Deletion of the GAS Regulatory Sequence of IFN- γ Induced Gene (ICAM-1) by Using CRISPR Cas9 to Decrease the Jak3/Stat3 Dependent IDO Expression to Repress Tumor Cells Development

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Abstract

IDO is a checkpoint molecule that regulates T-cell proliferation. Since IDO contributes to T cell death and creates an environment for cancer cells to grow, it is necessary to regulate IDO expression. Previous studies demonstrated that IFN-gamma-induced genes would indirectly activate IDO expression through the Jak/3 pathway, and ICAM-1 is one of the IFN-gamma-induced genes. This work investigates the effect of ICAM-1 on IDO expression and the effect of 2 GAS segment knockouts on ICAM-1 expression. CRISPR-Cas9, heat shock transformation, PCR, and restriction digestion are used to knock out the target sequence (GAS) and insert the successful knocked-out gene into the E. coli. RT-PCR is utilized to measure the expression level of ICAM-1 and IDO after the introduction of successfully knocked-out genes. The possible result of this work presents that both GAS gene knockouts are thriving; the new gene without GAS – 2787 bps expresses less ICAM-1 protein than the gene without GAS – 115 bps, and ICAM-1 does not affect IDO expression at all. Results of this work indicate that the GAS sequence that is farther away from the ICAM-1 RBS site might decrease the ICAM-1 expression level less than does GAS sequence that is nearer to RBS, and ICAM-1 might not have any direct impact on IDO expression, which means that tumor cell development cannot be inhibited by regulating ICAM-1 expression. Future studies could research more on functions of IFNgamma-induced genes other than ICAM-1 and their relationships with IDO expression.

Keywords: GAS sequence, IDO, IFN-γ, ICAM-1, Jak3/Stat3 pathway, CRISPR, gene knockout

I. Introduction

T cells are white blood cells that inhibit tumor cells by identifying them through attaching T cell receptors with major histocompatibility complex (MHC) of antigens on the surface of tumor cells [1]. Several checkpoint molecules modulate the immune response of T cells by either activating or inhibiting T cell proliferation, such as Indoleamine 2, and 3-dioxygenase 1 (IDO) [2]. Indoleamine 2, 3-dioxygenase 1 (IDO) is a rate-limiting enzyme that catalyzes the degradation of essential amino acids, and it plays a significant role in immunosuppressive mechanisms, such as it contributing to T cell death and suppressing T cell responses[3]. The expression of IDO from tumor cells generates an immunosuppressive tumor microenvironment to help the tumor to grow, so downregulation of IDO expression would be necessary to prevent cancer from developing [1].

Researchers found that the concentration of Interferon-y (IFN-y) has a positive relationship with IDO gene expression and IFN-y triggers the transcription of IDO through the Jak3/Stat3 pathway. As a start of the Jak3 pathway, IFN-γ is secreted by activated T cells as an immune response [4]. IFN-y then binds to receptors IFN-y R1 & IRN-y R2 on T cell surfaces which further leads to the phosphorylation of Jak (Janus) tyrosine kinases. After the phosphorylation, Jak kinases start to collect and phosphorylate a Stat3 dimer. The activated Stat3 dimer leaves the receptor and enters the nucleus to bind with the Gamma interferon activation site (GAS) of IFN-γ induced genes, which causes their transcription. The IDO gene is thus activated by IFN-γ transcription factors [5].

The IFN-y induced gene indirectly activated the expression of IDO. Since ICAM-1 is one of the 200 IFN-y mediated genes, ICAM-1 might activate the expression of IDO gene. The IFN- γ-mediated transcription is initiated by the promoter of the intercellular adhesion molecule (ICAM-1). IFN- γ activated site, the Gamma interferon activation site (GAS), is located throughout ICAM-1, which means that there are multiple GAS segments on ICAM-1 promoter (4.0 kb). Research presented that different GAS segments have different affinity in binding with Stat dimers. In a ICAM-1 gene, a GAS segment that locates 115 base pairs away from the translation initiation site and presents a strong affinity in binding with Stat dimers [6]. However, a GAS segment that locates farther away from the initial translation site with a 2787 base pairs distance shows lower affinity of binding with Stat1

dimers. All of the GAS sequences have a core sequence of 5′-TTNCNNNAA-3′ [7].

