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Induction of CD40 ligand expression in chronic myeloid leukemia cells

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1. Abstract

Chronic myeloid leukemia (CML) is a proliferative disorder in the myeloid linage of the hematopoiesis. It is characterized by the translocation of the long arms of chromosome 22 and 9 that leads to the formation of the Philadelphia chromosome and its fusion protein BCR-ABL. BCR-ABL is a constitutively expressed tyrosine kinase, which leads to uncontrolled proliferation and survival. The first line treatment for CML is tyrosine kinase inhibitors. However, some patients develop resistance and new alternatives are warranted.

Gene therapy using adenoviral gene transfer of the Th1 stimulator CD40L has shown promising results in previous studies conducted at the Uppsala University Hospital. In the present study, we aim to establish CD40L therapy for CML. We have evaluated gene transfer to CML cells using two types of gene vehicles as well as a protein transfer model using a genetically engineered bystander cell line. The results demonstrate that CML cells are resistant to Adenovirus serotype 5 vectors, but susceptible for gene transfer using a modified adenovector with a serotype 35 fiber and knob. The protein transfer model that has shown stable results in B cell leukemia showed conflicting results in the present study. The Ad5/35 vector was selected to evaluate the effect of CD40L on CML cells and its microenvironment. The CD40L gene was cloned into this vector system and virus production has begun.

In conclusion, CML cells are susceptible for gene transfer using an Ad5/35 vector. The human CD40L gene has been cloned into this vector and virus production is ongoing.

2. Table of contents

3. List of abbreviations

Ad5	Adenovirus serotype 5
APC	Antigen Presenting Cell
B-CLL	B Cell-Chronic Lymphocytic Leukemia
BCR	B Cell Receptor
CAR	Coxsackie Adenovirus Receptor
CD40L	CD40 Ligand
CML	Chronic Myeloid Leukemia
CsCl	Caesiumchloride
DCs	Dendritic Cells
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
ffu	Focus-forming unit
GM-CSF	Granulocyte Macrophage-colony Stimulating Factor
IFN- γ	Interferon γ
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharides
MHC I	Major Histocompatibility Complex Class I
MHC II	Major Histocompatibility Complex Class II
MQ	Milli-Q
NK-cells	Natural Killer cells
PBS	Phosphate Buffered Saline
RBC lys	Red Blood Cell lysis
TCR	T Cell Receptor
Th cell	T helper cell
TKI	Tyrosine Kinase Inhibitors
TLRs	Toll Like Receptors
TNF	Tumor Necrosis Factor

4. Background

4.1. The Immune System

The immune system is our defense against foreign substances such as microbes or macromolecules and it is commonly divided into innate immunity and adaptive immunity. The innate immunity is our first defense against infectious microbes and because it is already in place before the infection occurs they can respond rapidly to the microbe. Innate immunity consists of different cell types such as dendritic cells (DCs), macrophages, neutrophils, eosinophils, basophils and natural killer cells (NK-cells) but also of the complement system, signaling molecules and barriers such as the skin and the mucosa. The adaptive immunity needs more time to activate and respond to infections. Adaptive immunity consists of the antibody producing B cells and different subtypes of T cells. The adaptive responses often occur as a consequence of innate reactions because innate immune cells release factors that stimulate the adaptive cells. Vice versa, antibodies produced by the adaptive system are bound by innate cells to further enhance their responses to microbes [1].

4.2. Activation of lymphocytes

When a microbe enters the body it will encounter different phagocytic immune cells like macrophages and DCs residing in the mucosa. The phagocytic immune cells have several different toll like receptors (TLRs) by which they can recognize different common patterns on the microbe for example lipopolysaccharides (LPS). When the microbe binds TLRs and is phagocytosed by for example a DC, the DC is activated and migrates to a lymph node where it starts to break down the microbe to small peptides. The small peptides are then presented on the major histocompatibility complex class II (MHC II) to $CD4^+$ T cells. T cells have specific receptors, the T cell receptor (TCR), which recognizes MHC complexes with bound peptide. The TCR on one cell is highly specific and different from TCRs on other cells. This makes us able to detect a huge number of peptides from different microbes that we may encounter. The T cells that recognize the peptide presented on the DC are activated and start to proliferate. This activated cell is called T helper cell (Th cell) and it will provide help to activate for example cytotoxic T cells (CTLs), B cells and macrophages. DCs are able to cross present exogenous antigens via MHC class I as well. CTLs recognize peptides presented on MHC class I and the DCs will, hence, stimulate both Th cells and CTLs. For full activation, CTL activation requires help from Th cells that produces important cytokines such as IL-2. Th cells are as well important to stimulate B cells.

Antibodies produced by the activated B cells will bind to the microbes, which will then be either lysed by the complement system or phagocytosed by phagocytes. If the microbes are viruses or intracellular bacteria they will infect normal cells. Since all nucleated cells express MHC class I microbal peptides will be presented on MHC I to CTLs, which are able to kill the infected cells by induction of apoptosis [1]. Tumor cells can be eradicated by the same

mechanisms as the immune system eradicates virally infected cells. Therefore, it is of importance to understand the pathways of T cell activation to develop a potent immunotherapy for cancer.

For a T cell to get activated it needs two signals. The first signal comes from the interaction between the MHC with bound peptide and the TCR and the second signal comes from the interaction between costimulatory molecules such as CD28 on T cells and B7 on APCs. When the T cell is activated it starts expressing CD40 ligand (CD40L), which will further enhance the activation by binding to CD40 on APCs. CD40L is one of the most potent stimulators of DCs and will enhance the activation of so called Th1 cells that preferably help CTLs. B cells also needs two signals to respond to protein antigens. The first signal is the antigen binding to the B cell receptor (BCR) and the second is the interaction between CD40 on B cells and CD40L on T cells (Figure 1) [1].

