The Development of a CRISPR/Cas9 System with Nanoblades in Order to Study IBD-Related Genes in an *in vitro* Model

Arpie Bakhshian

Biomedical and Health Sciences

a. Rationale

In 2015, an estimated 1.3% of all adults in the United States (3 million) reported being diagnosed with Inflammatory Bowel Disease (IBD) (Abraham et al., 2009). IBD represents a group of intestinal disorders (Crohn's disease and Ulcerative colitis) that cause prolonged inflammation of the gastrointestinal tract. If not treated properly, this inflammation can result in life threatening complications, such as anemia, malnutrition, liver disease, and even colon cancer (CDC). Both Crohn's Disease and Ulcerative colitis are characterized by intestinal epithelial injury, including mucosal erosion, ulceration, cryptitis, and crypt abscess formation (Garcia-Carbonell et al., 2018).

According to genome association studies, there are mutations in specific genes correlated to the development of IBD in humans (Abraham et al., 2009). In order to devise potential treatment possibilities for Crohn's Disease and Ulcerative Colitis, additional research is required to comprehend the underlying processes involved in the manifestation of IBD, including the role of the intestinal epithelium, and the role of various genetic factors. In order to study different genes and their involvement in IBD, the goal of this experiment is to deliver a CRISPR/Cas9 system into a primary cell line involved with IBD. Previously, success in delivery of CRISPR/Cas9 systems *ex vivo* by either transfection or transduction into primary cell lines has been limited (Mangeot et al., 2019). Therefore, in an attempt to further understand the processes and cellular mechanisms in IBD, this experiment will study the efficacy of a novel method of delivery of CRISPR/Cas9 known as "Nanoblades" into Intestinal Epithelial Cells and Embryonic Stem Cells (targeted primary cells). In these primary cells, the CRISPR/Cas9 system will target the XBP1, CX3CR1, and TAP1 genes, which are susceptible genes in IBD.

Nanoblades, viral delivery vectors based on the Murine Leukemia Virus, deliver ribonucleoprotein cargo in a transient and rapid manner *in vivo* and *in vitro* without the delivery of a transgene, unlike other delivery methods. Nanoblade preparation is relatively simple and inexpensive and can be easily implemented in any laboratory equipped for cellular biology.

The Intestinal Epithelium at the interface between the intestinal microbiome and the lymphoid tissue associated with the gastrointestinal system plays a critical role in shaping the mucosal immune response (Abraham et al., 2009). Intestinal Epithelial Cells are part of a primary cell line that act as a physical barrier against excessive entry of bacteria and other antigens from the intestinal lumen into the circulation. In mouse models of IBD, several types of epithelial dysfunction have been shown to cause intestinal inflammation. These include defects in epithelial-cell development or proliferation, barrier function, cell-matrix adhesion, endoplasmic reticulum (ER) stress response, and epithelial restitution after injury (Abraham et al., 2009). This suggests that Intestinal Epithelial Cells play a major role in the immune response in the intestine, and in the development of IBD. Therefore, this experiment will focus on the use of Intestinal Epithelial Cells known as Caco2 Cells as target cells. Single-nucleotide polymorphisms (SNPs) will be induced in these cells by the delivery of a CRISPR/Cas9 system at specific loci to further study the inflammatory response in IBD.

Nanoblades will also be tested in other primary cells for genome editing. Targeting the same genes, Nanoblades will be used in Embryonic Stem Cells (ESC's). ESC's represent a major interest in research and gene therapy due to their proliferative potential and their capacity to colonize (Mangeot, et al., 2019). By using Embryonic Stem Cells as the target cells, if transfection is successful, they can be differentiated into Intestinal Organoids already containing mutations mirroring mutations in patients with IBD; these organoids would have the Epithelial Cell Line found in intestine, which would make them ideal to study inflammation in the intestine.

Consistent with predictions inferred from epidemiologic studies, genome association studies have identified genes associated solely with Crohn's disease and Ulcerative colitis, including the XBP1, CX3CR1, and TAP1 genes (Abraham et al., 2009). The XBP1 gene has been found to be vital in the ER stress response, which is necessary for the proper functioning of the immune system and in the cellular stress response. In a previous study, it was found that induction of ER stress *in vivo* by cell-specific XBP1 deletion would cause organ-specific inflammation, providing an explanation for the initiation of proinflammatory diseases (Kaser et al., 2008). It was also discovered that intestinal inflammation originated solely from XBP1 gene abnormalities in Intestinal Epithelial Cells (Kaser et al., 2008) .