My research question is that can we edit the GAS sequence of IFN-γ induced genes to lower the level of IDO expression thus preventing tumor cells from developing? Since GAS segments are part of the promoter sequence of IFN- γ induced genes and the transcription activity of that gene requires correct GAS sequences, we can utilize CRISPR-Cas9 to knock out the GAS gene sequences and replace them with a nonfunctional sequence by using homologous recombination [8].

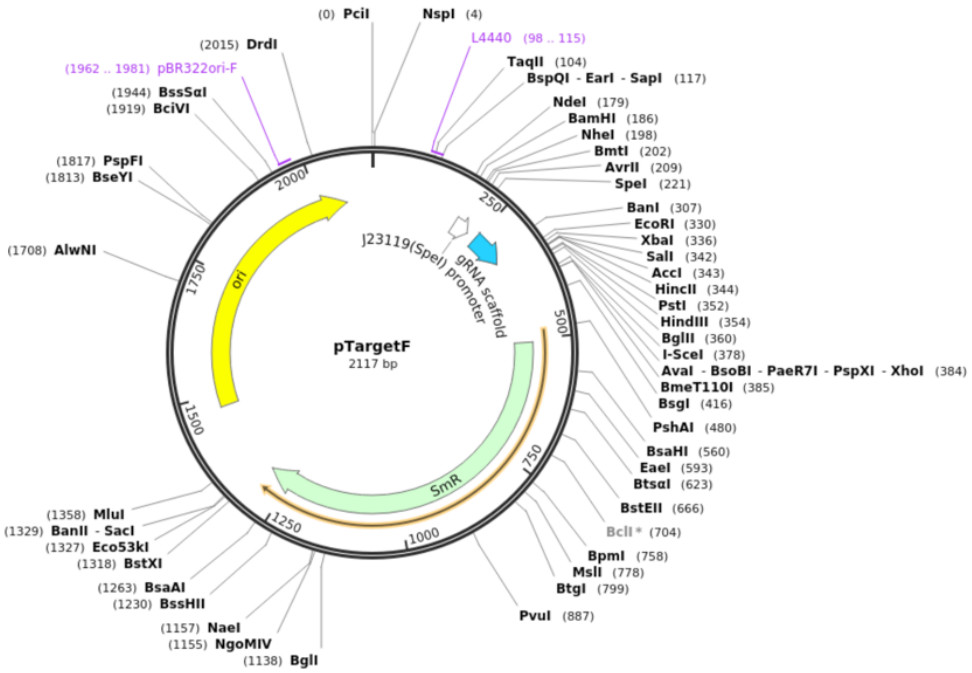
I predict that ICAM-1 expression can be reduced by knocking out different GAS segments on the ICAM-1 promoter and ICAM-1 can activate IDO expression. Using CRISPR mediated homologous recombination, will remove both or either of the 2 GAS sequences in the ICAM1 promoter and measure ICAM1 and IDO levels by RT-PCR. Negative control is a scrambled CRISPR targeting vector. The positive control is the introduction of LPA which is already known to increase ICAM 1 levels.

II. Materials and Methods:

This experiment has a positive control on the Lysophosphatidic acid (LPA) introduction to cells. LPA is known to enhance the expression of ICAM-1. The negative control is a scrambled CRISPR targeting vector, which means that the positive control does not receive treatment of Cas9 gene or sgRNA gene introduction.

A. Design sgRNA and clone it into pTarget vector:

Through the experiment we will get a plasmid of pTarget Mammalian Expression Vector System, which includes sgRNA, gene of Spectinomycin antibiotic resistance, J23119 promoter, as shown in Figure 1. The pTarget vector plasmid is designed to constitutively express scaffold guide RNA (sgRNA) which leads the Cas9 protein to cleave on the correct target site. To achieve this, the sgRNA sequence needs to be designed first. The designed sgRNA has a gene sequence of 5'-GTTTAAG AGCTATGCTGGAAACAGCATAGCAAGTTTAAAT AAGGCTAGTCCGTTATCAACTTGAAAA-3', in total of 4105 base pairs. The sgRNA is then replicated though polymerase chain reaction (PCR). The upstream primer sequence that is used for PCR is 5'-GAAAATTAATACG ACTCACTATAGGAAAAGAACAGGGAACAGATTGT TTAAGAGCTATGCTGGAAACAG-3'. The downstream primer sequence is 5'-GTTTAAGAGCTATGCTGGAAA CAGCATAGCAAGTTTAAATAAGGCTAGTCCGTTA TCAACTTGAAAAAGTGGCACCGAGTCGGTGC-3'. After amplification, sgRNA is then digested by restriction enzyme EcoR1. As shown below in Table 1, we combine 5 ul buffer, 1500 ug DNA, 1 ul EcoR1 and about 50 ul ddH2O in a test tube. The test tube needs to be Incubated at appropriate temperature (usually 37 °C) for 1 hour.