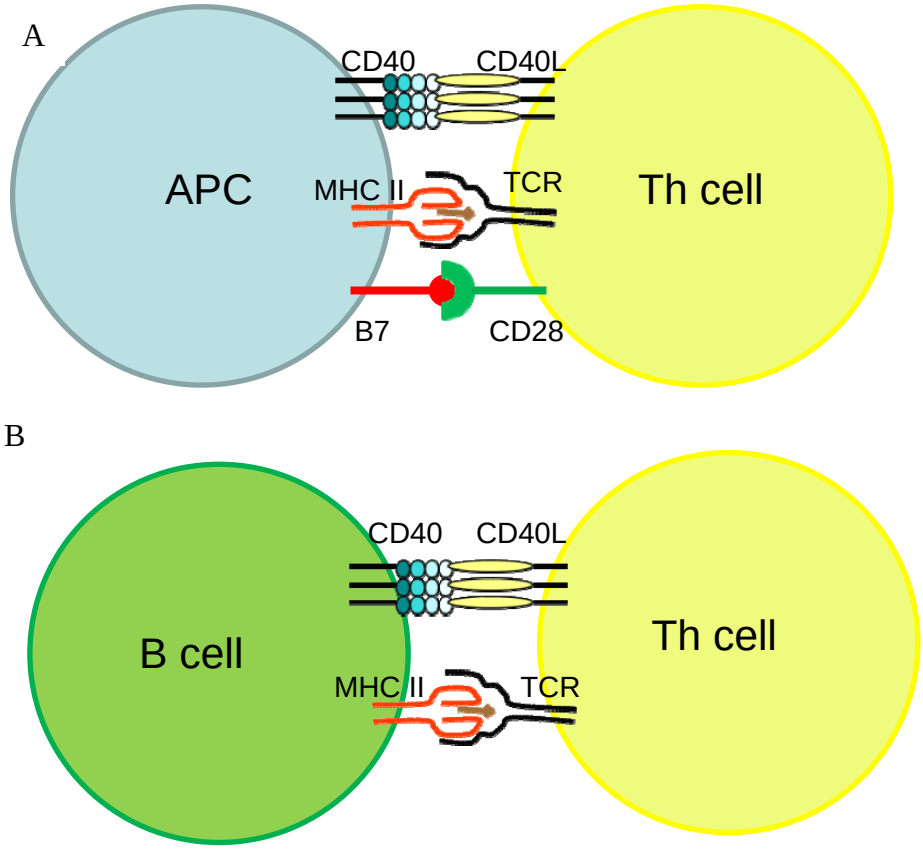


Figure 1. A: T helper (Th) cell gets activated by an antigen presenting cell (APC) that leads to upregulation of CD40L. B: B cell activation via CD40L by an activated T helper cell.

4.3. CD40/CD40L

CD40L or CD154 is a type II transmembrane protein which belongs to the tumor necrosis factor (TNF) gene superfamily [2]. It can function as a single molecule but is most effective as a trimer when it binds to trimerized CD40 [3]. CD40L was first thought only to be expressed on activated $CD4^+$ T cells but it has been shown that it is expressed on a variety of other activated cell types such as basophils, eosinophils, macrophages, NK-cells, B cells, plateletes, DCs, endothelial cells and epithelial cells [2]. For example platelets were thought to only be involved in hemostasis but it has been shown that they also have an important role as initiator of inflammation, partly by their release of prestored soluble CD40L [4].

CD40 is a member of TNF receptor gene superfamily and is mainly expressed on the same type of cells as CD40L with a couple of additions such as keratinocytes, fibroblast and carcinomas [2]. CD40 is constitutively expressed, but its expression can also be enhanced by cytokines such as interferon γ (IFN- γ), interleukin 1 (IL-1), IL-3, IL-4, TNF-α and granulocyte macrophage-colony stimulating factor (GM-CSF) [2].

CD40/CD40L interactions are very important in T cell dependent B cell activation; it initiates activation and proliferation of naïve B cells, isotype switching of Ig, formation of germinal centers, which leads to affinity maturation and the formation of memory B cells [1,2]. The importance of this interaction can be seen in persons with the X-linked hyper IgM syndrome, a sever immune deficiency, which is caused by a non functional CD40L gene and leads to recurring bacterial and viral infections and early lethality [5,6].

CD40/CD40L interaction has a very important role in the activation of DCs. When CD40L binds to CD40 on the DC it promotes a prosurvival signal in the DC and upregulates the expression of antigen presenting molecules such as MHC II, B7.1 and B7.2. DC activated by CD40/CD40L also increases their production of inflammatory cytokines especially IL-12 that promotes Th1 type of immunity [7].

Besides enhancing B cell activation and DC function, the CD40/CD40L interactions, has been shown to trigger apoptosis in carcinomas expressing CD40. The mechanism behind the increased apoptosis in tumor cells is still unknown since CD40 lacks intracellular death domains, however, the increased cell death is thought to involve Fas ligand and/or other death receptor ligands [8].

4.4. Adenoviral vectors

Adenovirus is a nonenveloped icosahedral virus with double stranded deoxyribonucleic acid (DNA). On the surface of the capsid the viral particle has a penton fiber and fiber knob, which is used to anchor it to the target cell (Figure 2) [12]. Over 50 different serotypes of adenovirus have been identified so far and they can be divided in to six subgroups, A to F. Adenovirus 5 (Ad5) belongs to group C and binds to the coxsackie adenovirus receptor (CAR) to attach to the target cell. Ad35 belongs to group B and use CD46 to attach to the target cell [13]. When the DNA has been delivered in to the cell the first gene to be transcribed is E1A. This gene activates the transcription of the early genes, which are involved in viral replication and immune evasion. The adenoviruses also have late genes, which are transcribed later (as the name suggest) and consist mostly of proteins involved in capsid formation [12]. The most common infections caused by adenoviruses are respiratory tract infections, conjunctivitis (pinkeye), hemorrhagic cystitis and gastroenteritis [12].

