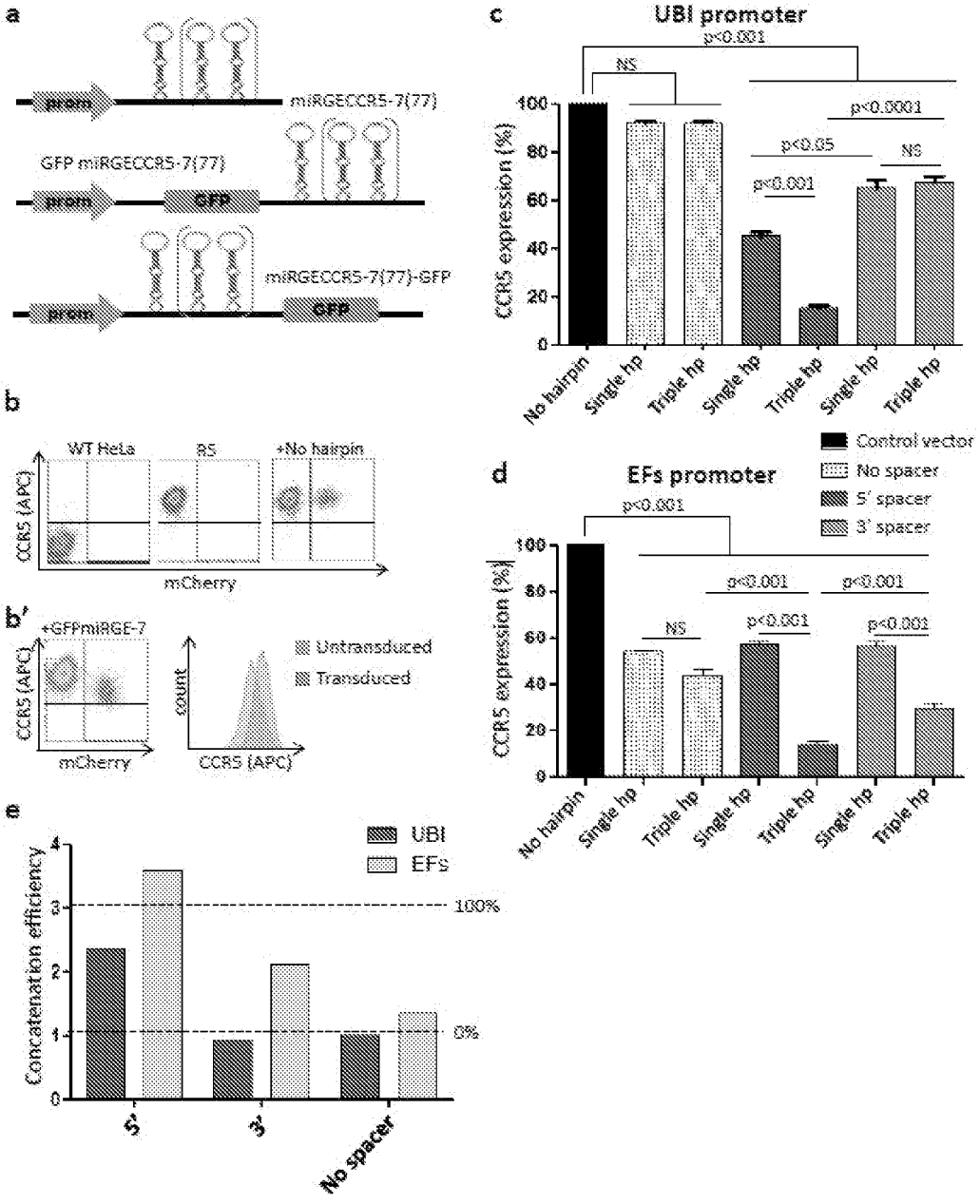

a
prom
miRGECCR5-7(77)
GFP miRGECCR5-7(77)
GFP
miRGECCR5-7(77)-GFP
b
WT HeLa
R5
+No hairpin
CCR5 (APC)
mCherry
b'
+GFPmiRGE-7
Untransduced
Transduced
count
c
UBI promoter
p<0.001
NS
p<0.0001
p<0.05
p<0.001
CCR5 expression (%)
No hairpin
Single hp
Triple hp
d
EFs promoter
Control vector
No spacer
5' spacer
3' spacer
e
UBI
EFs
Concatenation efficiency
100%
0%
5'
3'
No spacer

