG-3' (sense); 5'-TTG GCC GAT CAC CAG CCT CTC CAT-3' (antisense). Two additional PCR primers were designed to amplify the cDNA template covering the whole of exon 2 and the first 100 bp of exon 3. The sequences of the primers were: sense 5'-GGC CGT GAT CAG GAT GCC CTT CTC TC-3'; antisense 5'-CTG CCA TGT CAC AAT GAG GGC-3'. The underlined parts of the above primers represent Sau3A I cleavage sites.

After the two amplicons were digested with Sau3A I and purified from the agarose gel separately, they were ligated and subcloned to PCRII vector (Invitrogen, USA). Using the 294 bp insert as a template, an antisense RNA probe was transcribed with Sp6 RNA polymerase (Ambion, Canada) using [α-³²P] UTP (DuPont-New England Nuclear, USA). This antisense RNA transcript was hybridized to 20 µg of total RNA from activated human peripheral
lymphocytes or yeast RNA at 42°C for 18 hours in 80% deionized formamide, 100 mM sodium citrate (pH 6.4), 300 mM sodium acetate (pH 6.4), and 1 mM EDTA. Unhybridized RNA was digested with RNase A/T1 (Ambion) at 37°C for 30 min. RNase digested products were heat denatured and analyzed on a 6% polyacrylamide/8 M urea sequencing gel alongside an RNA century ladder (Ambion) labeled with the same radioisotope as a size marker.

2.3.10 Reverse transcriptase PCR

Human total RNA (5 µg) from brain, thymus, spleen, liver, small intestine and bone marrow were reverse transcribed using 1 µg of random hexamer (Life Technologies, Canada), 1 mM dNTP, 1 x reverse transcription buffer and MMLV (Life Technologies, Canada) at 37 °C for 1 hour and 90 °C for 10 min. 1/10th of the first-strand cDNA obtained was used for PCR, using a sense primer within exon 1 (5' - AGC AAG GAT GGA GAG GCT G- 3') and an antisense primer within exon 3 (5' - GGT ATT GAA GAG ACA CAT G- 3'). The semi-quantitative conditions used were: 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 57 °C for 1 min, 72 °C for 2 min; and finally extension for 10 min at 72 °C. After PCR amplification, 20 µl of each of the final reaction products was electrophoresed on a 1.2 % TAE-agarose gel with 0.5 µ g/ml EtBr.

2.3.11 Construction of promoter-reporter constructs

The previously mentioned 4 kb genomic Hind III fragment of the human CD200 5'-flanking region ligated into pBluescript II SK (-) (Stratagene, USA) was used for making promoter-reporter constructs. After digestion with Kpn I and BssH II, the 4 kb genomic DNA was cut into 1.2 kb and 2.8 kb fragments. The 1.2 kb fragment was subsequently excised from 1% TAE-agarose gel and purified using a Gel Extraction Kit (QIAGEN, Canada). Meanwhile, a
pGL-2 Basic vector (promoterless and enhancerless luciferase reporter vector) (Promega, USA) was cut with Kpn I and Mlu I respectively and gel purified using the same method. Ligation was performed using 150 ng of the purified 1.2 kb fragment and 250 ng of pGL-2 Basic vector in the presence of T4 DNA ligase (Life Technologies, Canada) at 14°C overnight. The ligation products were transformed into DH 10β E. Coli by electroporation using Cell-Porator Electroporation System I (Life Technologies, Canada). 20 µl of ElectroMax DH 10β cells (Life Technologies, Canada) was transferred to the electroporation cuvette along with 1 µl of the ligation mixture precipitated by 3M NaOAc and 100% ethanol. The electroporation was performed at 401V, 330µF capacitance, lowΩ, and 4kΩ (for Booster). Shocked cells were added to SOC media and shaken at 37 °C for 1 hour at 250 rpm. The cells were plated onto LB/ampicillin plates and incubated at 37 °C overnight.