The Tap 1 gene is also known for its susceptibility in IBD, especially in Ulcerative colitis. Located within the Major Histocompatibility Complex (MHC) class-I region, there are transporter associated with antigen processing (TAP) genes which encode for proteins involved in the transfer of antigenic peptides through the endoplasmic reticulum prior to their association with MHC class-I molecules (Miller et al., 2015). The function of these molecules is to display peptide fragments of proteins from within the cell to cytotoxic T cells; this will trigger an immediate response from the immune system against antigens displayed with the help of an MHC class I protein (Jongsma et al., 2013).

According to a study conducted by Leonardi et al., CX3CR1+ mononuclear phagocytes (MNPs) have been identified as being essential for the initiation of innate and adaptive immune responses to intestinal fungi. CX3CR1+ MNPs express antifungal receptors and activate antifungal responses in a Syk-dependent manner. In this study, genetic ablation of CX3CR1+ MNPs in mice led to changes in gut fungal communities and led to severe colitis that was rescued by antifungal treatment. In Crohn's disease patients, a missense mutation in the gene encoding CX3CR1 was identified and found to be associated with impaired antifungal responses. These results unravel a role of CX3CR1+ MNPs in mediating interactions between intestinal microbiota and host immunity at steady state and during inflammatory disease (Leonardi et al., 2018).

Therefore, to further understand susceptible genes in IBD, the XBP1, TAP1, and CX3CR1 genes will be studied in depth in Intestinal Epithelial Cells (Caco2 cells) and in Intestinal Organoids derived from Embryonic Stem Cells using a novel delivery technology known as Nanoblades. Since there is a limited understanding of the causes of inflammation in IBD, by inducing mutations in these genes using efficient and novel technology, we may be able to further understand the processes involved in intestinal damage, Intestinal-Epithelial-Cell damage, and the development of Crohn's disease and Ulcerative colitis, which could potentially lead to future applications of these methods in Immunology.

b. Research Question

Can Nanoblades be effectively used to transduce primary cell lines such as Intestinal Epithelial Cells and Embryonic Stem Cells in order to induce mutations in genes susceptible to IBD?

Hypothesis

Nanoblades can be used to transduce primary cell lines such as Intestinal Epithelial Cells and Embryonic Stem Cells in order to induce mutations in genes susceptible to IBD.

Expected Results

The expected results would show that Nanoblades caused Single Nucleotide Polymorphisms (SNPs) in Intestinal Epithelial Cells and Embryonic Stem Cells, and this would be shown through cleavage in the gel electrophoresis conducted. In order to display cleavage, two bands would need to be visible in one lane of a gel: the first band would represent the DNA of the cells that are not transduced and did not have any SNPs. The second band would represent the SNP in the targeted gene, meaning that the DNA is different for each strand. Since the two strands of DNA are different in sequence, they are also different in size and they appear at different intervals on the gel. Cleavage would mean that in IBD, Nanoblades can be used to transduce primary cell lines, which would help in the development of treatments and even a cure.

c. Procedure

This experiment will use both Nanoblades, a novel technology, and Lentiviral Vectors (LVs), a relatively common form of retrovirus used for gene editing in cells, in order to compare the efficacy of both types of gene editing in target cells. In this experiment, the target cells are Intestinal Epithelial Cells (Caco2 Cells) and Embryonic Stem Cells: primary cell lines. These two types of cells from primary cell lines are easy to maintain and are used for many avenues of scientific research. Although common, LV transfection is known to be incompatible with primary cell lines, which poses a problem. Therefore, in an attempt to efficiently induce mutations in these human cells, both Nanoblades and Lentiviral Vectors (LVs) will be used to target the genes XBP1, CX3CR1, and TAP1 to create random SNPs—random deletions across the gene sequence, usually in the beginning or middle of the gene's sequence.