FIG. 1

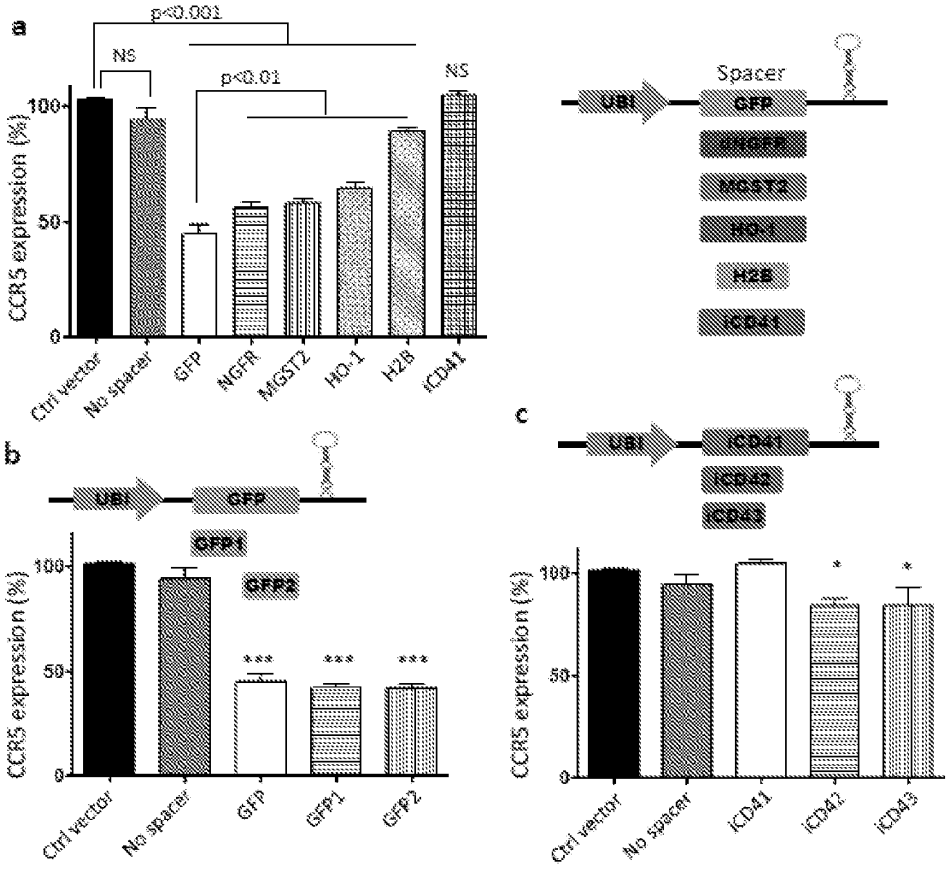

a
p<0.001
NS
p<0.01
NS
CCR5 expression (%)
100
50
0
Ctrl vector
No spacer
GFP
NGFR
MGST2
HO-1
H2B
ICD41
Spacer
UBI
GFP
NGFR
MGST2
HO-1
H2B
ICD41
b
UBI
GFP
GFP1
GFP2
***
***
***
CCR5 expression (%)
Ctrl vector
No spacer
GFP
GFP1
GFP2
c
UBI
ICD41
ICD42
ICD43
*
*
CCR5 expression (%)
Ctrl vector
No spacer
ICD41
ICD42
ICD43

