行政院國家科學委員會專題研究計畫　成果報告

HO-1/hDAF/HLA-DR 多基因轉殖豬細胞體外之功能測試(2/2)

計畫類別：整合型計畫
計畫編號：NSC92-2314-B-002-226-
執行期間：92 年 08 月 01 日至 93 年 07 月 31 日
執行單位：國立臺灣大學醫學院外科

計畫主持人：李章銘

計畫參與人員：李玉汶

報告類型：完整報告

處理方式：本計畫可公開查詢

中　華　民　國 93 年 11 月 9 日

附件一

行政院國家科學委員會補助專題研究計畫

■成果報告

□期中進度報告

異種器官供人體移植之可行性研究-使用多基因轉殖小鼠與豬器官

- HO-1/hDAF/HLA-DR 多基因轉殖豬細胞體外之功能測試

計畫類別： ■個別型計畫　　□整合型計畫
計畫編號：NSC 92-2314-B-002-226
執行期間： 九十一 年 八 月 一 日至 九十三 年 七 月 三十一 日

計畫主持人：李章銘醫師
共同主持人：
計畫參與人員：李玉汶小姐

成果報告類型(依經費核定清單規定繳交)：□精簡報告　■完整報告

本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

執行單位：臺大醫院外科

中　華　民　國　九十三　年　十一　月　八　日

中文摘要：

關鍵詞：基因轉殖豬，人類白血球抗原，人類延遲加速因子，第一型 Heme 氧化酵素

以豬器官來源的異種器官，為目前臨床移植所面臨的器官短缺的問題，提供了解決之道。但是其可行性，目前仍受限於人類對豬隻的異種抗體所誘發的超急性，及亞急性異種器官排斥，及物種間組織抗原歧異所引起的 T 細胞排斥。先前在我們所產製的 HLA DPW0401 基因轉殖豬中，發現人類 HLA DP 的抗原性，可完整表達在豬細胞表面，而相較於其他非基因轉殖同胞豬，HLA DPW0401 基因轉殖豬所引起人類周邊單核球(具 HLA DPW0401 基因型者)的增生反應，有減少的趨勢。

HO-1 是代謝 Heme 的主要酵素，而能保護細胞抵抗異種抗體所引發的細胞裂解及凋亡,及使異種移植器官在異種抗體存在之下，達到 ”適存化 ” 的狀態。而人類延遲加速因子(hDAF, CD55)為一補體活化鏈的調節者。目前發現 hDAF 的基因轉殖豬器官，包括心臟，腎臟及肝臟可在靈長類體內， 存活 7 天至 3 個月。

在過去一年中，我們已經成功地產製了 HLA-DR 及 HO-1 之基因轉殖豬，其 mRNA 及其蛋白的表達都已得到確定，針對 HO-1 保護作用的測試，我們已經成功地完成自然殺手細胞毒性測試的檢驗模式，我們發現殺手細胞與標的細胞於 5：1 的情形有最明顯的毒殺現象，之後我們將運用於 HO-1 轉殖豬細胞的測試，檢測此 HO-1 轉殖基因是否有保護作用。我們也完成了 HLA-DR 基因轉殖豬細胞對人類週邊單核球的增生測試，我們發覺這種基因轉殖豬細胞，與非基因轉殖同胞豬細胞相較，其引起的異種細胞免疫反應，並沒有明顯的差別，至於這些轉殖豬細胞，是否對於其他的異種免疫反應有保護作用，則有待進一步確定。