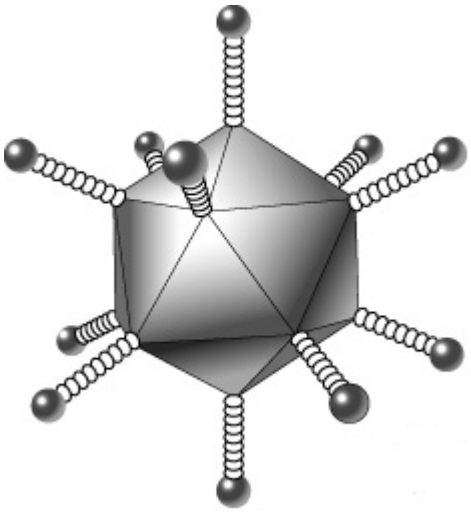


Figure 2 adenovirus. The structure of an adenoviral particle with the fibers and fiber knobs attached to the capsid. This picture was taken from http://commons.wikimedia.org/wiki/File:AdenovirusCat.png

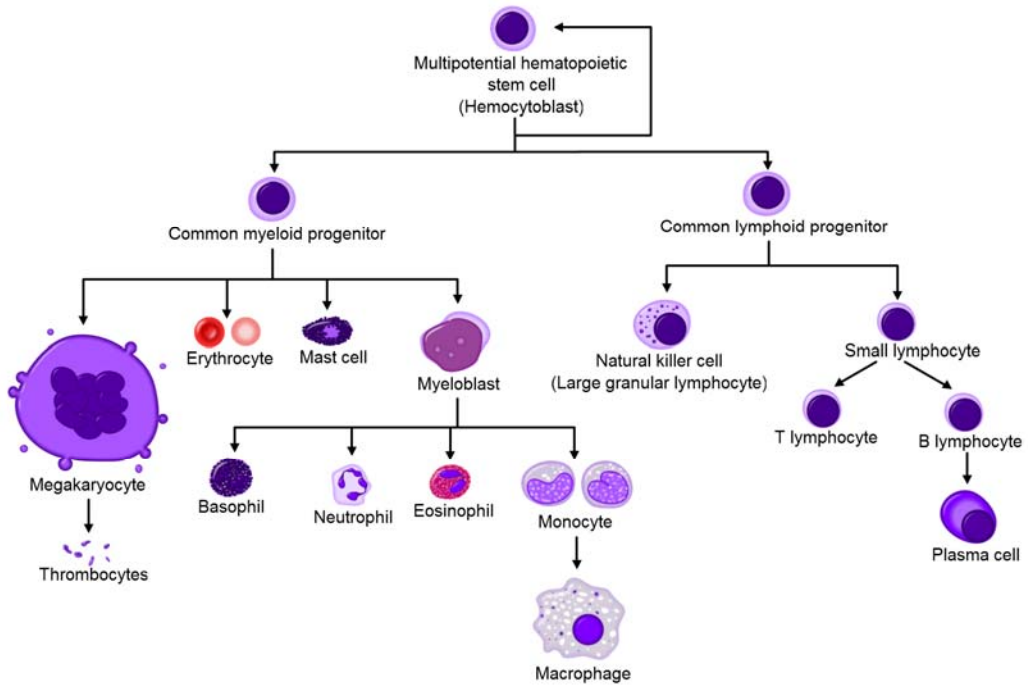


Figure 3 Hematopoeisis. The differentiation of different linages of immune cells. Picture taken from http://commons.wikimedia.org/wiki/File:Hematopoiesis_simple.png

4.5. Chronic myeloid leukemia

Chronic myeloid leukemia (CML) is a proliferative disorder in the myeloid linage of the hematopoiesis. The oncogenetic transformation occurs in a stem cell, but the transformed cells continue to differentiate and the patients have excess mature myeloid cells in their blood (Figure 3) [9]. Of all leukemia patients 10% have CML and the median age at diagnosis is in Sweden above 60 years. CML is characterized by the translocation of the long arms of chromosome 22 and 9 that leads to the formation of the Philadelphia chromosome and its fusion protein BCR-ABL (Figure 4) [9,10]. About 7% of all CML patients do not have the Philadelphia chromosome and the median survival years for those patients are much lower than patients with the Philadelphia chromosome [9]. ABL is a tyrosine kinase and normally it regulates several downstream target such as c-Myc, Jun and Akt, which play a big role in regulation of proliferation and survival of the normal cell. The fusion protein BCR-ABL is constitutively expressed, which leads to uncontrolled proliferation and survival of the malignant cells carrying the translocation. This gives the transformed cells a growth advantage that leads to the pathogenesis of CML [11]. The first line treatment for CML is tyrosine kinase inhibitors (TKI) that inhibits the tyrosine kinase BCR-ABL and thereby the increased proliferation and survival of transformed cells [11]. The introduction of TKIs has revolutionized the treatment for CML. However, not all patients are helped by TKIs because of resistance to the drug. Moreover, patients in a late phase of their disease often have a suboptimal response to the TKIs. Therefore, new therapies for patients that develop resistance and patients in late disease stages are needed. Immunotherapy could be a feasible option for these patients.