In order to create CRISPR/Cas9 Plasmids for both Nanoblades and LVs, the guide RNAs (sgRNAs) for each gene targeted will be selected in order to be incorporated onto the CRISPR/Cas9 plasmids. The sgRNA for XBP1 is sgRNA21, the sgRNA for CX3CR1 is sgRNA40, and the sgRNA for TAP1 is sgRNA33. The sgRNAs will be annealed and inserted onto pre-made CRISPR Cas-9 Plasmids. Next, they will be transformed into bacteria and plated on agar plates. To make sure the ligation is successful, colonies of bacteria will be collected randomly and digested to check if they will be positive for the ligation of the sgRNA. Gel electrophoresis will be run to check for colonies positive for the ligation, and this will be determined by a specific band that is visible on the gel.

The development of Nanoblades relies on the viral structural Gag polyprotein (a basic part of retroviruses), which multimerizes at the cell membrane and is sufficient, when expressed in cultured cells, to induce the release of Virus-like particles (VLPs) into the cell. When Gag is co-expressed together with a fusogenic viral envelope, pseudo-typed VLPs are produced that lack a viral genome but still retain their capacity to fuse with target cells and deliver the Gag protein into their cytoplasm (Mangeot et al., 2019). In order to create Nanoblades for this experiment, HEK-293T cells will be transfected with plasmids coding for Gag::Cas9, Gag-Pro-Pol, a sgRNA for each gene, viral envelopes, and Calcium phosphate as a transfection reagent. These transfected plasmids will then form into vesicles inside these T-cells which will then be released out of the cell membrane, resulting in the production of fusogenic VLPs being released in the culture medium, creating Nanoblades.

Lentiviral Vectors (LVs) will represent a mode of comparison to Nanoblades in this experiment. LVs are a common type of retrovirus used for transfection, but they do not have the transient mode of transport that the Nanoblades have. In order to create LV's in this experiment, HEK-293T cells will be once again transfected with three separate transfection systems: a transfer vector plasmid which contains genetic sequences necessary for the vector to infect the target cell and for the transfer of the therapeutic (or reporter) gene, retro-transcriptase and integrase packaging plasmids (8.9), which are required for the packaging of structural proteins, and an envelope (VSV-G) to provide stability to the vector by bringing together the particles that will be made by the packaging plasmid. Also transfected will be plasmids of interest, which are

the CRISPR/Cas9 plasmids annealed with different sgRNA depending on the gene, and Calcium phosphate as a transfection reagent.

By depositing the viruses created by the packaging cells and plasmids into cell media with Caco2 Cells, a transduction of the viruses into the Caco2 cells will be conducted with LV's, a control, and Nanoblades. For transduction of the Embryonic Stem Cells, the same procedures will be used as with Caco2 Cells. After each transduction, a genome extraction will be performed. The genomic extraction will be conducted by following a standard protocol for human or animal tissue in cultured cells. After the genome extraction, a Nanodrop Quantification will be conducted in order to determine the concentration of DNA obtained. The Nanodrop Quantification will be performed using a spectrometer machine that will measure the concentration of nucleic acids. After determining the concentrations of each sample, all samples will be diluted to 20 ng/uL in order to be used for the Polymerase Chain Reaction (PCR) and the SURVEYOR Nuclease Assay.

A PCR will then be conducted in order to determine if the WASp (control gene) and CX3CR1 gene will be transduced, and the percentage of transduction. Since two sgRNAs will be required to create SNPs in both these genes, the cleavage would be visible in the PCR alone. On the other hand, the XBP1 gene and the TAP1 gene require the use of a SURVEYOR Nuclease Assay because they both need only one sgRNA in order to induce SNPs. Each gene will be at a different temperature in order to have maximum efficiency.

In cells that will be transfected with a pair of sgRNAs to mediate a genomic (micro)deletion or inversion, indel mutations or SNPs can be detected by the SURVEYOR nuclease assay. This is an enzyme mismatch cleavage assay, or endonuclease, used to detect single base mismatches or small insertions or deletions (indels). In this experiment, the SURVEYOR will be used in order to detect SNPs in Caco2 Cells and Embryonic Stem Cells. In order to detect SNPs, the SURVEYOR program requires the heating and separation of the two strands of DNA, which are then cooled and reannealed in order to detect mutations on one strand of the DNA, or SNP. In order to see the results from this assay, an agarose gel electrophoresis will be performed to visually detect cleavage (two bands of DNA). If cleavage is present, it is evident that there are SNPs in the targeted cells, and the delivery of the CRISPR/Cas9 system is successful, and cleavage efficiencies between Nanoblades and LVs will be compared.