Abstract：

Keywords: Transgenic Pig , Xenotransplantation, HLA, HO-1, hDAF

Pig to human xenotransplatation is a promising strategy to overcome the organ shortage in clinical transplantation. However, the application is hampered by the xenoreactive antibody mediated hyperacute rejection, delayed xenograft rejection and MHC disparity associated T cell rejection. Previous, we have produced the HLA DPW0401 transgenic pig, which was shown equipped with the human HLA antigenecity and, as compared to the non-transgenic litermate pig, induced a minor cellular response to the human PBMCs that came from the HLA DPW 0401(+) human. HO-1 (heme oxygenase-1) is the main enzyme metabolizing the heme, which has been demonstrated to offer a protective effect for the xenograft to achieve accommodation under the presence of xenoreactive antibodies, and help to attenuate xenoantibody mediated celll lysis and apoptosis. To test the protective effect of HO-1 exogene from the NK related cytotoxicity, we have establish the optimal condition for this cytotoxic assay using the NK cell line, K562. The cell killing efficacy will be maximal when the effector/target ratio being 5: 1, 47% of cell death of the total cell population. We have also successfully cultured the aortic endothelial cells of pig (PAEC) from the non-transgenic pig. The culture of the PAEC of the transgenic pig is ongoing now. Because the HLA-DR 1501 has been successfully produced, we also test the xenogenic MLC for the HLA DR transgenic pig. We found that the tendency about xenogenic cellular response was not evidently different between the HLADR transgenic and non-transgenic littermate control pis PBMC. Whether this transgenic pig cell can obtain protective effect in another process of xenogenic immune response required to be examined further.

Background

Xenotransplantation with graft organ from pig is a promising strategy to overcome the shortage of organ, which has been the main restriction for clinical transplantation (1,2). The xenoreactive antibody-mediated hyperacute rejection (HAR) and delayed xenograft rejection (DXR) (or acute vascular rejection) are the major obstacles to impede the survival of xenofgraft organ in human (3-7). Various strategies have been developed to suppress the HAR or DXR successfully, including using organs from transgenic pigs of human Decay Accelerating Factor (8,9), depletion, or suppression of production of xenoreactive antibodies (10-14). The cellular response induced by differences of MHC between disconcordant species can provide a continuous detrimental effect to graft organ, even though the xenograft has gone through the stages of HAR or acute vascular rejection. It has been demonstrated that the porcine MHC molecules can effectively induce a strong human T cell response, through direct or indirect antigen recognition (15-17). The human NK cell associated anti-porcine cytotoxicity was also maintained, which was T cell and antibody-independent (18).

Heme oxygenase (HO) is responsible for the metabolism of hemin and can be detected in all mammalian tissues. At least three isoenzymes, i.e., HO-1, HO-2, and HO-3, were included in the HO family (20-2). HO-1 is a stress-inducible protein which can be up-regulated by stimulation by cytokines, heavy metals, hypoxia, or oxygen-free radical (23-5). HO-2 is a constitutively expressed protein, particularly aboundant in brain, testis, or liver of mammals (26). The role of HO-3 is relatively obscured, which is believed to be a regulator rather than a direct catalyst in the heme processing (22). HO-1 can catalyze the conversion of heme to biliverdin, Iron, and carbon monooxide (27). Under usual circumstance, which is rich in biliverdine reductase, the biliverdin can be rapidly transformed into bilirubin, the more familiar form. The CO can help to maintain the vascular tone in small and median sized arteries, or aorta (28-9). Bilirubin is an antioxidant (30) and may be important in maintaining the viability of cells under stress environment. Although the iron is toxic to the cells by creating detrimental reactive oxygen species, the iron can be neutralized by ferritin right away after its production (27). Ferritin is a strong antioxidant that protects the cells from oxidative stress. Its expression can in turn be up-regulated by iron through binding to its mRNA iron-responsive elements (31-2). Blocking the activity of HO-1 by tin mesoporphyrin can deteriorate renal function of rat kidney after ischemia and induce an extensive injury in the renal tubule (33). Up-regulation of HO-1 by hemin can also ameliorate the halothane-induced hepatotoxicity (34). Recently it was found that the expression of HO-1 in the endothelium of genograft could essential for the xenograft accommodation. The mouse heart xenotransplant can survived long-term in the rats receiving CVF and CyA. However, the heart from a HO-1 deficient mouse was eventually rejected three to seven days after transplantation in rats under the similar treatment (35). Based on these observations, we expect that through expression of human HO-1 on the porcine cells might also contribute to the survival porcine xenograft.