FIG. 2

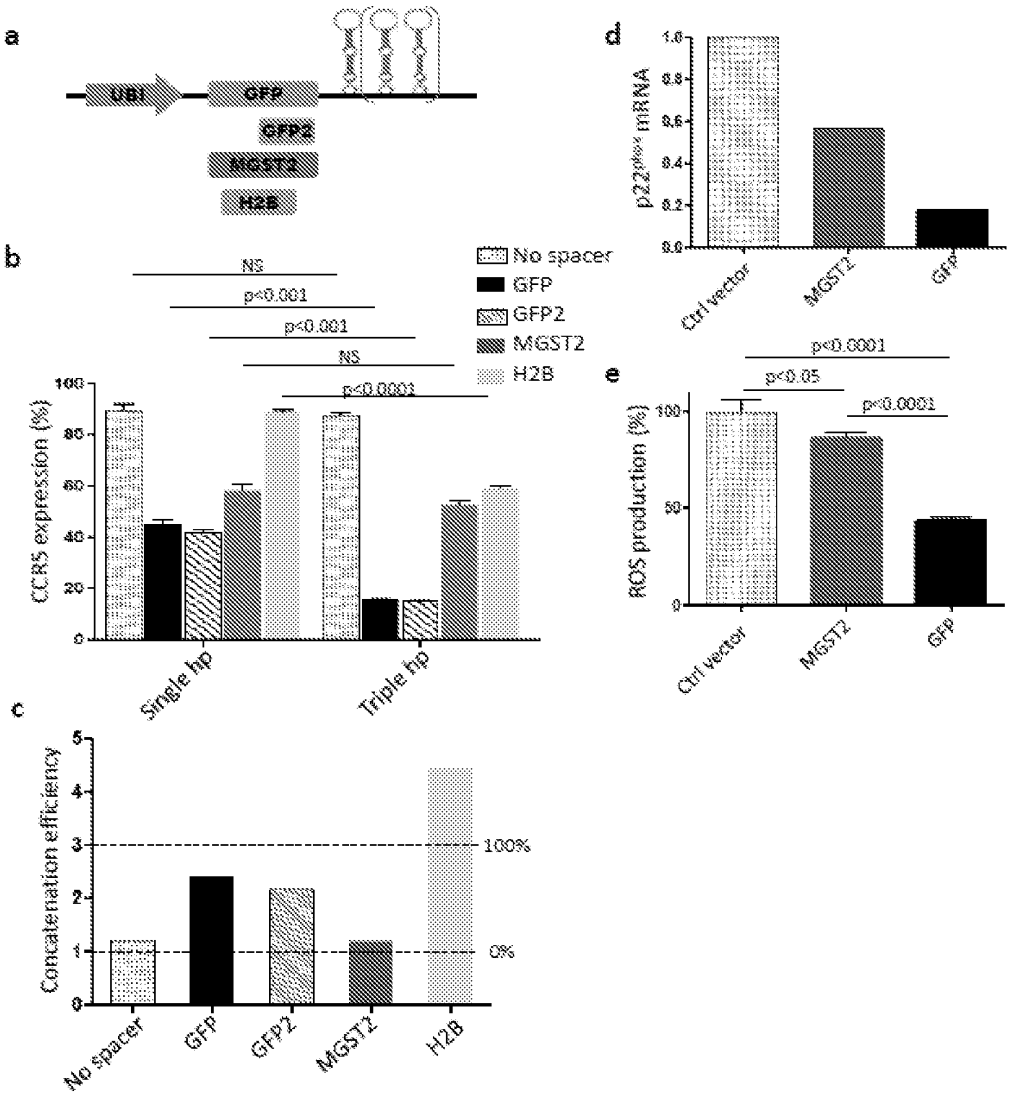

a
UBI
GFP
GFP2
MGST2
H2B
b
No spacer
GFP
GFP2
MGST2
H2B
NS
p<0.001
p<0.001
NS
p<0.0001
CCR5 expression (%)
Single hp
Triple hp
c
Concatenation efficiency
100%
0%
No spacer
GFP
GFP2
MGST2
H2B
d
p22phox mRNA
Ctrl vector
MGST2
GFP
e
p<0.0001
p<0.05
p<0.0001
ROS production (%)
Ctrl vector
MGST2
GFP

FIG. 3

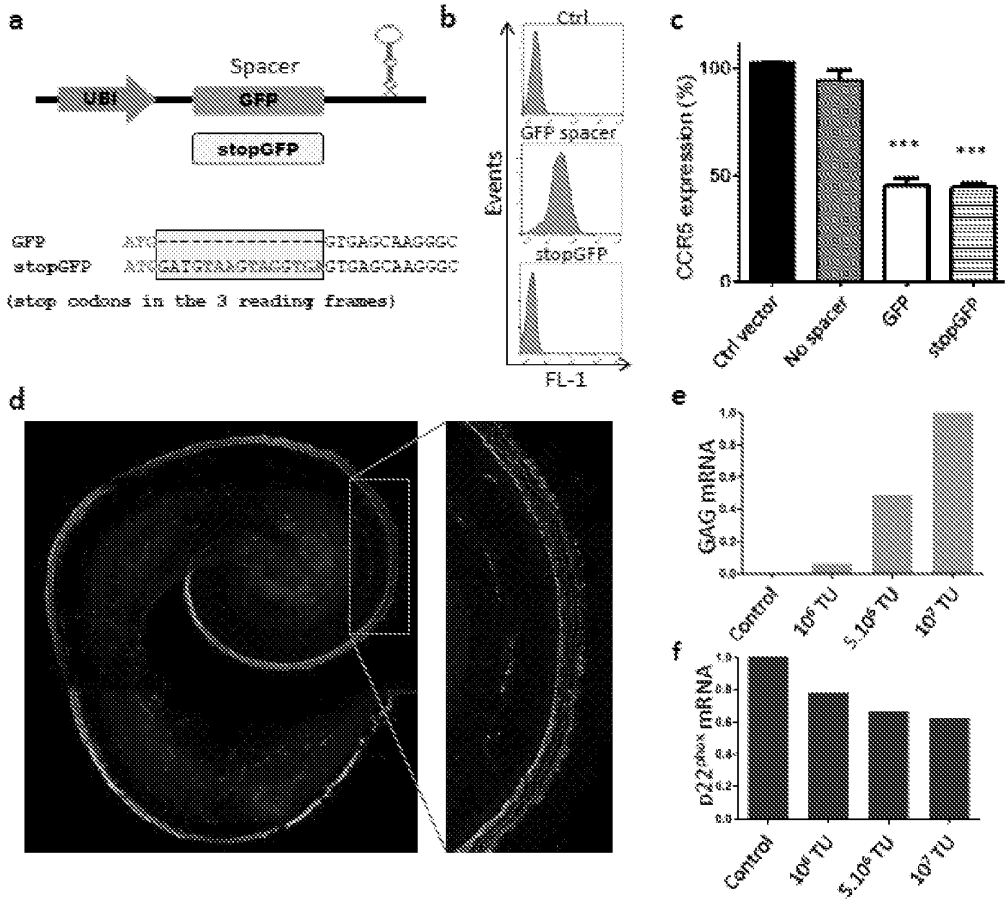

a
Spacer
UBI
GFP
stopGFP
GFP
stopGFP
(stop codons in the 3 reading frames)
b
Ctrl
GFP spacer
stopGFP
Events
FL-1
c
CCR5 expression (%)
100
50
0
***
***
Ctrl vector
No spacer
GFP
stopGFP
d
e
GAG mRNA
Control
10⁶ TU
5.10⁶ TU
10⁷ TU
f
Control
10⁶ TU
5.10⁶ TU
10⁷ TU