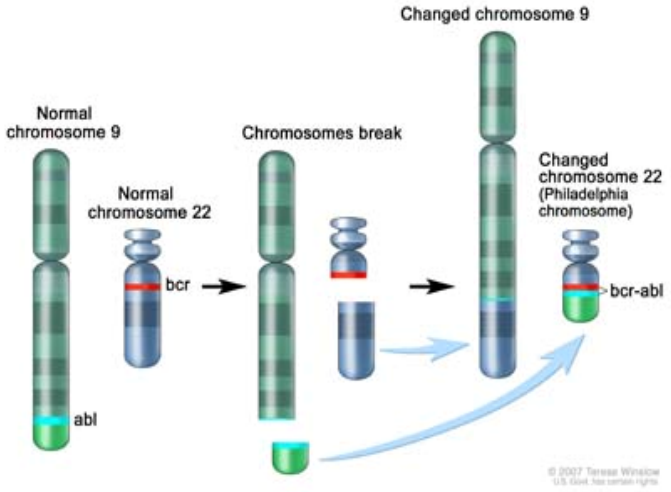


Figure 4 Philadelphia chromosome. Chromosome 9 and 22 before and after translocation. Picture taken from http://visualsonline.cancer.gov/preview.cfm?imageid=7153&fileformat=jpg

4.6. Modified adenoviral vectors for gene delivery

Modified adenoviral vectors are commonly used as gene delivery vehicles in gene therapy especially for treatment of cancer. A serotype 5 vector has been used to deliver the CD40L gene into the tumor to evoke anti-tumor immune responses. The vector has been evaluated in mice, dogs and in a human clinical trial [14]. To restrain the expression of the transgene to the transduced cells only the adenoviral vectors used for gene delivery are rendered replication-defective by deletion of the E1 gene. E1A is the first gene transcribed and it activates the transcription of the other viral genes required for viral replication. To amplify viral particles before transduction modified cell lines, which have the missing gene, are used.

Ad5 have been used to mediate gene delivery in to malignant hematopoietic cells but with limited success and that is due to the fact that malignant hematopoietic cells do not express the CAR, which is needed for Ad5 to attach to the cell. The fiber and fiber knob of Ad5 can be replaced with the fiber and fiber knob from Ad35 (Ad5F35) that attach to CD46, which is expressed on malignant hematopoietic cells [15,16].

4.7. Aim

The aim of this project was to induce expression of CD40L in CML cells as a potential immunotherapy for CML.

The specific goals were to:

1) Evaluate vector transfer of the CD40L gene into CML cells
2) Evaluate protein transfer via a CD40L engineered bystander cell line

5. Materials and Methods

5.1. Recombinant Adenovirus production and purification

5.1.1. Primary cells and cell lines

Blood was drawn from newly diagnosed CML patients. Blood samples were centrifuged at 250 x g for 5 minutes and the plasma was removed. To obtain immune cells and tumor cells from the samples red blood cells were lysed. Red blood cell (RBC) lysis buffer (NH_4Cl, $KHCO_3$, EDTA, pH 7.4) was added to the blood and incubated for 5 minutes at room temperature. After incubation blood samples were centrifuged at 250 x g for 5 minutes. For complete lysis of red blood cells more RBC lysis buffer was added. The samples were washed in phosphate buffered saline (PBS) two times and then cryopreserved in fetal bovine serum (FBS; Invitrogen™, Carlsbad, USA) containing 10% dimethyl sulfoxide (DMSO; Apoteket AB, Uppsala). The human bladder cancer cell line KU1919 was used as a bystander cell line for co-culturing with CML cells in RPMI 1640 media (Invitrogen™) containing 10% FBS, 1% penicillin and streptomycin (Invitrogen™) and 0.1% sodium pyruvate (Invitrogen™). For production of the adenoviral vector the human embryonic kidney cell line 293 (CruCell, Leiden, Netherlands) or the human embryonic retinoblast cell line 911 (ATCC, Rockville, USA) was used. Cell lines for adenoviral vector production were cultured in DMEM (Invitrogen™) containing 10% FBS, 1% penicillin and streptomycin and 0.1% sodium pyruvate.

5.1.2. Adenoviral vectors

The pAdEasy-1 (Figure 5) [16] with fiber tail domain replaced with Ad5 and fiber shaft and knob domain replaced with Ad35 containing soluble human CD40L (Ad5F35 shCD40L) were kindly provided by Lisa Christiansson at Uppsala University and Ad5F35mock were kindly provided by Berith Nilsson at Uppsala University.

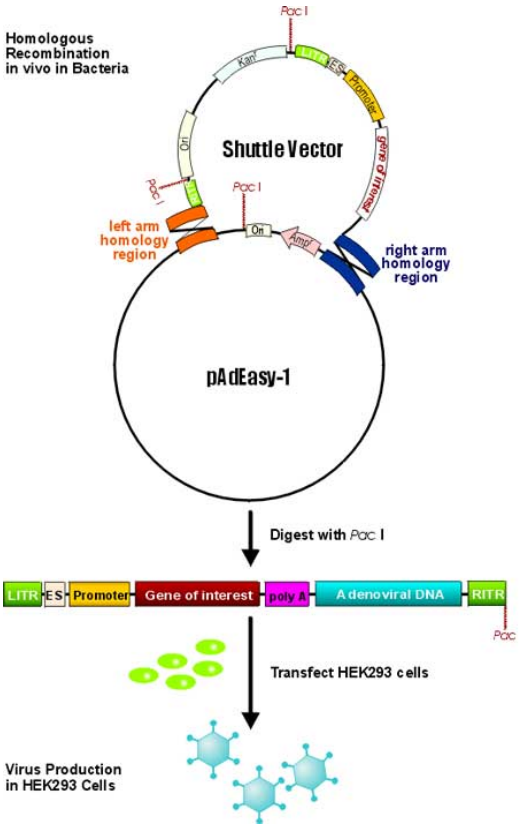


Figure 5. Shuttle vector and pAdEasy-1. Picture taken from http://www.biocompare.com/images/bc/006/ArticleImages/adez1.JPG

5.1.3. Linearization and concentration of plasmid DNA

One day prior to cell transfection the recombinant adenoviral vector was linearized with PacI (New England Biolabs, Ipswich, UK) for 16 hours at 37°C and heat inactivated for 20 minutes at 65°C. A small sample of the vectors were analysed on a 1% agarose gel to verify linearization. The linearized vector was precipitated with 1/10 volume of 3 M sodium acetat, pH 5.2 and 2.5 volume of ice-cold 99% ethanol and incubated for 20 minutes at -80°C. After incubation the tubes were centrifuged at 15700 x g, 4°C for 20 minutes. The supernatant were removed and the pellet washed with 70% ethanol and centrifuged again at 15700 x g, 4°C for 20 minutes. The supernatant was removed and the pellet was air dried for 10 minutes and then dissolved in Milli-Q water.