Materials and Methods

Establishing the NK cell cytotoxicity assay .

This assay was based on a dual fluorescent staining of target cells. The dye, DIOC18 (3,3'-dioctadecyloxacarbocyanine perchlorate) is a carbocyanine membrane dye that label

cellmembrane by inserting two lond (C18 carbon) hydrocarbonchains into the lipid bilayers, is used to stain the membrane of the target cells. Propidium Iodide (PI) is used to label dead cells. The K562 cell line was used as the effector cell to test the protective effect of HO-1 transgene for the NK associated cytotoxicity (Gong et al., 1994). The procedure was listed as below

1. Wash the tatget cells in PBS and re-suspend in PBS at a concentration of 1X 10^6 cell/ml.
2. Stain the target cell with DIOC 18 10 ul per 1X 10^6 cells
3. Incubation of the cells for 20 min at 37^{o}C, 5% CO2
4. Wash the cells twice with buffer solution.and resuspend in complete culture media at a concentration of 1X 106 cells/ml
5. Prepare the target and effector cells and made a co-culture of them with the desire ratio, i.e. E:T: 1:5; 1:2.5; 1:1; 1:0.5; 1:0.2. Incubate the co-culture for 4 hours.
6. Centrifuge the cell mixture at 250 g for 5 min after wash, discard suernatant.
7. Washed and re-suspend the cultured cells
8. Label the cells with Propidium iodide (5 ul/per test) incubate at room temperature in dark.
9. Analysis with flowcytometry.

Culture of porcine endothelial cells(PAEC)：

1) Treat the porcine aorta with collagenase (2.5 mg/ml; Sigma).
2) Seeding of PAECs in 25cm^2 gelatin-coated culture flasks in RPMI. 1640 medium (Gibco, Paisleg UK), supplemented with 10% heat-inactivated FCS(Life Technology), 25mM sodium bicarbonate(Flow, Les Ulis, France), 2mM glutamine (Flow), 1 mM sodium pyruvate (Flow), 60 μg/ml tylocin(Life Technologies), 50 IU/ml penicillin (Flow), and 50 μg/ml streptomycin (Flow) (referred as culture medium)
3) Subculture the PAEC with treatment of trypsin-EDTA(Life Technologies)-with the use of 2nd to 8th subculture
4) Incubate the PAEC with recombinant human TNF-α (Genzyme , Boston, MA) (100U/ml fo36 hours), the optimal condition for inducing MHC expression in previous report.(24)

Mixed lymphocyte culture：

1. Dilution the heparinized human blood with double volume of HBSS
2. Overlayer the above mentioned mixture with 10ml Ficoll-paque
3. Centrifuge with 1500 rpm for 30 minutes
4. Harvest the mononuclear cell layer in the interface
5. Wash with HBSS for three times (PBMC)
6. Incubate the PBMC for 4 hours at 37^o C in 75-cm^2 plastic flask (Falcon #3023, Becton Dickinson Labware, Lincoln Park, NJ) to deplete the adherent cells.
7. Recover the adherent cells with rubber policemen and analysis with flowcytometry
8. Incubate non-adherent cells for 60 min at 37o C on nylon wool (Fenwal Laboratories, Deerfield, IL) and then gently elute enrich the T cells

9. Treat the T cells with 1:100 dilution of the ascitic fluid of a murine anti-porcine macrophage- and granulocyte-specific antibody for at 4°C for 30 min.
10. Add rabbit complement 9HLA-ABC, Pel Freeze, Roger, AR) at 1:8 dilution and incubate for 45 min at 37°C
11. Wash the cells with culture media
12. Prepare the responder and stimulator cells
    a) responder cells adjust the concentration to $1x10^6$ cells/ml
    b) stimulator cell:(PBMC) adjust the concentration to $2x10^6$ cells/ml
    c) Irradiate the stimulator cells with 4500 cGy and incubate in 37°C, 5% CO_2 for 20 minutes.
    d)Wash the stimulator cells with HBSS for three times
    e)Adjust the concentration of stimulator cells to $1x10^6$ cells/ml
13. Add into each well with 100 μl of stimulator and responder cells respectively and done in triplicate
14. Incubate in 37° C and 5% CO_2 for 6 days
15. Add 20 μl H^3 thymidine incubating for 6 hours.
16. Harvest with glass fiber and dry in air
17. Add scintillating cocktail
18. Count the cpm with β-counter.