FIG. 4

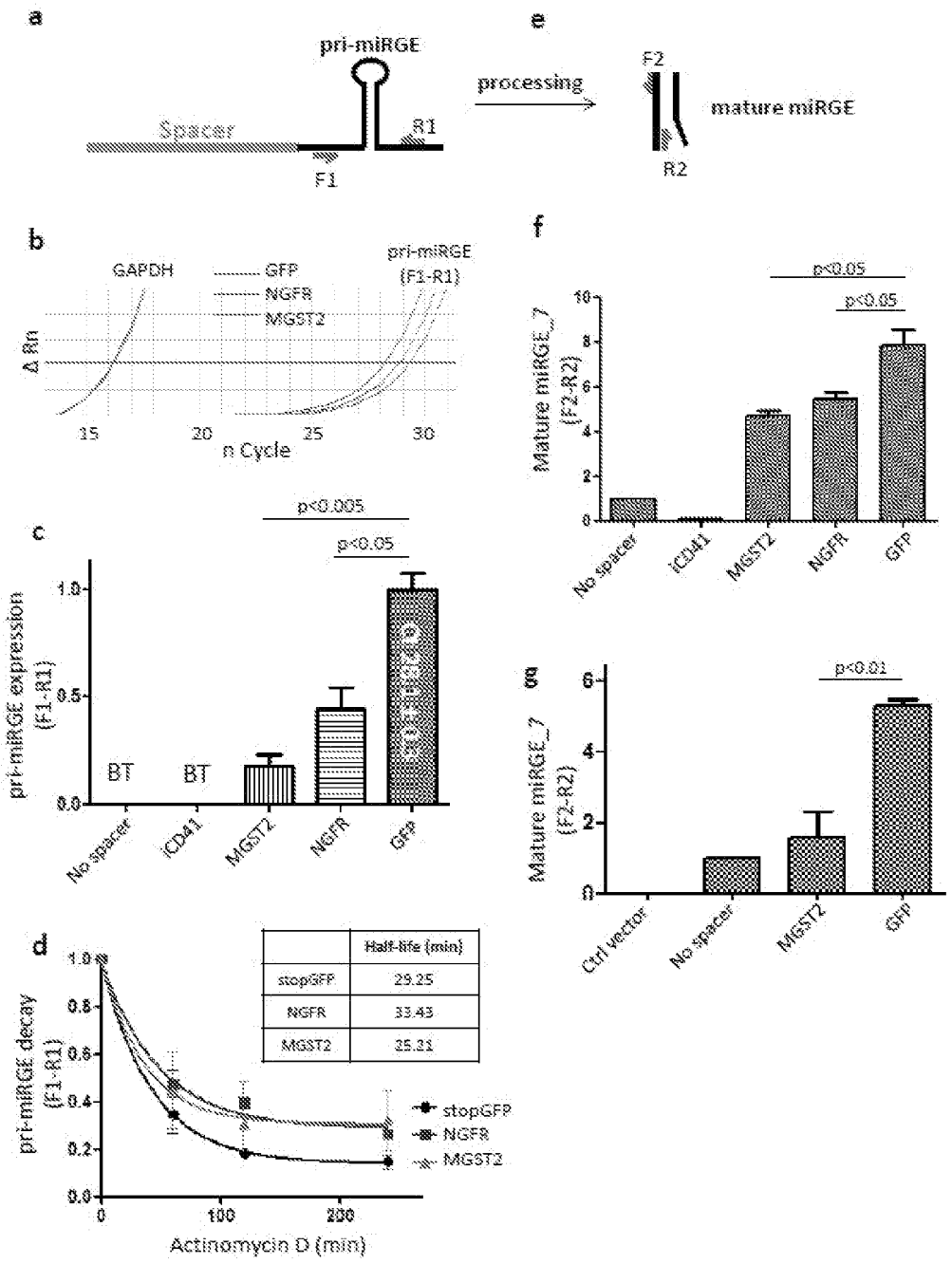

a
pri-miRGE
Spacer
R1
F1
e
processing
F2
mature miRGE
R2
b
GAPDH
GFP
NGFR
MGST2
pri-miRGE (F1-R1)
ΔRn
15
20
25
30
n Cycle
f
p<0.05
p<0.05
Mature miRGE_7 (F2-R2)
No spacer
iCD41
MGST2
NGFR
GFP
c
p<0.005
p<0.05
pri-miRGE expression (F1-R1)
BT
BT
No spacer
iCD41
MGST2
NGFR
GFP
g
p<0.01
Mature miRGE_7 (F2-R2)
Ctrl vector
No spacer
MGST2
GFP
d
pri-miRGE decay (F1-R1)
Half-life (min)
stopGFP 29.25
NGFR 33.43
MGST2 35.31
stopGFP
NGFR
MGST2
Actinomycin D (min)