5.1.4. Cell transfection

Before transfection Opti-MEM (Invitrogen™) mixed with purified plasmid were drop wise added to Opti-MEM with lipofectamine (Invitrogen™) and incubated for 30 minutes in room temperature. After incubation DMEM was added and then the tubes were centrifuged at 250 x g for 5 minutes and the supernatant removed. The medium from 293 cells with a confluence of 60-70% cultured in 60 mm Petri dishes (Sarstedt, Numbrecht, Germany) was removed and the plasmid/liposome complex was added. The plates were incubated in 37°C, 7% CO_2 for 4 hours. Following incubation, DMEM with 5% FBS, 1% penicillin and streptomycin was added and then the plates were incubated in 37°C, 7% CO_2 for 9 days before harvest.

5.1.5. Harvest of adenoviral particles

Cells and medium were collected using a cell scraper (Corning Incorporated, New York, USA) and transferred to a tube and centrifuged at 110 x g for 5 minutes. The supernatant was removed and the cell pellet resuspended in 1 ml 0.1 M Tris-HCl, pH 8.0. For collection of viral particles the cells were frozen in liquid nitrogen, thawed in a 37°C water bath and vortexed vigorously; this was repeated 4 times in total. The cells were then centrifuged at 12000 x g for 8 minutes. The supernatant containing the virus particles was either used directly or stored in 4% sucrose in -80°C freezer.

5.1.6. Amplification of viral particles

Virus particles were amplified in three amplification rounds. In the first amplification virus containing supernatant was mixed with DMEM containing 10% FBS, 1% penicillin and streptomycin and 0.1% sodium pyruvate and then added to 293 or 911 cells with a confluence of 70-80% in 10 cm Petri dishes (Sarstedt) and incubated for 2 hours at 37°C, 7% CO_2. After incubation DMEM was added and the plate was incubated again. When at least 50% of the cells had detached from the plate or become round (after approximately 7-10 days) the virus particles were harvested as described above.

For the second round of amplification virus containing supernatant from the previous step were mixed with DMEM containing 10% FBS, 1% penicillin and streptomycin and 0.1% sodium pyruvate. The mixture were added to 293 or 911 cells with a confluence of 70-80% in two 15 cm Petri dishes (Sarstedt) and then incubated for 2 hours at 37°C, 7% CO_2. After incubation DMEM was added and the plate was incubated again. When at least 50% of the cells had detached from the plate or become round (after approximately 7-10 days) the virus particles were harvested as described above.

For the third amplification step virus containing supernatant from the previous step were mixed with DMEM containing 10% FBS, 1% penicillin and streptomycin and 0.1% sodium pyruvate and then added to 293 or 911 cells with a confluence of 70-80% in five 25x25 cm plates (Corning Incorporated) and then incubated for 2 hours at 37°C, 7% CO_2. After incubation DMEM was added and the plates were incubated again. When at least 50% of the cells had detached from the plate or become round (after approximately 7-10 days) the virus particles were harvested as described above.

In all amplification steps one plate with cells not infected with virus was kept to be able to compare the morphology of infected and uninfected cells.

5.1.7. Purification of viral particles

Virus containing supernatant from the last amplification step was mixed with DMEM containing 10% FBS, 1% penicillin and streptomycin and 0.1% sodium pyruvate. A Caesiumchloride (CsCl) gradient was created in an Ultra clear tube (Beckman Coulter, Brea,

USA) by pipetting CsCl with the density of 1.2g/ml on top of CsCl with the density of 1.4g/ml. The diluted virus was pipetted to the gradient and the tubes were centrifuged in an ultracentrifuge (Ultrapro 80, Sorvall) at 35000 rpm at 4°C without brakes. Two bands will form and the lower contains the viruses. We did not see any band so we did not go any further in the purification steps.

5.2. Transfer of CD40L into CML cells via a bystander cell line

5.2.1. Membrane staining

To be able to distinguish between KU1919 cells and CML cells in a flow cytometry staining the KU1919 cells were fluorescently labeled with the kit PKH2 green fluorescent cell linker kit (Sigma-Aldrich, Missouri, USA). The adherent KU1919 cells were detached with cell dissociation buffer (Invitrogen™), washed with RPMI 1640 media containing 10% FBS, 1% penicillin and streptomycin and 0.1% sodium pyruvate and centrifuged at 250 x g for 5 minutes. Two tubes were prepared one containing diluent A from the kit together with the KU1919 cells and the other tube containing $4x10^{-6}$ M PKH2 dye. The contents of the two tubes were mixed thoroughly and incubated at room temperature for 25 minutes. To stop the reaction FBS was added and the tube was incubated for 1 minute. The labeled KU1919 cells were washed three times in RPMI 1640 media and centrifuged at 250 x g for 10 minutes to remove excess label. Cells where then cultured at 37°C, 5% CO_2 for 24 hours.

5.2.2. Transduction of bystander cell line

Two hundred thousand KU1919 membrane labeled cells were transduced with 400 focus-forming units (ffu)/cell with Ad5 containing human CD40L (Ad5hCD40L), which was kindly provided by Lisa Christiansson at Uppsala university and Ad5 not containing CD40L (Ad5mock), which was kindly provided by Lina Liljenfeldt and Linda Sandin at Uppsala University. The transduced cells were incubated in RPMI 1640 media containing 10% FBS, 1% penicillin and streptomycin and 0.1% sodium pyruvate at 37°C, 5% CO_2 for 1 hour before washing with RPMI 1640 media and plating on a 6-well plate (Sarstedt). After transduction cells were cultured at 37°C, 5% CO_2 for 24 hours before coculture with CML cells.