Cellular Proliferative Assay (Xenogenic MLC)

Responders: PBMCs from the persons with the same genotype of the HLAII exogene expressed on the transgenic pig cells.

Stimulators (porcine PBMCs, porcine aortic endothelial cells (PAEC), and porcine Islet cells):

a. Cells from non-transgenic littermate pig
b. Cells from DP transgenic pig
c. Cells from DR transgenic pig
d. Cells from DP + DR transgenic pig

Results and discussion:

The establishment of NK cytotoxicity assay

Using the NK cell line, K 526, we will be able to evalaue the protective effect of the HO-1 exogene from the NK related cytotoxicity. We have already established the optimal condition to assay the

cytoxicity. The effector/ target ratio of 5/1 will produce a maximal efficacy of NK cytotoxicity with 46% of the cells stained with propidium iodide. About 27% of cell killing was found when the effector / target ratio being 1 / 1 or 2 / 1. The efficacy was not satisfactory when the target cell was more than the effector cells. This will provide a useful tool to evaluate the HO-1 exogene effect. The detail of the results were demonstrated in Fig 1-7. Next step we will test the system using the swine PBMC, PAEC and islet cell as the target cells.

Culture of the PAEC

At the present time, we have susceccfully produced the HLA DR 1501 transgenic pig. The PAEC of the non-transgenic pig has been harvested and cultured successfully. The culture PAEC of the HLA DR transgenic pig was ongoing at the present time.

Xenogenic MLC for the HLA DR transgenic pig.

Xenogenic MLC with coculture of the human PBMC and the transgenic and non-transgenic swine PBMC was performed. After seven days of culture, the CPM of the human responder cells did not produced differences of cellular proliferation to the stimulator cells from the non-transgenic or transgenic pig. The results are demonstrated in the figures of 8 and 9.

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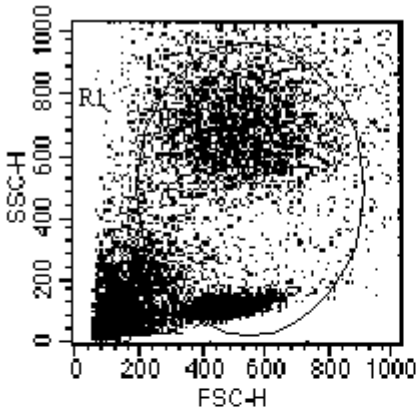
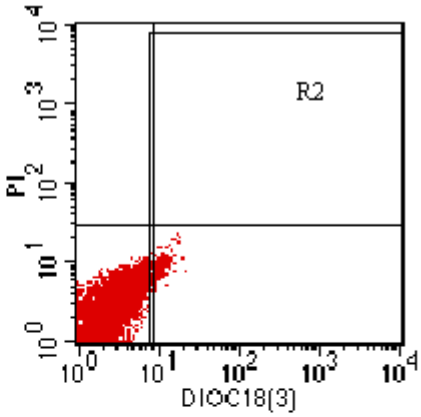
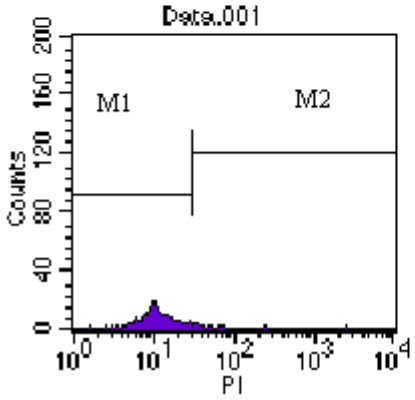
Cytotoxicity assay of the NK cell line, K562