FIG. 5

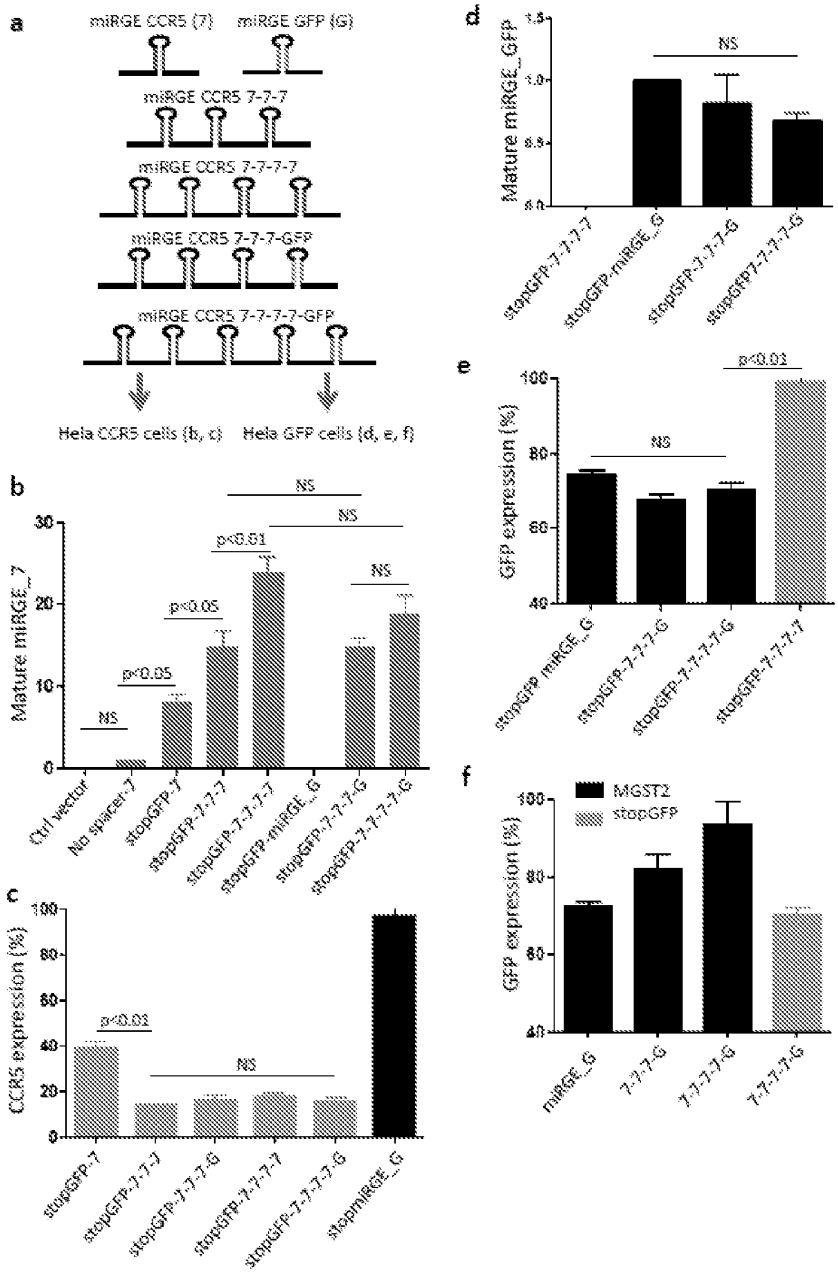

a
miRGE CCR5 (7)
miRGE GFP (G)
miRGE CCR5 7-7-7
miRGE CCR5 7-7-7-7
miRGE CCR5 7-7-7-GFP
miRGE CCR5 7-7-7-7-GFP
Hela CCR5 cells (b, c)
Hela GFP cells (d, e, f)
b
Mature miRGE_7
NS
p<0.05
p<0.01
Ctrl vector
No spacer-7
stopGFP-7
stopGFP-7-7-7
stopGFP-7-7-7-7
stopGFP-miRGE_G
stopGFP-7-7-7-G
stopGFP-7-7-7-7-G
c
CCR5 expression (%)
stopGFP-7
stopGFP-7-7-7
stopGFP-7-7-7-G
stopGFP-7-7-7-7
stopGFP-7-7-7-7-G
stopmiRGE_G
d
Mature miRGE_GFP
stopGFP-7-7-7-7
stopGFP-miRGE_G
stopGFP-7-7-7-G
stopGFP7-7-7-7-G
e
GFP expression (%)
stopGFP miRGE_G
stopGFP-7-7-7-G
stopGFP-7-7-7-7-G
stopGFP-7-7-7-7
f
MGST2
stopGFP
GFP expression (%)
miRGE_G
7-7-7-G
7-7-7-7-G
7-7-7-7-G

FIG. 6

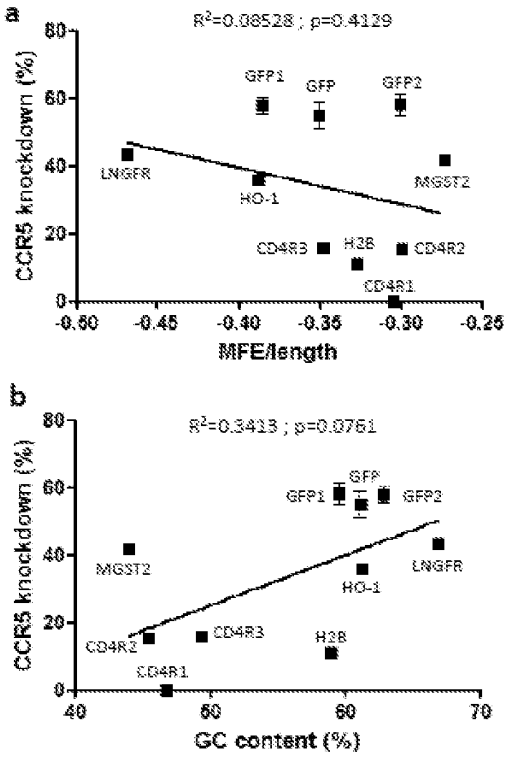

a
R²=0.08528 ; p=0.4139
CCR5 knockdown (%)
GFP1
GFP
GFP2
LNGFR
HO-1
MGST2
CD4R3
H3B
CD4R2
CD4R1
MFE/length
b
R²=0.3413 ; p=0.0761
CCR5 knockdown (%)
GC content (%)