5.2.3. Coculture

Cryopreserved primary cells from CML patients, where erythrocytes had been removed before cryopreservation, were thawed and washed. Dead cells were removed with a dead cell removal kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufactures instructions. Briefly thawed cell suspensions were centrifuged at 250 x g for 5 minutes. The cells were then resuspended and incubated with dead cell removal microbeads for 15 minutes in room temperature. MS positive selection columns (Miltenyi Biotecs) were placed in a MACS separator (Miltenyi Biotecs) and rinsed with binding buffer. The cells were added to the columns where labeled dead cells where retained in the column and unlabeled live

cells passed through. The columns were then rinsed with additional binding buffer. Live cells were counted and $1x10^6$ cells were cocultured with bystander KU1919 cells, either untransduced, transduced with Ad5Mock or Ad5CD40L for 24 hours.

5.2.4. Flow cytometry

Cocultured cells were collected, centrifuged at 250 x g for 5 minutes and divided in to three FACS tubes. To one of the tubes no antibody was added (negative control) and in the other two CD40L-PE (Biolegend, San Diego, USA) and anti-mouse IgG1 (isotype control, Biolegend) were added respectively. The tubes were incubated for 15 minutes at room temperature and cells were washed in PBS before analyzing CD40L expression by flow cytometry (FACS Calibur, BD, LSRII, BD).

6. Results

6.1. Adenoviral transduction of CML cells

Previous results from our lab has shown that CML cells are resistant to transduction with Ad5 containing the human CD40L gene (Figure 6).

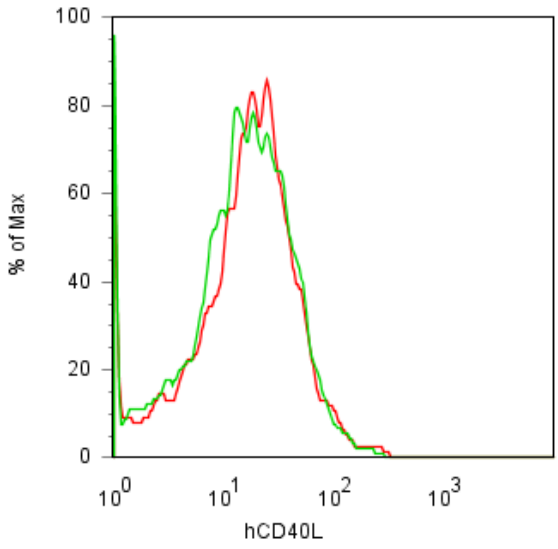


Figure 6. Red: Ad5hCD40L transduced CML patient cells. Green: Untransduced CML patient cells. Kindly provided by Lisa

6.2. Transfer of CD40L into CML cells via a bystander cell line

To find other means for transfer of CD40L into CML cells we cocultured CML cells with a bystander cell line expressing CD40L. The bystander cell line KU1919 was fluorescently labeled and transduced with AdhCD40L or AdMock. As an extra control some cells were also left untransduced. The transduced bystander cells were then cocultured with CML cells.
Coculturing CML cells with the bystander cell line expressing CD40L increased the expression of CD40L in CML cells. CML cells cocultured with bystander cells transduced with mock vector did not have increased CD40L expression (Figure 7).

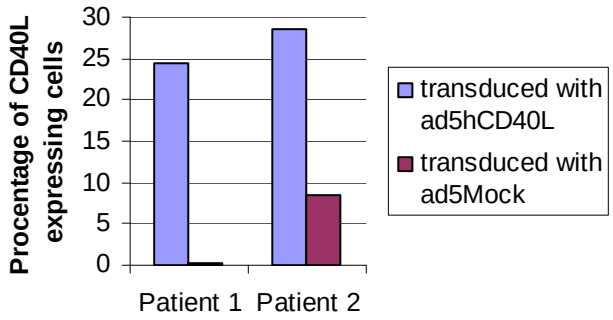
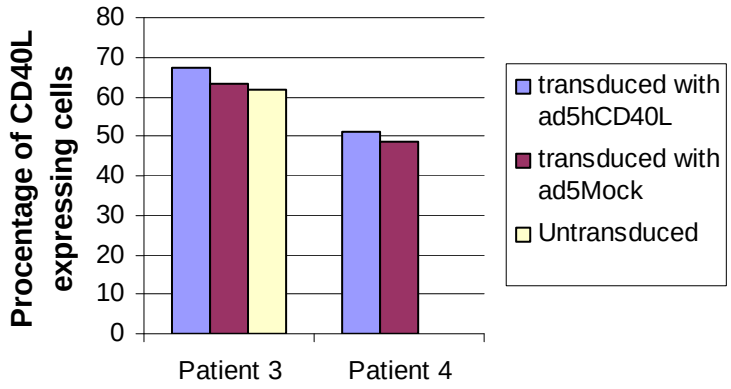


Figure 7. CD40L expression was evaluated by flow cytometry in CML cells from two patients cocultured with bystander cells. Bystander cells were transduced with Ad5CD40L or Ad5Mock.

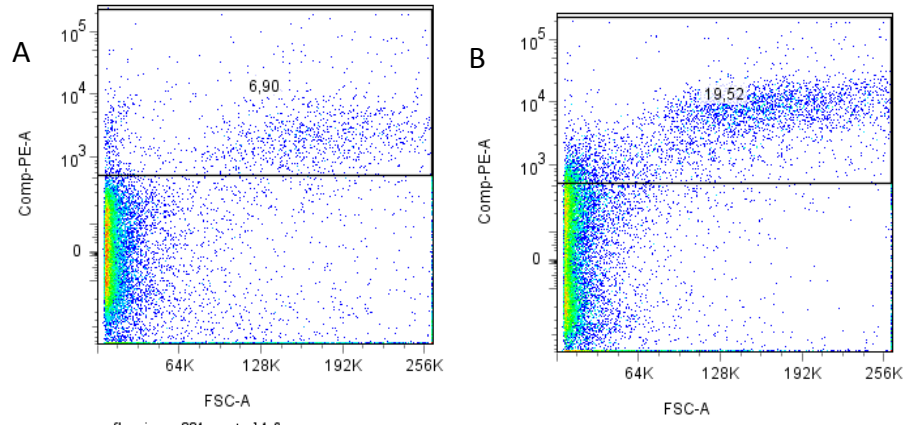
The experiment was repeated (Figure 8) with two new CML patient samples. This experiment showed expression of CD40L on all CML cells independently if they had been cocultured with Ad5CD40L, AdMock transduced or untransduced bystander cells.