*The graph is from Addgene, a store where plasmids are sold. The plasmid shown above is a complete plasmid that contains anything required to replicate, transcribe and translate, such as primers, promoters, RBS site…[9].

Figure 1. pTarget plasmid vector that encodes for sgRNA

TABLE I. The amount of substances added during restriction cloning

Buffer	5 ul
DNA	1500 ug
EcoR1	1 ul
ddH2O	Up to 50 ul

After restriction digestion, ligation needs to be performed to connect sgRNA with the pTarget plasmid vector on their sticky ends. A T4 DNA ligase enzyme is used during the process to join sgRNA and pTarget plasmid together. As demonstrated in the table below,

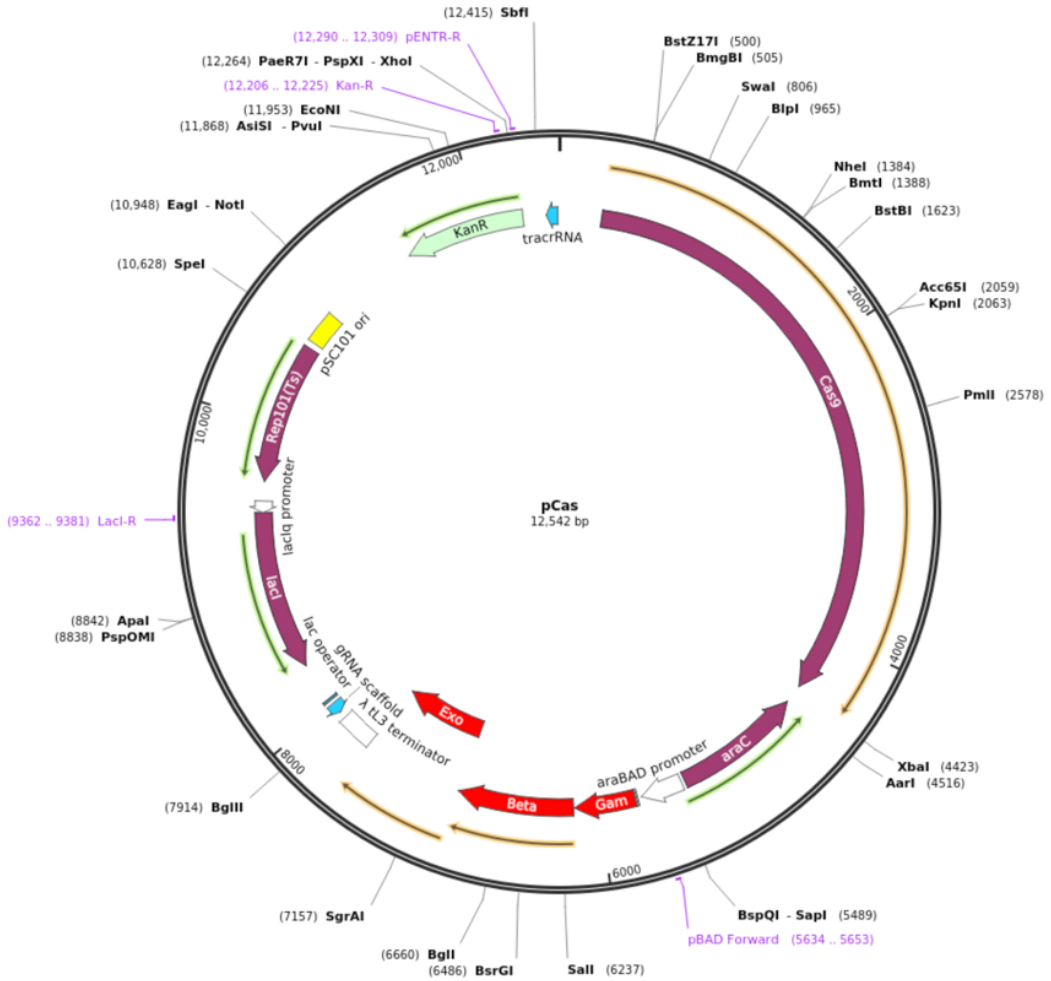
Table 2 shows that T4 buffer of 2 ul, pTarget DNA of 1 ul, DNA of sg RNA of 2 ul, T4 DNA ligase of 1 ul and ddH2O of 13 ul are combined in a test tube. The combined liquid needs to be Incubated at room temperature for 2hr, or at 16°C overnight.