Fig1: Target cells w/o stain



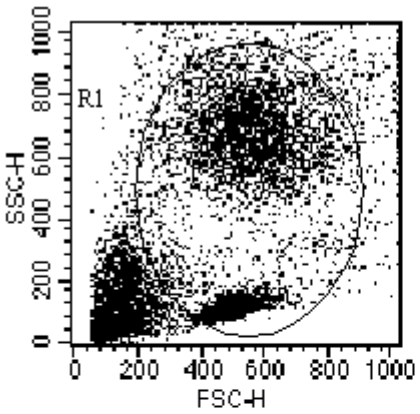


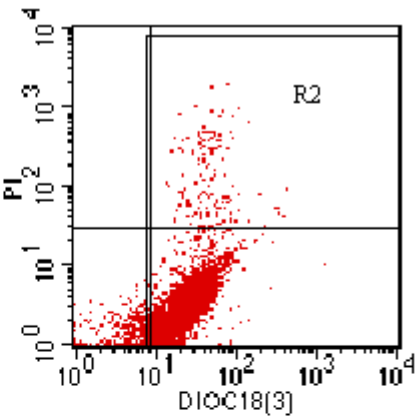


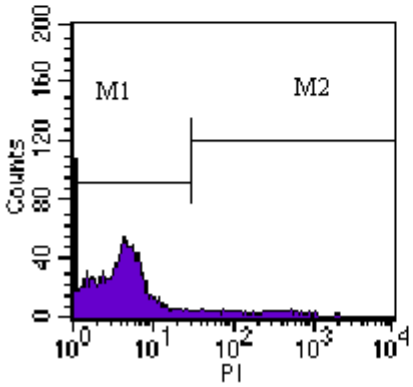
Gate: G2

Marker	Events	% Gated
All	850	100.00
M1	830	97.65
M2	20	2.35

Fig 2: Target cells stained with DIOC18 (3)/PI



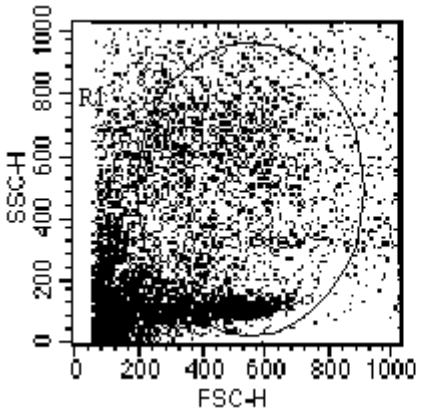


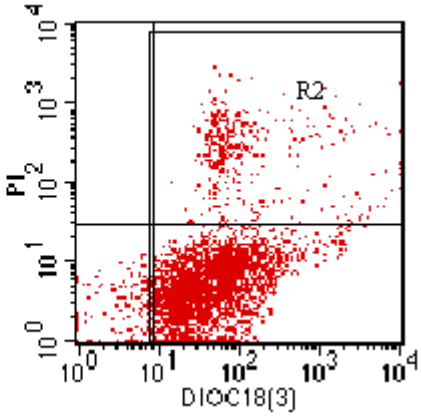