Twelve colonies were randomly picked and subjected to Hind III digestion to confirm the size of the inserts. Two clones containing 1.2 kb inserts were sequenced using the ABI PRISM™ dRhodamine Terminator Cycle Sequencing Reading Reaction Kit (Model 377, PE Applied Biosystems, USA). pGL2-1 primer (5'-TGT ATC TTA TGG TAC TGT AAC TG-3') and pGL2-2 primer (5'-CTT TAT GTT TTT GGC GTC TTC CA-3'), flanking the multiple cloning sites in the pGL-2 Basic vector, were used for 5' to 3' and 3' to 5' sequencing, respectively. The conditions for the sequencing reaction included 96 °C for 3 min and 25 cycles at 96 °C for 20 sec, 50 °C for 10 sec and 60 °C for 4 min. Extension products were purified by ethanol/ MgCl₂ precipitation and electrophoresed on the ABI PRISM 310. The sequences were analyzed using the DNAsis for windows, sequence analysis software (Hitachi Software Engineering America Ltd, USA).
2.3.12 Transfection and luciferase assays

DNA of the 1.2 kb CD200/ pGL-2 reporter construct (pGL2 -1075/+39) was purified using a double CsCl method. The plasmid construct was transfected into HeLa cells and dendritic cells (DC2.4) using a GenePorter (Gene Therapy Inc., USA) reagent. The pGL2 control vector (containing both SV40 promoter and enhancer) was used as a positive control; pGL2 Basic (promoter-less, enhancer-less) was used as a negative control. The CMV-based β-galactosidase reporter vector was used as an internal control for transfection efficiency. Luciferase activity was measured over a 10 second time course using the Monolight luminometer (Analytical Luminescence Laboratory, USA) and the Calbiochem reagent system with 25 μl of cell lysate. β-galactosidase activity was measured using the chlorophenol red-β-galactosidase-based (Boehringer Mannheim) assay as described elsewhere (82). Protein concentrations of cell extracts were measured using the bicinchoninic acid Protein Assay Reagent Kit (Pierce, USA). The luciferase activity of each sample was normalized to β-galactosidase activity and protein concentration per sample.

2.4 Results

2.4.1. Isolation of genomic clones bearing a 5'-flanking region of human CD200 gene:

A human P1 artificial chromosome (PAC) library was screened by PCR using two primers designed to amplify a 275-bp DNA fragment within exon 3 (Ig V-like domain) of CD200. McCaughan et al, working with human CD200 genomic DNA, previously named the exons encoding the leader peptide and the Ig V-like domain exon 1 and exon 2 respectively, but they mentioned that there might be a further unidentified 5’ exon in this gene since no start codon in
‘exon 1’ was detected(78). Borriello et al reported the genomic sequence of *murine CD200* and identified an exon 1 of 12 bp which was located approximately 2 kb 5’ upstream of exon 2 (75). Following this nomenclature, we have named the exon encoding the Ig V-like domain exon 3 rather than exon 2 in the subsequent discussion. PCR primers for exon 2 could not be used to screen the Genome Systems PAC library as the amplicon encoded was too short.

Using the V-region PCR primers, two positive clones each with inserts of size ~150 kb were obtained from Genome Systems, USA. PAC DNA from these clones was isolated and used for subsequent studies. To confirm that the CD200 gene was included in the clones, both PCR and dot blot approaches were used. After PCR amplification of PAC DNA with the same set of the primers (primer1 and primer 2), an amplicon with the expected size was detected on agarose gel (Fig. 2). In addition PAC DNAs were dot blotted and hybridized separately with oligo 1 and oligo 2, using DNA from an “empty” PBK vector as a negative control. The PAC DNA hybridized with either oligo 1 or oligo 2 (Fig. 3). In addition, an antisense oligonucleotide (oligo 3) corresponding to human EST database sequences 5’-CAG ACT GCC CAT CCT TGC TC-3’ was used in the same experiment and showed a strong positive result, but only with the PAC 2 clone(Fig. 4). Since this probe was constructed to detect exon 1, we presume that fortuitously in the PAC 1 clone the V-region exon of CD200 “sits” at the 5’ end of the cloned DNA (see Fig. 5). Accordingly, all subsequent studies were performed with DNA from the PAC 2 clone.

To generate a sublibrary for further analysis, DNA from the positive clone was digested separately with *Hind III* and *BamH I* and analyzed by southern hybridization with the same probe (oligo 3). 4 kb *Hind III* and 10 kb *BamH I* fragments were detected (Fig. 6). For ease of subsequent manipulation, the 4 kb fragment was ligated to pBluescript SK II (-) and transformed
Figure 2. PCR amplification of two human PAC clones with primer pairs located within the human CD200 exon 3 (V-like region). A 275 bp fragment was detected in both PAC clones shown. A 100 bp DNA ladder from GIBCO/BRL was used as marker for the gel.
Figure 3. Dot blot hybridization of the two human PAC clones (Figure 2) with oligo 1 and oligo 2 (see Materials and Methods). DNA from the PBK vector was used as a negative control.
Probe: human CD200 exon 1 (oligo 3)