TABLE II. Amount of substances added for the DNA ligation process

T4 buffer	2 ul
pTarget DNA	1 ul
DNA of sgRNA	2 ul
T4 DNA ligase	1 ul
ddH2O	13 ul

B. Get pCas vector plasmid:

Another plasmid with lambda red gene, Cas9 and Kanamycin resistant gene is needed to be designed. The sequence of Cas9 gene is 5'-ATGGATAAAAAATACAG… GAGTCAGCTGGGCGGTGAC-3', in total 4104 base pairs [10]. The Cas9 gene is used to express Cas9 protein which can be directed to cut off GAS segments (target sequences). Lambda red gene encodes phage-derived proteins that are utilized to facilitate the homologous recombination after the gene knockout. As shown in Figure 2, both Lambda red gene and Cas9 gene are incorporated in the pCas vector.



*(The pCas plasmid vector shown above is from the Snapgene website)

Figure 2. pCas plasmid vector that encodes for the Cas9 protein and Lambda red protein

C. Design repair template

The repair template is composed of single-stranded oligonucleotides. It contains left & right homology arms, three stop codons and a restriction enzyme gene site. The repair template is designed to replace the target gene and PAM site, so that the DNA can still function without the target gene expression and Cas9 binding as sgRNA will no longer recognize the binding site once PAM site is eliminated.

D. Design plasmids each with its target gene

The experiment designs a plasmid with a GAS segment that is 2787 bps away from the ribosome binding site (RBS) of ICAM-1 (target gene 1) and ICAM-1 gene. The gene sequence of the target gene 1 is 5′- TTTCTGAAA-3′. We need to design another plasmid that contains GAS segment that is 115 bps away from the translation site of ICAM-1 (target gene 2). The gene sequence of the target gene 2 is on the promoter of ICAM-1 and has a gene sequence of 5'-TTCCGGGAA-3'. The gene sequence of target gene 1 and gene 2 has a slight difference of 3 nucleotide differences but may cause a large difference in protein expression result. For the positive control, we design a plasmid with both GAS sequences, ICAM-1 gene and LPA gene.

E. Identify the PAM site on the target gene sequence

A protospacer adjacent motif (PAM) site is essential for the sgRNA to recognize in order to lead the Cas9 protein to cleave. The PAM site has 3 nucleotides that follow the 5'-NGG-3' form. Therefore, the PAM site for target gene 2 would be CGG on 5'-TTCCGGAA-3'. However, since target gene 1 does not have NGG form nucleotides, PAM site on the homology arm can be used, which is AGG on 5'-TTTCCTTGAAAAACGCAGATCCTAGAGGATCC-3′ (The underlined part in the gene sequence is the target 2 gene sequence).

F. Transformation of plasmids with target genes

DH5alpha of 30ul is added with 10 ul plasmid 1 (plasmid with the target gene 1) in a test tube. The test tube is placed on ice for 30 minutes. After that, the tube is immediately placed in a 42 degree Celsius water for 85 seconds. The tube is then placed on ice for 1.5 hours. Transformed DH5alpha is grown on a kanamycin plate. Every colony on the plate represents cells that successfully engulf plasmid 1. We then pick 98 colonies up and amplify them through PCR. The 98 test tubes are kept in -80°C refrigerator. We repeat the above process for plasmid 2, positive control plasmid, and negative control plasmid. The experiment is repeated for 3 times and each result is recorded.

G. Transform pCas plasmid vector into E. coli

Electroporation is used to transform the pCas plasmid vector into the E. coli cells. Electrical pauses are applied to create holes on the cell membrane, so that pCas plasmid can pass through the membrane. The transformed cells are then grown at 36 degree Celsius on a kanamycin plate. The experiment is repeated for 3 times and each result is recorded.