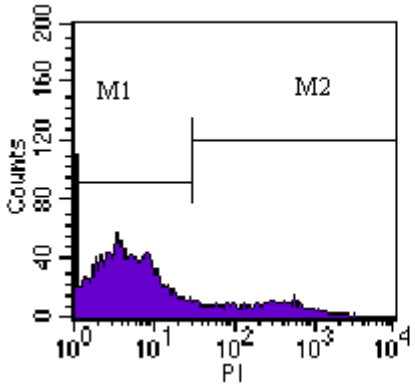
Gate: G2

Marker	Events	% Gated
All	6610	100.00
M1	6315	95.54
M2	297	4.49

Fig 3: E:T 1:5



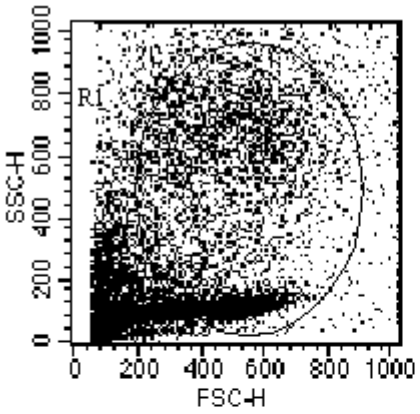


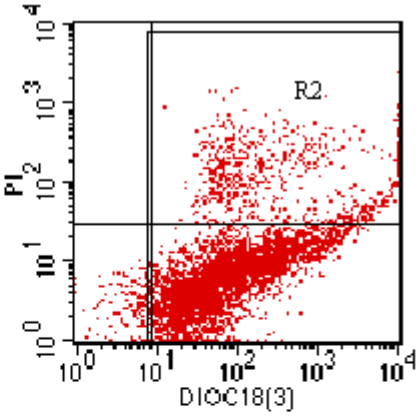


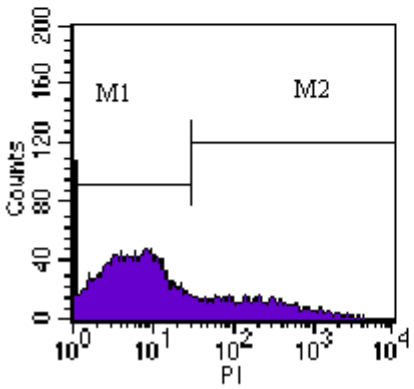
Gate: G2

Marker	Events	% Gated
All	10655	100.00
M1	9114	85.54
M2	1546	14.51

Fig4: E:T 1: 2.5