Figure 4. Dot blot hybridization of the two human PAC clones with an antisense oligo 3 corresponding to the human EST database sequence 5'-CAGACTGCCCATCCTTGCTC-3' (CD200 exon 1). DNA from the PBK vector was used as a negative control.
Figure 5: Schematic to map likely position of CD200 gene on PAC 1 and PAC 2. Oligo 1, 2 and 3 are the probes respectively used for dot blot.
**Figure 6.** Southern hybridization of the *Hind* III and *BamH* I digested human genomic fragments with oligo 3. A 4 kb *Hind* III and a 10 kb *BamH* I genomic fragment were identified.
into DH 10β cells by electroporation. The sublibrary was screened with the same antisense oligo
3, and 16 positive clones were obtained. Sequencing of 4 randomly selected clones gave identical
results and indicated that a 1.1 kb 5’-flanking region of CD200 was contained in the 4 kb HindIII
fragment.

Primer walking technology was used to sequence the PAC DNA, with a new antisense
primer close to the 5’ end of the 1.1 kb 5’-flanking region. This yielded a further 400 bp of
sequence upstream of the documented 1.1kb 5’-flanking region.

2.4.2. Determination of the transcription initiation site of CD200:

Three different methods were used to identify the transcriptional initiation sites of the human
CD200 gene, namely 5’-RACE, primer extension and RNase protection. For 5’-RACE, a Smart
Race cDNA amplification system (Clontech) was used to amplify 5’ cDNA ends of the CD200
gene, using as starting material total RNA extracted from activated human peripheral
lymphocytes. First-strand synthesis was primed using an oligo (dT) primer. After reverse
transcriptase reached the end of the mRNA, it added several dC residues. The Smart II oligo,
whose terminal stretch of dG residues annealed to the tail of the cDNA, then served as an
extended template for RT. An antisense primer located in the middle of exon 3 was also used for
the 5’RACE PCR. After TA-cloning of the RACE products, 27 independent clones were picked
and sequenced. This technique revealed a major transcriptional initiation site located 58 nt
upstream of the translation start site and designated +1 (Fig. 7). These studies also revealed
alternative mRNA splicing in the human CD200 gene (see below), confirming previous reports
for murine CD200(75).

For primer extension analysis, the same source of RNA was primed with an antisense primer
Mapping of transcriptional start site for HuCD200 by 5'RACE

Figure 7. 5' RACE mapping of the human CD200 transcription initiation site. The nucleotide sequence of a portion of human CD200 5'-flanking region and the whole of exon 1 (box) is shown. A major transcription initiation site is located at 58 nt 5' upstream of the translational start site. X's represent mapping of a clone with transcriptional start site at this location.
located in exon 1 and reverse transcribed. A sequencing reaction using CD200 genomic DNA and the same primer as was used for RT, was performed. These studies detected a transcriptional initiation site 28 nt upstream of the translational start site (Fig. 8). This matched one of the transcriptional initiation sites detected by 5'RACE and represents the site closest to the translation start site.

An RNase protection assay was performed as described in the Materials and Methods. Data for this study are shown in Fig. 9, along with data using a commercial yeast RNA control (Ambion, USA). These data confirm the major transcriptional start site at -58nt.

2.4.3. Alternative mRNA splicing of the human CD200 gene:

Two fragments persisted in the agarose gel even upon nested PCR in 5'RACE. To examine whether this represented alternative mRNA splicing for CD200, RT-PCR was performed using a sense primer located at exon 1 and an antisense primer within exon 3. Two transcripts were detected in human brain, thymus, spleen, liver, bone marrow, small intestine, and peripheral lymphocytes (Fig. 10). The larger transcript was much more abundant than the smaller one in human brain only. Sequence analysis showed that the smaller transcript eliminates exon 2, resulting in a frame shift and premature translation termination, as reported for the murine CD200 gene(75).

Interestingly, a second AUG codon is located 120 nt downstream of this first stop codon, and in a perfect Kozak context. Theoretically this could be a site for reinitiation of translation, and recent evidence from our laboratory suggests physiological expression of a truncated CD200 product (CD200ₐ) does occur, with significant functional consequences.