Figur 8. CD40L expression as shown by flow cytometry in CML cells from two patients cocultured with bystander cells. Bystander cells were transduced with Ad5CD40L, Ad5Mock or untransduced.

To evaluate the CD40L expression of untransduced KU1919 membrane labeled (Figure 10) and not membrane labeled (Figure 11) KU1919 cells were stained with anti human CD40L

antibody. Flow cytometry analysis showed CD40L expression in untransduced KU1919 cells both on membrane labeled and unlabeled cells.



Figur 10. . CD40L expression in KU1919 cells which were membrane labeled and untransduced as shown by flow cytometry. A has been stained with isotype control anti-mouse IgG1 and B with CD40L-PE.

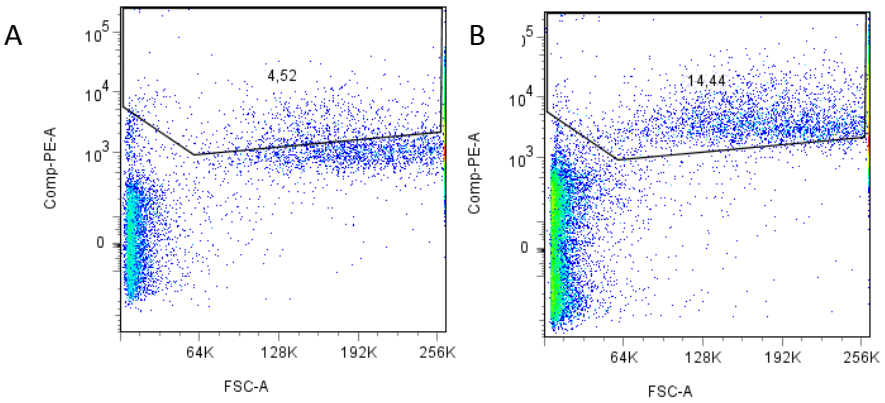


Figure 11. CD40L expression in KU1919 cells which were untransduced and not membrane labeled. A has been stained with isotype control anti-mouse IgG1 and B with CD40L-PE.

To evaluate a new bystander cell line for coculture 293 were stained for CD40L expression. (Figure 12). Analysis by flow cytometry showed no CD40L expression on 293 cells so we decided to continue with these cells.

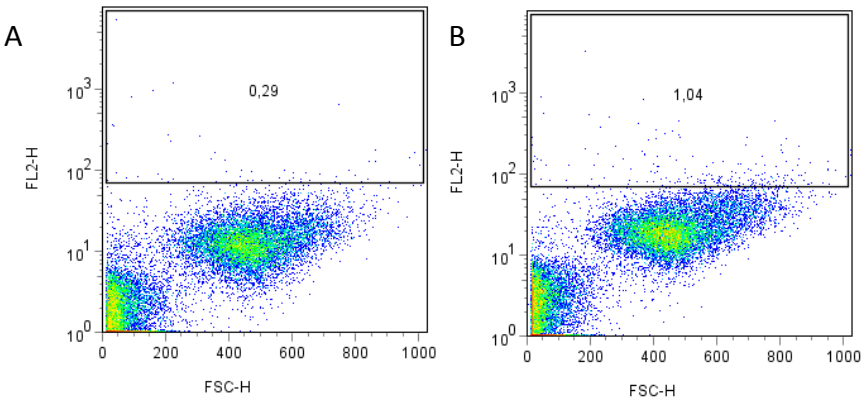


Figure 12. CD40L expression in 293 cells which were untransduced and not membrane labeled. A has been stained with isotype control anti-mouse IgG1 and B with CD40L-PE.

293 cells were membrane labeled, transduced with Ad5CD40L or Ad5Mock and cocultured with CML cells. CD40L expression on CML cells was increased both on cells cocultured with Ad5CD40L transduced cells as well as Ad5Mock transduced cells (Figure 13). Because of the difficulties to control the bystander protein transfer model we continued to evaluate the possibility to use a direct gene transfer to CML cells.

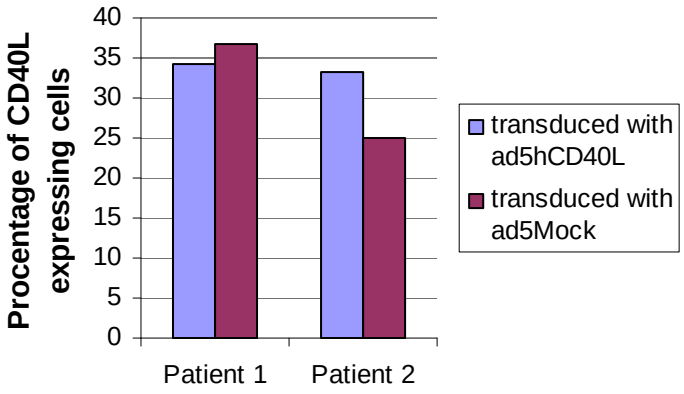


Figure 13. CD40L expression in CML cells from two patients cocultured with bystander cells. Bystander cells were transduced with Ad5CD40L or Ad5Mock.

6.3. Transduction of CML cells with a modified adenoviral vector

Results by the coworkers in the lab showed that a modified Ad5 vector with the fiber knob and shaft exchanged for the serotype 35 expressing GFP was able to transduce CML patient cells.