Risk and Safety

To perform the experiment there is some risk involved, but most is prevented by taking safety precautions. Potential risks could be due to handling bacteria used during the creation of plasmids, the chemicals involved in certain protocols, and cells during maintenance and transfection. However, the risks could be reduced by wearing gloves, goggles, a lab coat, working under a hood, and by following institutional rules.

Data Analysis

The data from this experiment will be analyzed using the percentages of cleavages form the SURVEYOR Assay that will be conducted. By determining the percentages of cleavage in Intestinal Epithelial Cells and Embryonic Stem Cells, statistical significance tests can be conducted in order to determine if Nanoblades were an effective method of transduction as opposed to Lentiviral Vectors. If this should be true, Nanoblades can be used in research to study the effects of mutations in certain genes in IBD and potentially in other primary cell lines.

d. Bibliography

Abraham, Clara, and Judy H. Cho. "Inflammatory Bowel Disease." *New England Journal of Medicine*, vol. 361, no. 21, 2009, pp. 2066–2078., doi:10.1056/nejmra0804647.

Andersen, Mads Hald, et al. "Cytotoxic T Cells." *Journal of Investigative Dermatology*, vol. 126, no. 1, 2006, pp. 32–41., doi:10.1038/sj.jid.5700001.

Endert, Peter Van. *Antigen Processing: Methods and Protocols*. Humana Press, 2019.

Garcia-Carbonell, Ricard, et al. "Elevated A20 Promotes TNF-Induced and RIPK1-Dependent

Intestinal Epithelial Cell Death." *Proceedings of the National Academy of Sciences*, vol. 115, no. 39, 2018, doi:10.1073/pnas.1810584115.

Kaser, Arthur, et al. "XBP1 Links ER Stress to Intestinal Inflammation and Confers Genetic Risk for Human Inflammatory Bowel Disease." *Cell*, vol. 134, no. 5, 2008, pp. 743–756., doi:10.1016/j.cell.2008.07.021.

Lin, Jonathan H., et al. "Endoplasmic Reticulum Stress in Disease Pathogenesis." *Annual Review of Pathology: Mechanisms of Disease*, vol. 3, no. 1, 2008, pp. 399–425., doi:10.1146/annurev.pathmechdis.3.121806.151434.

Mangeot, P.E., Risson, V., Fusil, F. *et al.* Genome editing in primary cells and in vivo using viral-derived Nanoblades loaded with Cas9-sgRNA ribonucleoproteins. *Nat Commun* **10,** 45 (2019) doi:10.1038/s41467-018-07845-z

Mitra, Sahana, and Hyung Don Ryoo. "The Unfolded Protein Response in Metazoan Development." *Journal of Cell Science*, vol. 132, no. 5, 2019, doi:10.1242/jcs.217216.

Ran, F. Ann, et al. "Genome Engineering Using the CRISPR-Cas9 System." *Naure Protocols*, vol. 8, no. 11, 24 Oct. 2013, pp. 2281-308, DOI:10.1038/gt.2013.43.

Shaffer, A.l, et al. "XBP1, Downstream of Blimp-1, Expands the Secretory Apparatus and Other Organelles, and Increases Protein Synthesis in Plasma Cell Differentiation." *Immunity*, vol. 21, no. 1, 2004, pp. 81–93., doi:10.1016/j.immuni.2004.06.010.

Wagnerova, A., and R. Gardik. "In Vivo Reprogramming in Inflammatory Bowel Disease." *NCBI*, DOI:10.1038/gt.2013.43. Accessed 13 Nov. 2019.

Wells, James M., and Jason R. Spence. "How to Make an Intestine." *The Company of Biologists*, vol. 2014, no. 141, DOI:10.1242/dev.097386. Accessed 13 Nov. 2019.

Post Summary

No Changes Were Made.