2.4.4. Characterization of the 5'-flanking region:
Figure 8. Mapping of the human CD200 transcription initiation site by primer extension analysis. An oligonucleotide corresponding to the 3' end of exon 1 of human CD200 was hybridized to RNA extracted from activated peripheral lymphocytes (PBL) and extended with reverse transcriptase. A sequencing reaction using the same oligonucleotide primer was used as size marker. Yeast RNA was used as a negative control.
Figure 9: RNase protection assay to document transcriptional start site for CD200. mRNA was obtained from human PBL. Lane 1 used an RNA probe for human CD200 from −100 to +194 with an additional 106 bp of vector sequence to the Not1 cleavage site in the vector. Lane 2 is yeast RNA hybridized with the same probe with subsequent addition of RNase (negative control). Lane 3 is yeast RNA labelled with probe in the absence of RNase (positive control...probe size ~400bp). Estimated transcriptional start site from lane 1 (fragment hybridizing with size 252bp) is -58.
Alternate mRNA splicing of the human CD200 gene.

Figure 10. RNA samples from the following tissues are shown: 1-3, brain; 4-6, thymus; 7-9, spleen; 10-12, liver; 13-15, small intestine; 16-18, bone marrow; 19, no DNA.

The 5'/3' primer pairs used in PCR were as follows:
1,4,7,10,13,16: 5'exon 1, 3' exon 3;
2,5,8,11,14,17: nested primers, 5' exon 1, 3' exon 3;
3,6,9,12,15,18,19: 5' and 3' primers exon 3;

The lane shown as M: represents a 100 bp DNA ladder (GIBCO/BRL)

Note that only in brain tissue is there no significant expression of a smaller CD200 mRNA transcript.
a. Identification of transcription factor binding sites: several putative transcription factor binding sites are located in the 5'-flanking region of the CD200 gene described (Fig. 1), including:

- a TATA box surrogate (AGGAA(83)) 56 nt upstream from the major transcriptional initiation site;
- a GATA-1 (WGATAR(84)) site at nt -612;
- 2 Ap-2-like (CCCMNSSS(76)) elements located at nt -1390 and -200;
- 3 Putative Sp1 binding sites (KRGGCKRRK, GGGCGG(82)) at nt -1297, -790 and -635;
- Multiple TCF-1, bHLH regions;
- 7 potential binding sites for the Ets family (TTCC, GGAA (82)) are located between nt -252 and -52. Ets proteins regulate transcriptional initiation, and some members of this family cooperate in transcription with Ap-1(85).

Conspicuous by their absence were sites for NFkB, GAS, STATs, IRF-1, NFAT etc. This may be due to cloning of only a limited (1.1 kb) region of the promoter to date.

b. Repetitive sequences in this region: An antisense Alu repetitive sequence was identified from nt -1249 to -974. Alu sequences represent 5-6 % of the total human genome(86). Dinucleotide (dG-dA)$_n$ repeats were detected from nt -937 to -842 and from nt -562 to -516. The potential for polymorphism in this region, and its role in human disease, is under investigation.

2.4.5 Analysis of functional promoter activity of the CD200 gene:

To examine if the cloned 5'-flanking region of the human CD200 gene has constitutive promoter activity a promoter/reporter construct pGL2-1075/+39 (Fig. 12) was transiently transfected into Hela cells and dendritic cells (DC2.4). The latter is a polyoma-virus transformed murine dendritic cell line of C57BL/6 origin which was a kind gift from Dr.K.Rock (NIH,
Figure 11. Complete nucleotide sequence of the 5'-flanking region of the human CD200 gene. The beginning of the first exon is shown in **BOLD**, and the boundary denoting the commencement of the first intron for this gene is marked as the termination of **BOLD**. Nucleotides are numbered relative to the transcription initiation site (+1). Putative transcription factor binding sites and repetitive sequences are underlined. The amino acid sequence translated from the first exon is shown below the nucleotide sequence.
murine dendritic cell line of C57BL/6 origin which was a kind gift from Dr.K.Rock (NIH, Bethesda, MD, USA). A pGL2-control vector bearing an SV40 promoter/enhancer was used as a positive control, and the pGL2-Basic vector lacking both promoter and enhancer was used as a negative control. Cells were co-transfected with pRSV-β-gal to control for transfection efficiency. Results shown in Figs 12 and 13 confirm that this construct does indeed have constitutive promoter activity. Interestingly, since HeLa cells do not express detectable constitutive expression of CD200, these data might indicate a gene repressor for CD200 which is absent in the promoter construct used.