H. PCR

After that, we pick up colonies that are present in the growth medium since these cells express Cas9 gene. We need to mix 98 cell colonies in 98 test tubes in total. As shown in Table 3, each cell colony is added to a test tube with 15 ul Taq polymerase mix, 1 ul VF, 1 ul Vr and 13 ul ddH2O, as shown in the table below. The experiment is repeated for 3 times and each result is recorded.

TABLE III. Amount of substances added for PCR

Taq polymerase mix	15 ul
VF (forward primer)	1 ul
VR (reverse primer)	1 ul
ddH2O	13 ul

I. Gel Electrophoresis

We add 3 ul from each of the 98 test tubes into the Gel with dye and check the result shown in the ultraviolet scanning machine. (See Results) The experiment is repeated for 3 times and each result is recorded.

J. Transform pTarget vector and template repair vector by using Electroporation

Using Electroporation, we transform the pTarget vector and template repair vector at the same time into 97 test tubes that include our negative control and the 96 test groups. During the transformation, lambda red gene is induced, so homologous recombination of the plasmid that contains the target 1 gene and repair template can possibly occur in 96 test tubes. Once the repair segment in combined with our target plasmids, successfully edited genes will not be re-edited by Cas9 protein since PAM site is eliminated. However, DNA will not function or die if the repair template fails to combine with our target plasmids. Transformants are replicated and picked into a kanamycin plate and a chloramphenicol plate. Colonies grow on the kanamycin plate but not on the chloramphenicol plate indicating that the gene knockout is successful. The experiment is repeated for 3 times and

each result is recorded.

K. RT-PCR

The experiment is performed according to the guide on Thermo Fisher Scientific. RT buffers, dNTPs, designed primers, reverse transcriptase and DNA polymerase are all bought on Thermo Fisher Scientific. We add LPA and the ICAM-1 gene & promoter that has successfully knocked out GAS segment (-2787 bps) together with the above materials and measure the level of RNA expression of IDO. Another RT-PCR test is done by adding LPA and the ICAM-1 gene & promoter that has successfully knocked out GAS segment (-115 bps). The above process is repeated for the positive and negative control group. The experiment is repeated for 3 times and each result is recorded.

III. Statistical analysis

Three Chi-square tests are performed in total for the study. The first Chi-square test examines the relationship between ICAM-1 and IDO expression. If the p value is greater than 0.05, then we have convincing evidence that there is an association between ICAM-1 and IDO expression. The second Chi-square test examines the relationship between GAS 1 (-115 bps) and ICAM-1 expression. If the p value is greater than 0.05, then we have convincing evidence that there is an association between GAS segments and ICAM-1 expression. The third test examines the relationship between GAS 2 (-2787 bps) and ICAM-1 expression. The Confidence level is 95 % for all three tests.

A. Possible Results

TABLE IV. Possible Results Table

Measured substance:	Result 1	Result 2	Result 3	Result 4	Result 5
ICAM-1 reduced with 1 GAS KO (-2787 bps)	+	-	-	-	-
ICAM-1 reduced with 1 GAS KO (-115 bps)	+	+	+	-	-
ICAM-1 reduced with both GAS KO	+	-	+	-	+
Supporting Hypothesis?	Fully Support	Not Support	Fully Support	Not Support	Partially Support

*Positive sign indicates experimental results close to positive control's.
*Negative sign indicates experimental results close to negative control's.
Positive control: the Lysophosphatidic acid (LPA) introduction to cells. LPA is known to enhance the expression of ICAM-1.
Negative Control: Scrambled CRISPR targeting vector