FIG. 7

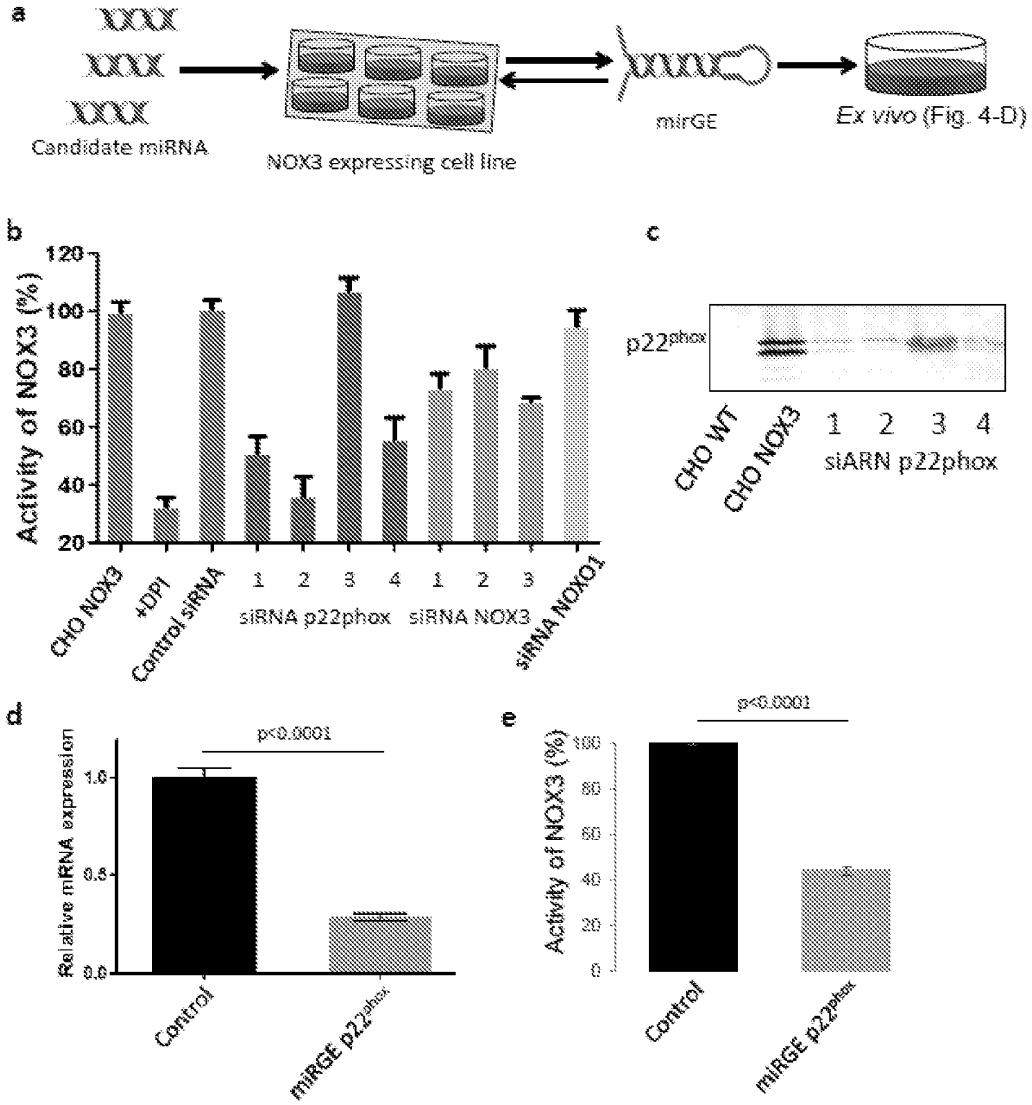


FIG. 8

Concatenation potency = CP (%) Knockdown potency = KP (%)	E= Concatenation efficiency with 3 hairpins
$CP=(KP)^E$	If E=3 concatenation efficiency is 100%
$E=\ln(CP)/\ln(KP)$	If E=1 concatenation efficiency is 0%

FIG. 9

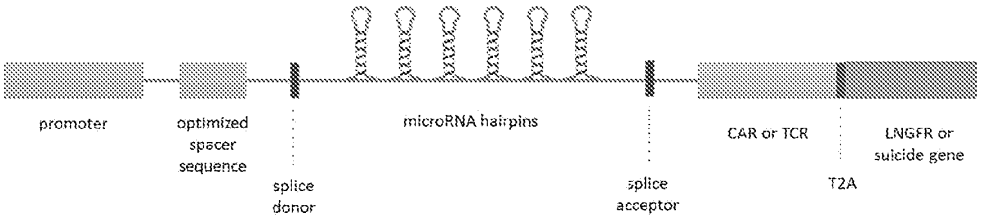

promoter
optimized spacer sequence
splice donor
microRNA hairpins
splice acceptor
CAR or TCR
T2A
LNGFR or suicide gene