2.4.6. Genomic organization of human CD200:

The human CD200 gene is located on human chromosome 3(78). We found that the human CD200 5'-flanking region, together with exon1 and 2, completely matched a human chromosome 3 clone in the human database (accession number: AC068958), and documented the existence of a 7.7 kb intron between exon1 and 2. Combining our data with published results allows us to record the complete genomic organization of the human CD200 gene as shown in Fig. 14.

2.5 Discussion

Cell surface molecules of the integrin/immunoglobulin superfamily provide auxiliary signals to lymphocytes activated by engagement of antigen-specific receptors. Such co-stimulatory signals can regulate, amongst other functions, the production of cytokines which can in turn modulate cell:cell interactions. Studies from our laboratory have indicated that pre-transplant portal venous (pv) infusion of antigen-specific dendritic cells suppresses graft rejection(14), in association with regulation of cytokine production(87, 88).
Figure 12. Promoter activity of human CD200 gene assessed in Hela cells. A promoter/reporter construct, pGL2 -1075/+39, was generated and transiently transfected into Hela cells. A pGL2-control vector bearing the SV40 promoter/enhancer was used as a positive control, while a pGL2 Basic vector, lacking promoter and enhancer, was used as a negative control. Luciferase activity (mean±SD) was normalized to pRSV-β-gal and protein concentration.
Human CD200 promoter activity in dendritic cell line DC2.4

Figure 13. Promoter activity of human CD200 gene assessed in a mouse dendritic cell line (DC 2.4). The promoter/reporter construct pGL2-1075/+39 was transiently transfected into DC 2.4. Controls were as for Figure 12. Data show mean (+SD) of triplicate measurements.
Figure 14. Genomic organization of human CD200. E, exon; Int, intron; L, leader peptide; V, Ig V-like domain; C, Ig C-like domain; TY, transmembrane and cytoplasmic domain.

☐: untranslated region; ■: coding sequence
Using a PCR-based subtractive hybridization method we isolated a molecule, CD200, whose expression was altered following PV immunization(42, 47). We speculated that CD200 delivered a general and physiologically important regulatory signal to the immune system, an hypothesis which was supported independently by recent findings from Hoek et al in a CD200 "knockout mouse" which was found to have increased susceptibility to autoimmune disease(55). These findings suggest that understanding the regulation of expression of CD200 will play an important role in developing novel insight into the therapy of human disease.

In the present study, we have cloned and characterized the 5'-flanking region of the human CD200. The structure of the 5'-flanking region is interesting in several aspects. There is no canonical TATA box within the normally expected distance (25 to 30 bp) of any of the potential transcriptional start sites. A number of genes lack obvious TATA boxes, and in general, multiple transcription start sites exist in such a TATA-less promoter. TATA-less promoters have been divided into two classes. One class, found in constitutively expressed genes, consists of GC-rich promoters, which (see Smale, 1989(89)) usually contain several transcription start sites spread over a fairly large region and several Sp1-binding sites. The CD200 promoter region described lacks the consensus "initiator" sequence (PyPyA+INT/APyPy) described by Smale (89). Another class has no apparent TATA boxes and is not GC-rich. Many of these latter promoters are not constitutively active but rather are inducible during differentiation and development (see Sehgal, 1988(90)). The reported CD200 gene (above) features elements of both classes, which is consistent with reports that the expression of the CD200 is both constitutive(43) and inducible(49).

Despite the absence of a TATA box, a number of putative regulatory cis-acting elements exist in the 5'-flanking region of the human CD200. Two Sp1-binding sites were identified in the
5'-flanking region. Spl is one of the first cellular transcription factors to be identified and cloned in virtue of its binding to a G-rich motif in the SV40 early promoter. This protein binds to G-rich sequences present in a variety of cellular and viral promoters and stimulates their transcriptional activity (91). The interactions between Spl and Ets-like proteins also seems to play an important role in transcriptional activation when a TATA box is not present in the promoter region of a gene (92), and as noted earlier, seven potential binding sites for the Ets family were found in the proximity of the major transcriptional start site. The first member of the Ets gene family was discovered a decade ago, and subsequently a series of cellular Ets genes were isolated including Ets-1, Ets-2, Erg, Elk-1, Sap-1, PEA-3, PU.1, Fli-1, Pok/Yan, and Etv-1. Ets genes have been reported to play a role in T cell development and regulate cellular growth and differentiation (93). Sharrocks et al suggested that Ets-domain proteins function as either transcriptional activators or repressors and that their activities are regulated by signal transduction pathways, including the MAP kinase pathways (94). Two Ap-2 binding sites are also present in the 5'-flanking region of CD200. Ap-2 is a site-specific DNA binding protein that can activate transcription (95), and Ap-2 binding have been demonstrated in the cis-regulatory regions of several viral and cellular genes. It is interesting that no STAT sites or NFkB sites were identified in the promoter region sequenced. In keeping with data on the human CD80 promoter region (67) this does not negate the possibility that such sites exist in as yet unsequenced regions (more 5') of the currently described promoter region.