Possible Result 1: The target gene 1 and target gene 2 knockouts are successful since no false cleavage or mutation occurs.
In the experiment, CRISPR Cas9 does not have an off-target effect since sgRNA successfully recognizes and binds to the PAM site of both genes that need to be knocked out. During the replication, transformation, restriction enzyme digestion and ligation processes, the plasmid in E. coli might not mutate, so the binding of Cas9 protein on target sequences is likely successful.
Possible Result 2: The insertion of the repair template is not successful for target gene 2.
Because the PAM site on target gene 2 cannot be found and we instead use the PAM site on the homology arm, the CRISPR might falsely recognize the PAM site and knock the unwanted gene out, so that the whole DNA dies. However, A PAM site sequence can be found on target gene 1, so it is likely that target gene 1 can be knockout successfully.
Possible Result 3: The new gene without target gene 2 expresses less ICAM-1 protein than does the new gene without target gene 1.
Because target gene 2 is farther from the translation initiation site than target gene 1, it's possible that target gene 1 has a larger difference in influencing the expression level of protein of ICAM-1.
Possible Result 4: ICAM-1 does not affect IDO expression at all.
IDO expression is indirectly activated by IFN-gamma induced genes, but there are thousands of IFN-gamma induced genes, so ICAM-1 might not be the one that increases the IDO expression.
Possible Result 5: Both new genes without target gene 1 & 2 express only a little after CRISPR cleavage.
If both target genes 1 and 2 are crucial in the ICAM-1 promoter for initiating the transcription of ICAM-1 gene, then ICAM-1 gene cannot be transcribed when target genes 1 and 2 are knocked out.

IV. Discussion

Previous studies report that IFN-gamma induced genes indirectly activate the IDO expression through the Jak3/Stat3 pathway on tumor cells. There are thousands of IFN- γ induced genes, and ICAM-1 is one of them. To test the effect of ICAM-1 on IDO expression and the effects of 2 GAS segments on ICAM-1 gene expression, this study knocks out 2 GAS segments separately on ICAM-1 promoter by using CRISPR Cas9 and introduce the successfully knocked out genes on ICAM-1 plasmid with IDO. This study is significant and highly applicable since IDO expression contributes to tumor development and regulating IDO expression might possibly suppress tumor cells from growing.

Possible result 1 is reasonable that both knockouts support the hypothesis and no off-target effect occurs since sgRNA recognizes the PAM site on target region with a high accuracy. Success in the experiment indicates that the ICAM-1 gene expression can be regulated easily. Moreover, it is unlikely that a mutation occurs in the core region of a gene sequence, so that GAS sequences do not change over the experiment, which also exhibit stability of cell proliferation.

The experiment of possible result 2 that does not support the hypothesis is possible because of the PAM site on target gene 2 (GAS sequence -2787 bps). Since no gene sequence on the target gene is in the 5'-NGG-3' form, we choose the PAM site a few nucleotides away from the target gene, which means that Cas9 might cleave both the target GAS sequence and unwanted sequences out. The cleavage of unwanted sequences might result in the dysfunction of the promoter of ICAM-1. Thus ICAM-1 transcription cannot be activated.

Possible result 3 is consistent with experimental results of the previous studies that GAS sequences with different distances from the initial translation site tend to have a difference of influence on ICAM-1 protein expression level, thus possible result 3 supports the hypothesis. Previous studies demonstrate that GAS sequence that is 2787 base pairs away from the initial translation site will likely bind with Stat dimers with low affinity and the transcription of ICAM-1 cannot be activated without the presence of GAS sequence that is 115 bps from the RBS site. We can possibly introduce a mutation in GAS sequence to see if ICAM-1 expression is reduced or increased and combine the knocking out and mutation adding to control the ICAM-1 expression in a wider range in the future.

Possible result 4 does not support the hypothesis and it is possibly because IFN-gamma induced genes are more than 200 and ICAM-1 is only one of them. ICAM-1 only has little probability to be the only one that activates the IDO expression. However, in future studies, more IFN-gamma induced genes may be tested to see their relationships with IDO expression and how GAS sequences on each of them affect their expression level.

Possible result 5 likely occurs because the GAS is on the promoter sequence of ICAM-1 and the editing on promoter might directly result in the failure in transcription activation. Therefore, the next study could be conducted for testing the effect of replacement of the entire promoter of ICAM-1 on the ICAM-1 expression.

V. Conclusion

In General, this study is conducted to test the effect of ICAM-1 on IDO expression by introducing them in the same cell and utilize RT-PCR to measure their expression levels. Additionally, the effects of 2 GAS sequences on ICAM-1 are also examined through CRISPR Cas9 gene knock out and homologous recombination. Results of this study indicate that the GAS sequence that is farther away from the ICAM-1 RBS site might decrease the ICAM-1 expression level less than does GAS sequence that is nearer from RBS, and ICAM-1 might not have any direct impact on IDO expression.

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