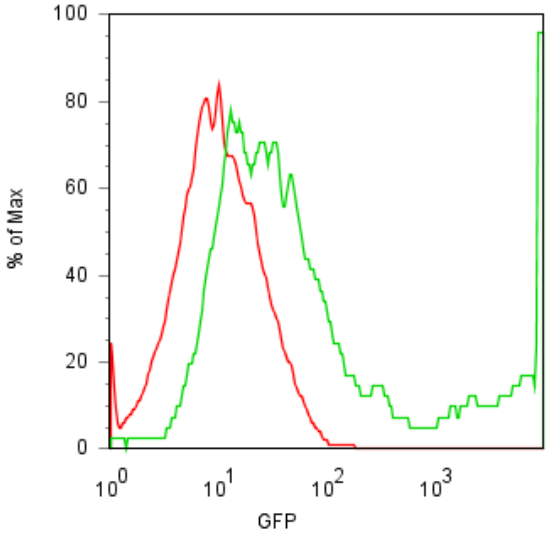


Figure 14. Green: Ad5F35GFP transduced CML patient cells. Red: Untransduced CML patient cells. Kindly provided by Lisa Christiansson.

6.4. Recombinant Adenovirus production and purification

Because of the successful gene transfer using the Ad5F35- GFP vector we decided to produce Ad5F35shCD40L and Ad5F35Mock. The adenoviral particles were produced in 293 and 911 cells with three amplification rounds before purification. In each amplification round a difference in morphology between treated and untreated cells (as a sign of viral production and replication) was seen after 7 to 10 days. Cells were harvested and lysed and supernatant was used to infect new producer cells. The change in morphology was seen 1-2 days earlier in cells producing the Ad5F35shCD40L compared to cell producing the Ad5F35Mock. When purifying the adenoviral particles no viral particle band was formed indicating we were not able to produce neither Ad5F35hCD40L nor Ad5F35Mock. New cultures are ongoing.

7. Discussion

As a potential immunotherapy for CML we wanted to induce the expression of CD40L in CML cells. Transduction of malignant cells with Ad5hCD40L has been evaluated as an immunotherapy in other cancers [17]. Previous results from our lab and others show that CML cells cannot be transduced with Ad5 carrying the CD40L gene [16]. To transfer CD40L into CML cells we cocultured them with Ad5hCD40L transduced bystander cells. This method to transfer CD40L into malignant cells has been used by Biagi et al to transfer CD40L into B-CLL cells in a clinical trial [18]. When coculturing the CML cells with engineered bystander cells we detected an upregulation of CD40L expression on CML cells cocultured with Ad5hCD40L transduced bystander cells, but not in CML cells that had been cocultured with

Ad5Mock transduced bystander cells. When repeating the experiment, however, we saw approximately the same amount of CD40L expression in CML cells cocultured with bystander cells transduced with Ad5hCD40L Ad5Mock or untransduced cells. To be able to separate the CML cells and the tumor cells in a FACS-plot we fluorescently labeled the membrane of the bystander cells. We hypothesized that the membrane labeling would somehow upregulate the CD40L expression on all bystander cells and to evaluate this we stained for CD40L expression on KU1919 cell that were membrane labeled but not transduced with CD40L. These cells expressed CD40L. Moreover, when analyzing the CD40L expression on KU1919 cells that had not been membrane labeled we detected the same CD40L expression as on membrane labeled cells and we concluded that the increased CD40L expression was not dependent on membrane labeling. In earlier experiments KU1919 has not been shown to have CD40L expression. CD40L is induced on multiple cells upon stress so it is likely that something in the culturing procedure made them express CD40L.

To avoid CD40L expression on untransduced cells we investigated if another cell line could be utilized. We next evaluated the 293 cell line that did not expressed CD40L on untransduced cells. When the coculturing experiment was repeated with the 293 cells we again detected expression of CD40L in CML cells cocultured with both Ad5hCD40L and Ad5Mock transduced bystander cells. We hypothesize that the enhanced CD40L expression on CML cells has nothing to do with CD40L transfer from the bystander cell line but instead that the CML cells reacts to the bystander cells (which are allogeneic) and thereby get activated and express CD40L. This could potentially be a good approach to develop a CD40L expressing CML cell vaccine. But because of the difficulties to evaluate the cellular mechanisms we decided to try direct gene transfer by using the Ad5F35 vector instead.

We and others [16] has previously shown that CML cells can be transduced with the modified adenoviral vector Ad5F35 expressing the GFP gene. To evaluate if Ad5F35 could be used to transfer also the CD40L gene into CML cells we wanted to produce an adenoviral vector carrying the hCD40L gene. As a control we wanted to produce an Ad5F35 not expressing a transgene (AdMock). Ad5F35shCD40L and Ad5F35Mock were produced according to a protocol used in our lab for production of Ad5 vectors. The viral particles are produced in producer cells and harvested when the cells start to change morphology (due to accumulation of viral particles in the cells), they become round and deattach from the culture dish. According to the protocol the time until the cells start to change morphology in Ad5 production is 3-4 days, but in our production this took 7-10 day. This could be because Ad5 and Ad35 attach to different cell surface molecules and thereby the infection of the producer cell may take longer time for the Ad35 than the Ad5. It may as well be a problem with our producer cell line.

After three rounds of amplification viral particles can be purified on a cesium gradient. When we tried to purify our viral particles we did not get a band containing the particles. The

reason for not being able to produce viral particles is not known. It is possible that the concentration of viruses was too low. This theory fit with the fact that the cells did not change their morphology until day 7-10. The change in morphology may have been induced by other causes than viral load. For example, CD40L can have a toxic effect on the cells which could make them change their morphology because of cytotoxicity. That would explain why Ad5F35shCD40L changed their morphology before Ad5F35Mock. To solve the problem of virus production we have started new production cultures. We will evaluate another producer cell line and also different time points of virus harvest.

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