Two dinucleotide (dG-dA), tandem repeats (microsatellites) were identified in the 5'-flanking region of human CD200. Microsatellite loci can both gain and lose repeats through slipped strand mispairing during DNA synthesis, and indeed unit number variability in the repeats has been associated with the occurrence of specific genetic diseases. Knowledge of the functional
constraints imposed upon the repeats sheds light on their potential use as molecular clocks for monitoring genome evolution (96), but to date polymorphism in this region has not been investigated.

An antisense Alu repetitive sequence was also documented in the 5'-flanking region of the CD200 gene. Alu elements have amplified in primate genomes through a RNA-dependent mechanism, termed retroposition, and have reached a copy number in excess of 500,000 copies per human genome. These elements have been proposed to have a number of functions in the human genome, and have certainly have had a major impact on genomic architecture. In addition, some genetic diseases and malignancies have been reported to be associated with unequal homologous recombination between Alu repeats (97). Our data suggests that both (dG-dA), and Alu repeats may provide polymorphic linkage markers at the CD200 locus.

Transcription initiation sites were mapped using 5' -RACE, an RNase protection assay and primer extension methodology. One major transcription initiation site and several minor initiation sites were documented in the 5'-flanking region, a feature common in TATA-less promoters. We do not yet know if these potential multiple initiation sites account for the different splice forms of CD200 referred to earlier. We have also identified the previously unknown exon 1 of the human CD200 which contains 12 nucleotides encoding 4 amino acids. Comparison with the murine and rat homologues, revealed two amino acid differences from that reported for murine CD200 and three from rat CD200.

Promoter activity of the CD200 construct was confirmed in HeLa cells and a murine dendritic cell line, DC2.4 (see Figs. 12 and 13). Note that the failure to detect constitutive CD200 expression in HeLa cells, despite activity from the promoter construct, implies the existence of gene silencers for CD200 which lie outside of the cloned region. Furthermore, since the relative
activity of the CD200 promoter construct in DC2.4 cells exceeded that seen in HeLa cells, we assume there is a preferential tissue distribution for constitutive expression of CD200.

Based on our results from RT-PCR, alternative mRNA splicing of the human CD200 occurs mainly in lymphoid tissues but not in brain. In addition, although the alternative mRNA splicing results in a frame shift and premature translation termination, we note the existence of another downstream AUG start codon in a perfect Kozak context (-3 A and +4 G relative to the first translational start codon A). Kozak suggested that when the first codon is followed shortly by a terminator codon, creating a small ORF (5'-mini-cistron), the 40S ribosomal subunit can remain bound to the mRNA, resume scanning, and potentially reinitiate at another AUG codon downstream (98). We have recently confirmed that reinitiate of translation does indeed occur for CD200 (both mouse and human), resulting in expression of a truncated CD200 (CD200\textsubscript{t}), which is an antagonist to CD200 functional activity (Gorzynski et al, in press). This suggests that regulation of relative expression of CD200 and CD200\textsubscript{t} will also be important physiologically.

Mapping the promoter region of human CD200 has allowed us to discern the genomic organization of the human CD200 gene, and map a large (7.7kb) intron 1. In such a large intron, we speculate other regulatory DNA elements (enhancers or silencers) may exist.

Analysis of the human CD200 promoter region should allow us to gain insight into the molecular mechanisms by which this gene is regulated. The factors that regulate CD200 expression, relative to expression of costimulator molecules such as CD80/CD86 and CD40, may represent useful therapeutic targets (for transplantation, autoimmunity, etc).
CHAPTER 3

Summary and future research on regulation of CD200 gene expression
3.1 Summary:

The primary goal of this research project was to clone and characterize the 5' flanking region of the human CD200 promoter. The ultimate goal was to develop a detailed understanding of both the constitutive and inducible transcriptional regulation of expression of this molecule.