FIG. 10

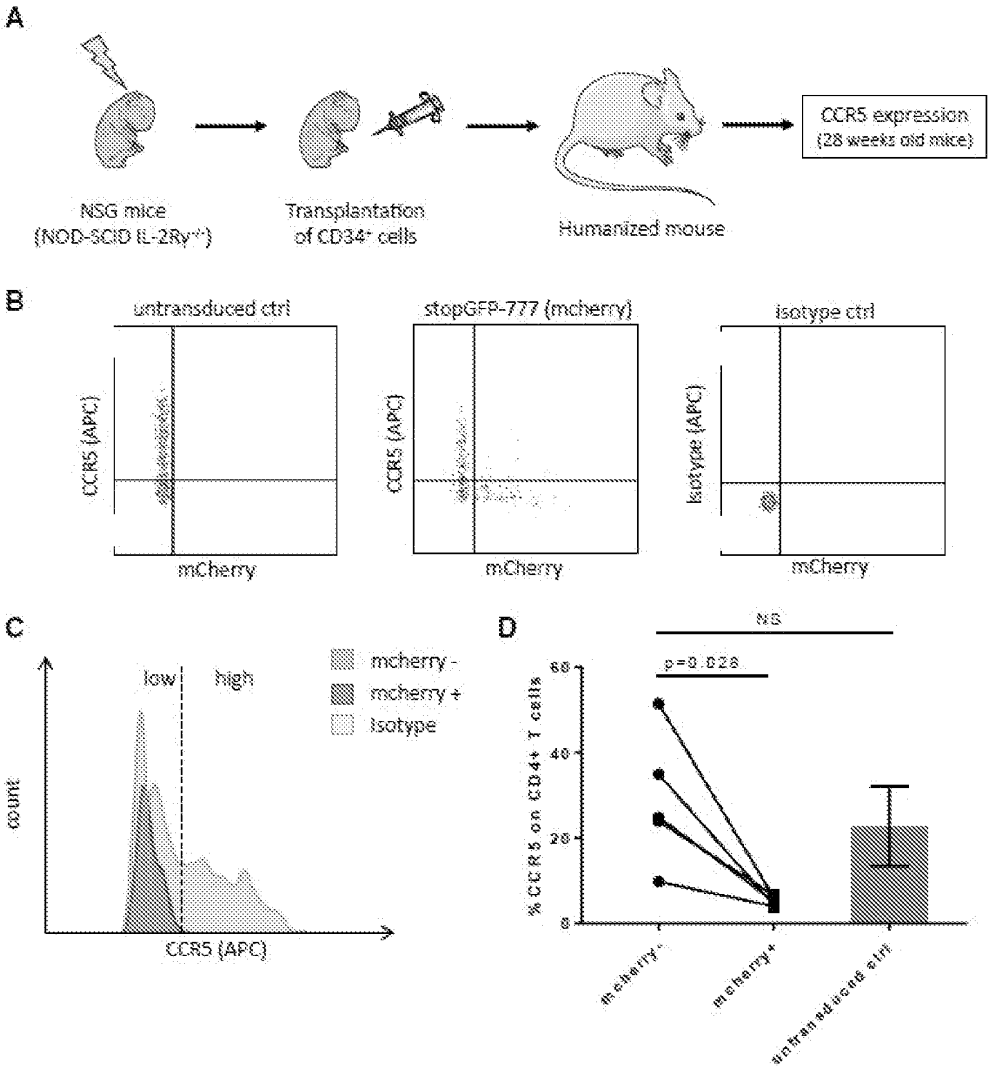

A
NSG mice (NOD-SCID IL-2Rγ-/-)
Transplantation of CD34+ cells
Humanized mouse
CCR5 expression (28 weeks old mice)
B
untransduced ctrl
stopGFP-777 (mcherry)
isotype ctrl
CCR5 (APC)
isotype (APC)
mCherry
C
low
high
mcherry -
mcherry +
isotype
count
CCR5 (APC)
D
NS
p=0.028
% CCR5 on CD4+ T cells
60
40
20
0
mcherry -
mcherry +
untransduced ctrl