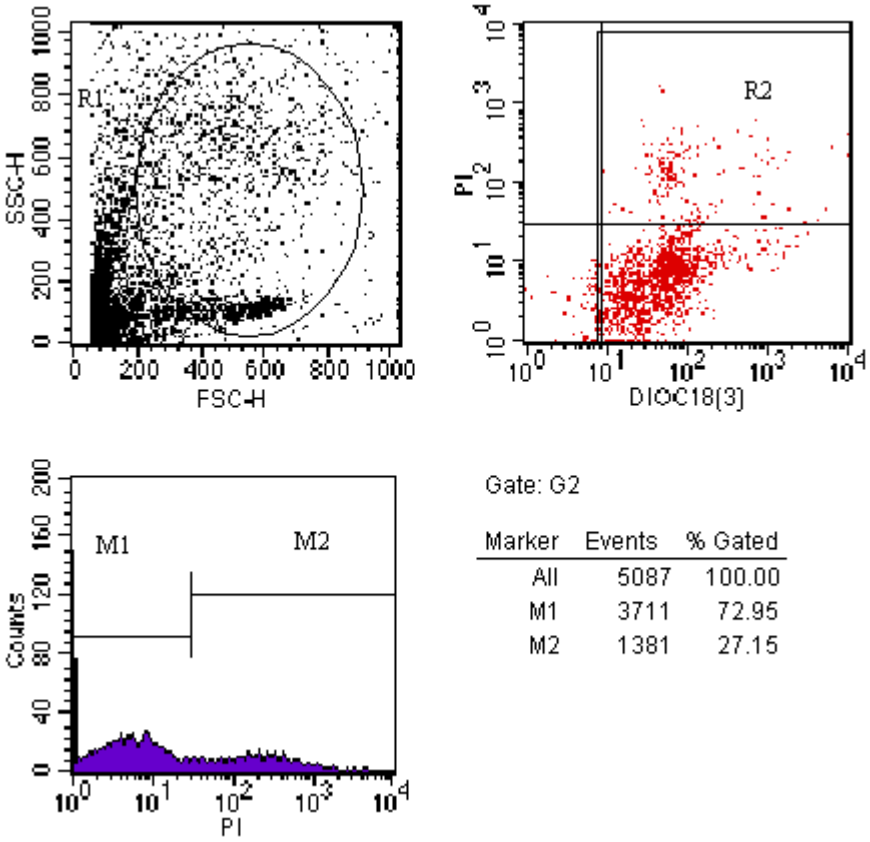




Gate: G2

Marker	Events	% Gated
All	12389	100.00
M1	9692	78.23
M2	2710	21.87

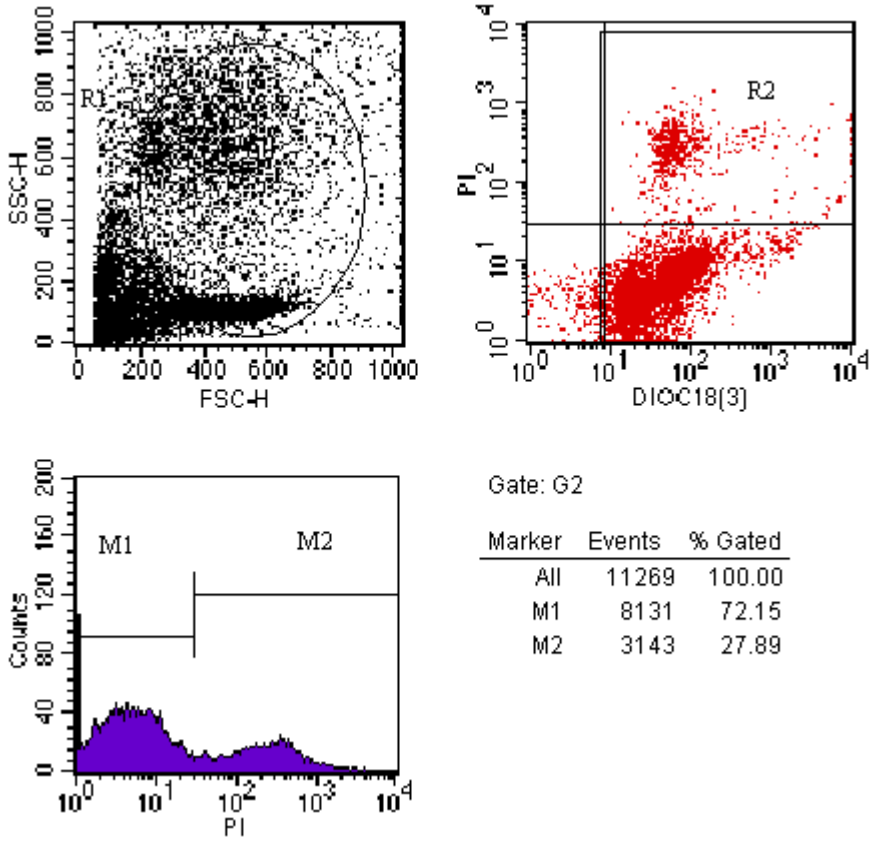
Fig 5: E:T 1: 1



Gate: G2

Marker	Events	% Gated
All	5087	100.00
M1	3711	72.95
M2	1381	27.15

Fig 6: E:T 1: 0.5



Gate: G2

Marker	Events	% Gated
All	11269	100.00
M1	8131	72.15
M2	3143	27.89

Fig 7: E:T 1: 0.2