While we were the first report a cDNA clone for the full-length of murine CD200(42), the cloning and sequencing of human CD200 was reported several years earlier(78). Nevertheless, comparison of the reported DNA sequences of genomic human and murine CD200 suggested that the human sequence was incomplete, with additional 5' DNA yet to be documented. Work in the previous chapter has resolved this anomaly, and shown that indeed a previously undescribed exon 1 for human CD200 exists some 7.7 kb upstream of the previously named exon 1, and the region upstream of this “new” exon 1 contains the human CD200 promoter.

We have mapped by DNA sequencing, some 1100 bp of the human CD200 promoter, showing both by 5'RACE and RNase protection assays that the transcriptional start site is at position -58 (with respect to the ATG codon). It is worthy of note that primer extension assays detected this transcriptional start site also, but in addition several other minor sites, which may or may not be of significance. Multiple sites for binding of transcription factors were identified, including Ap-2, Sp1, Ets (which can interact with Ap-1 (82), TCF-1, bHLH and a GATA-1 (WGATAR) site. Seven potential binding sites for the Ets family (TTCC, GGAA) were found between nt -253 and -53, and it is known that Ets proteins regulate transcriptional initiation. The functional significance of these binding sites for known transcription factors remains to be explored.
There are a number of other features of interest in the DNA sequence we described. An antisense Alu repetitive sequence was identified from nt -1249 to -974. Alu sequences represent 5-6% of the total human genome (86). In addition, dinucleotide (dG-dA), repeats were detected from nt -938 to -843 and from nt -563 to -517. Polymorphism in such dinucleotide repeats in other genes of importance in immunoregulation (e.g. interferon gamma, IFNγ, and the costimulator molecule, CTLA4), have been investigated for their role in the genetic susceptibility to certain diseases (99, 100). The significance of the dinucleotide repeats in this region is still under investigation.

Finally, conclusive proof that the region described really does represent a promoter region for human CD200 was provided with functional studies, using luciferase-reporter constructs, which showed the 1.1kb region cloned did indeed contain constitutive promoter activity in cells capable of expressing CD200. This was best observed with endothelial cells (unpublished data), since, for technical reasons, we, along with other groups, have found it extraordinarily difficult to transfet fresh or cultured dendritic cells (the primary expression site for CD200). Sequential deletion constructs have yet to be performed which would identify the existence of critical promoter elements in the construct described in Chapter 2.

3.2 Future goals for this project:

3.2.1. The role of different transcription factors in regulation of CD200 expression:

In order to explore whether the transcription factor, a site of binding for which exists at this region is of importance in CD200 gene expression under basal or constitutive conditions, the following studies will be performed, initially in non-activated cells, and then in cells activated to increase expression of CD200 (by IL-4, LPS).
DNase 1 in vitro footprinting will be used initially to examine the transcription factor(s) responsible for CD200 expression under different conditions. Nuclear extracts from non-expressing cells, or control (or cytokine-stimulated) DC will be incubated with cDNA fragments containing the DNA region of interest with/without DNase 1 (0.01 to 0.5 Kunitz units). Protection in a subregion of the sequence is indicative of an interaction of a transcription factor with its DNA target sequence. In those experiments looking at activated cells, these studies will be performed using different times of cytokine stimulation, to characterize the tempo of production of the relevant transcription factors.

Further confirmation that protection in the footprint analysis does indeed reflect binding of an (inducible) transcription factor(s) will be sought using electromobility gel shift assays (EMSA), and using radiolabeled oligonucleotides representing the protected region. Using the same extracts as used for footprint analysis, we anticipate that induction of binding to the labeled oligonucleotide will be seen in comparison to extracts of control cells. 10ug of nuclear extracts will be pre-incubated with 2ug of double-stranded poly (dI-dC) (Pharmacia Biotech) prior to addition of 2ng of the radiolabeled probe (sp. act. approx. 50,000 cpm/ng). After 30 min incubation (rt) in 200 mM HEPES (pH7.8), 10mM EDTA, 10mM DTT (final volume 10ul) samples are analysed on 6% non-denaturing polyacrylamide gels. The specificity of binding will be documented using a 100-fold excess of cold oligonucleotide for this region (preincubation for 10 min prior to addition of radiolabeled probe).

Finally, we will explore whether the binding seen reflects that of previously characterized transcription factors (STATs, NFkB etc) by supershift analysis using transcription factor specific antibodies. As an example, in such studies 10ug nuclear protein will be incubated with 1ug
specific antibody on ice for 1 hr prior to addition of radiolabeled probe. Specificity in the
supershift assay will be examined by competition with an excess of oligonucleotide containing
the relevant transcription factor consensus sequence or a mutated oligonucleotide (lacking this
motif).

3.2.2. Comparison of promoter regions of CD40, CD80, CD86 and CD200:

At the outset it was argued that understanding the control of expression of CD200 might
help shed light on the regulation of relative expression of CD40, CD80, CD86 vs CD200 in
APC. This has fundamental importance in immunology, since there are numerous data now
suggesting that increased expression of the three former are implicated in the induction of
inflammatory reactions., while our laboratory has argued that increased expression of the latter
(CD200) counters this effect. With our newly described characterization of the human CD200
promoter, we can begin to address this question. An interesting experimental approach will be to
explore the effect of reciprocal translocation of regions of e.g. the CD40 promoter and CD200
promoter on the inducibility of these molecules in DC and their subsequent activities as APC.

3.2.3. Regulation of differential expression of full-length CD200 vs CD200-

During this research, we observed by Northern analysis expression of at least 2 mRNAs
for CD200 in material isolated from lymphohaematopoietic tissue (spleen, thymus, bone marrow,
liver), but not brain-this was reported independently by others also(75). Sequence analysis
confirmed that the smaller mRNA band corresponded to a splice variant of CD200, missing exon
2. This is associated with an early, in frame, stop codon in the mRNA(75).

However, 120bp downstream of this stop codon there lies an in-frame second consensus
Kozak sequence. Translation initiation at this location would result in expression of a truncated CD200 protein (CD200\textsubscript{r}). This truncated molecule would lack an intra-molecular S-S bond, and an N-glycosylation site conserved in the extracellular NH\textsubscript{2} domain across rat, mouse and human CD200 molecules. Our laboratory has recently shown that CHO cells expressing a cDNA for CD200\textsubscript{r} block immunosuppression by CHO cells expressing full-length CD200 (unpublished). Thus in vivo regulation of mRNA splicing, or translation of CD200/CD200\textsubscript{r}, or exogenous delivery of CD200\textsubscript{r}, following construction of an immunoadhesin as done for CD200 itself, may have profound effects on immunoregulation in vivo.

To begin we will assess the control of relative expression of CD200/CD200\textsubscript{r} by cytokines. We have already documented that heterogeneity of DC is associated with differential expression of CD80/CD86 and CD200(81). Expression of these molecules is in turn regulated by endogenous cytokines. We have found that LPS stimulated, macrophage-derived, cytokines augment expression of CD200 (RMG-unpublished). In order to examine a role for cytokines in regulation of mRNA splicing, and/or translation of CD200/CD200\textsubscript{r}, fresh splenic DC will be incubated with the cytokines IFN\textgamma, IL-1 or IL-4, alone, or in combination, for 24hrs. Thereafter cells will be stained with FITC or PE-coupled mAbs to CD80/CD86/CD200/CD200\textsubscript{r} or, as control, DEC205. mRNA expression for GAPDH (control gene) or CD200/CD200\textsubscript{r} will also be examined. We predict that expression of CD200\textsubscript{r} will correlate with CD80 expression (increased in rejection), and inversely with CD200 expression.

3.2.4. Regulation of mRNA stability as a means of controlling CD200 expression levels:

All of the data to date has considered mRNA levels measured under steady state conditions. No attempt has been made to ask whether changes in expression of CD200 mRNA
expression results from altered induction (increased transcription) or from decreased degradation (altered stability). This becomes a key area to address, since it fundamentally changes the way we think about the manner in which changes in CD200 might be produced to induce immunoregulation. As a first step, the mRNA half-life for CD200 in resting cells (DC) will be measured, followed by analysis of this same half life after induction of increased expression with cytokines. No changes in half-life imply that a “pure” induction of transcription occurs to increase CD200 expression. A decreased half-life (with our existing evidence for increased translational expression of CD200!) following cytokine addition implies that mRNA stability is reduced, while transcription is much increased. In contrast an increased half life might lead us to suspect that increased transcription was not responsible for the increased mRNA expression induced by cytokines, but rather that their site of action was elsewhere (in DNA flanking regions controlling RNA stability). We would proceed to map these regions using deletional constructs, much in the fashion used to map the essential promoter region of CD200.
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