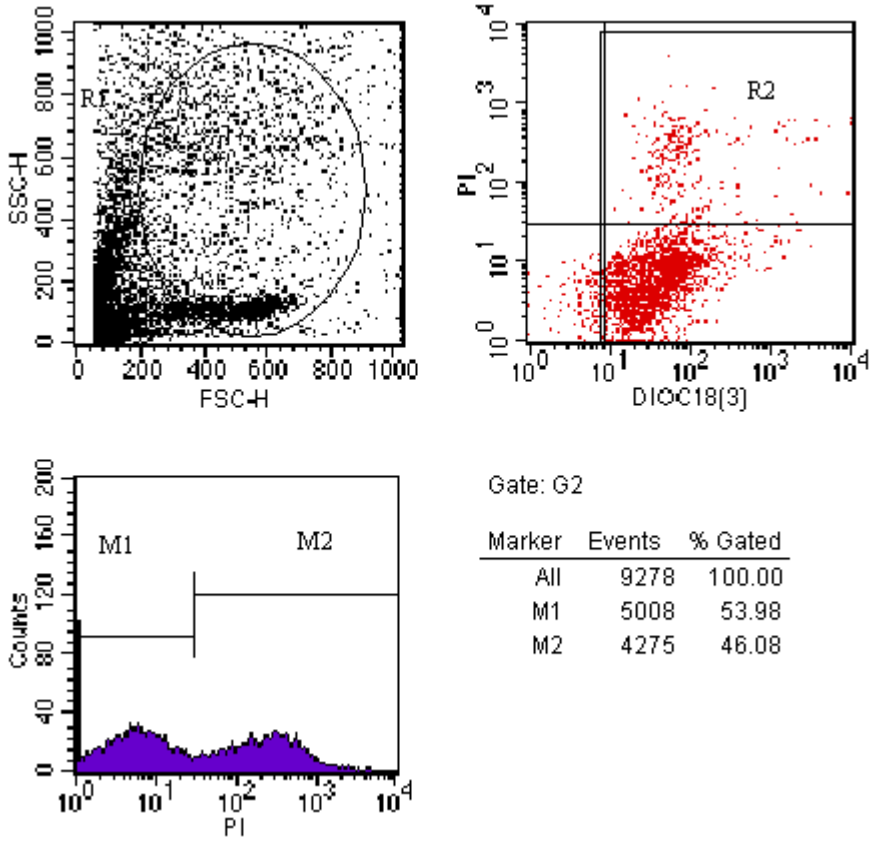


Fig 8: Xenogenic MLC for the HLA-DRw15 transgenic pig using PBMC from HLADRw15 (+) human **(representive of one of thre experiments)**.

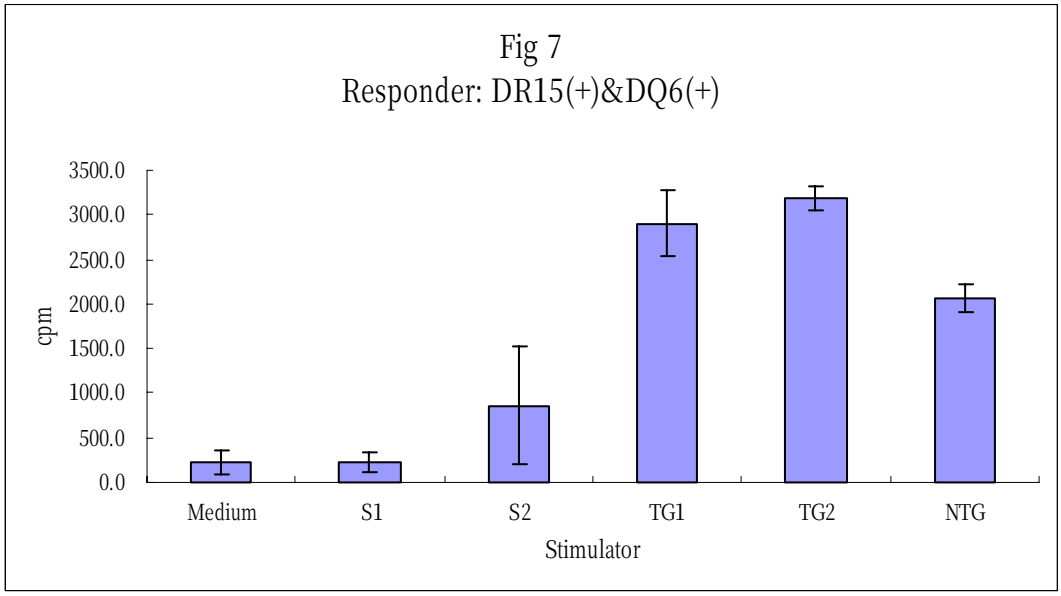


Fig 8: Xenogenic MLC for the HLA-DRw15 transgenic pig using PBMC from HLADRw15 (+) human **(representive of one of thre experiments)**.