FIG. 11

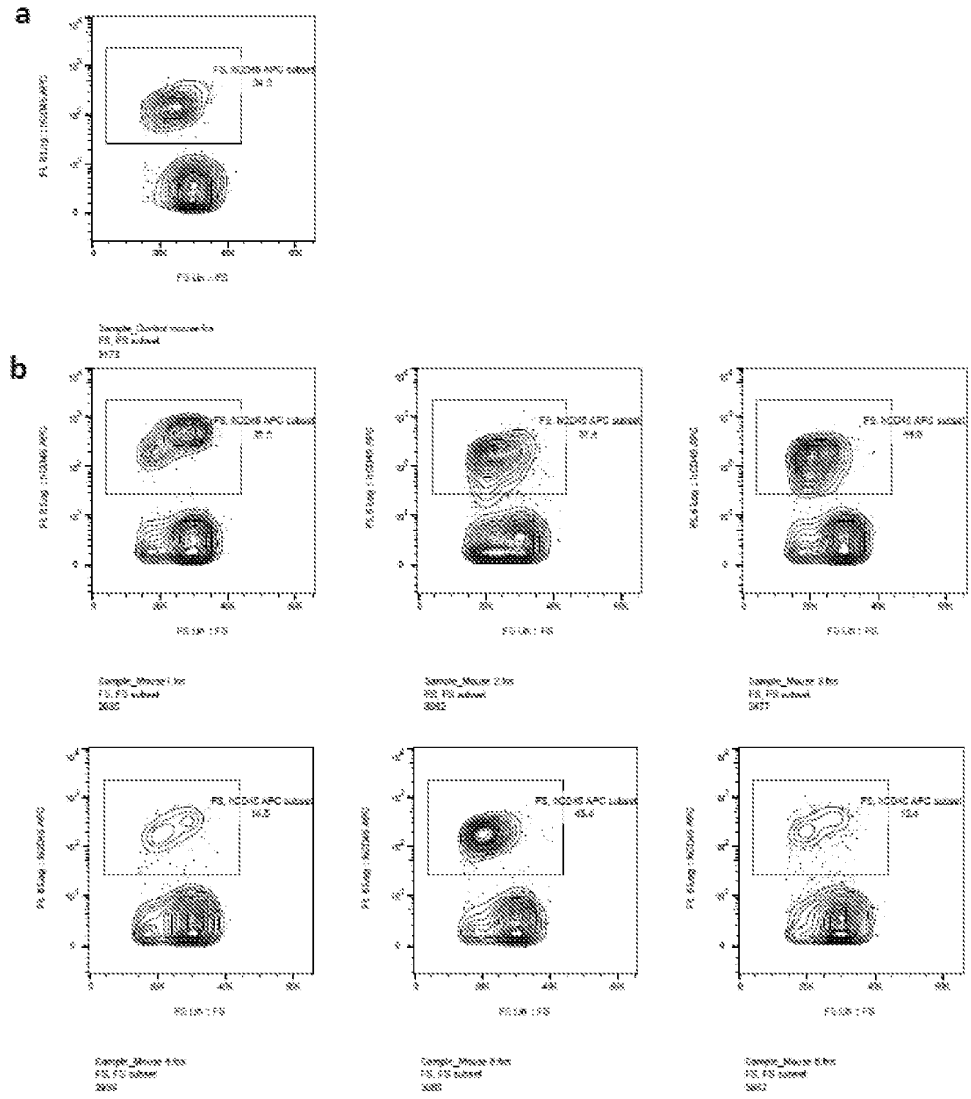


FIG. 12

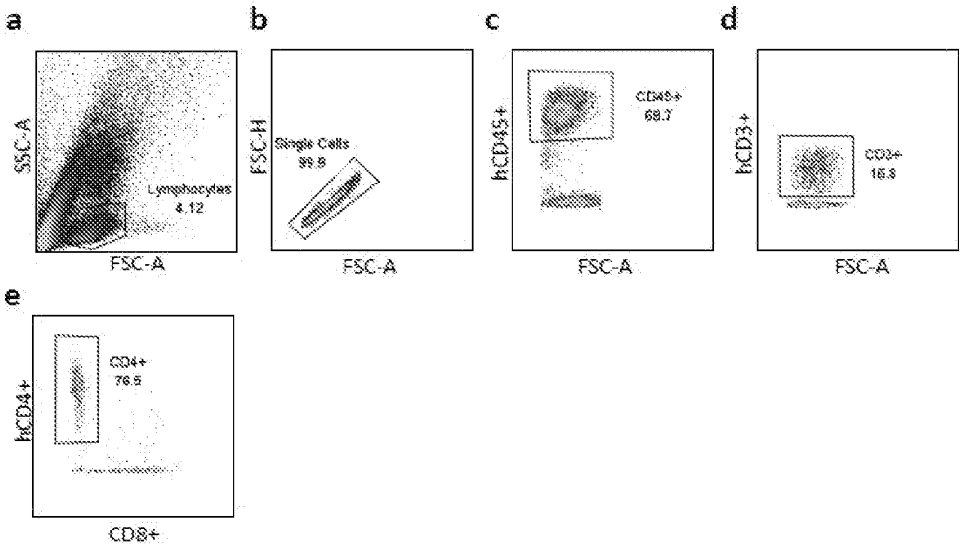

a
SSC-A
FSC-A
Lymphocytes
4.12
b
FSC-H
Single Cells
99.8
FSC-A
c
hCD45+
CD45+
69.7
FSC-A
d
hCD3+
CD3+
16.8
FSC-A
e
hCD4+
CD4+
76.5
CD8+

FIG. 13

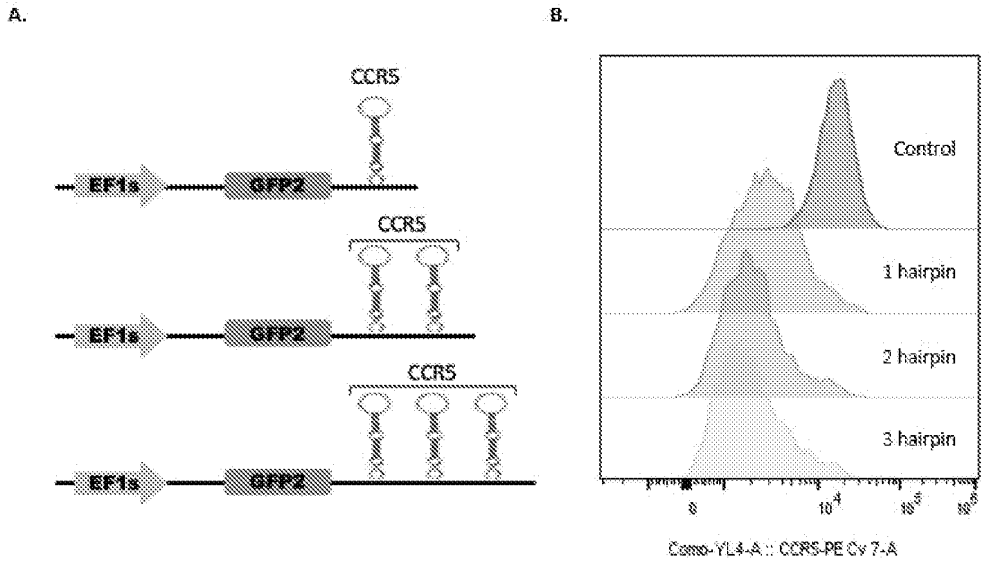

A.
CCR5
EF1α
GFP2
CCR5
EF1α
GFP2
CCR5
EF1α
GFP2
B.
Control
1 hairpin
2 hairpin
3 hairpin
Comp-YL4-A :: CCR5-PE Cy 7-A
C.
Relative CCR5 expression
1.0
0.8
0.6
0.4
0.2
0.0
Control
1 hairpin
2 hairpin
3 hairpin

FIG. 14

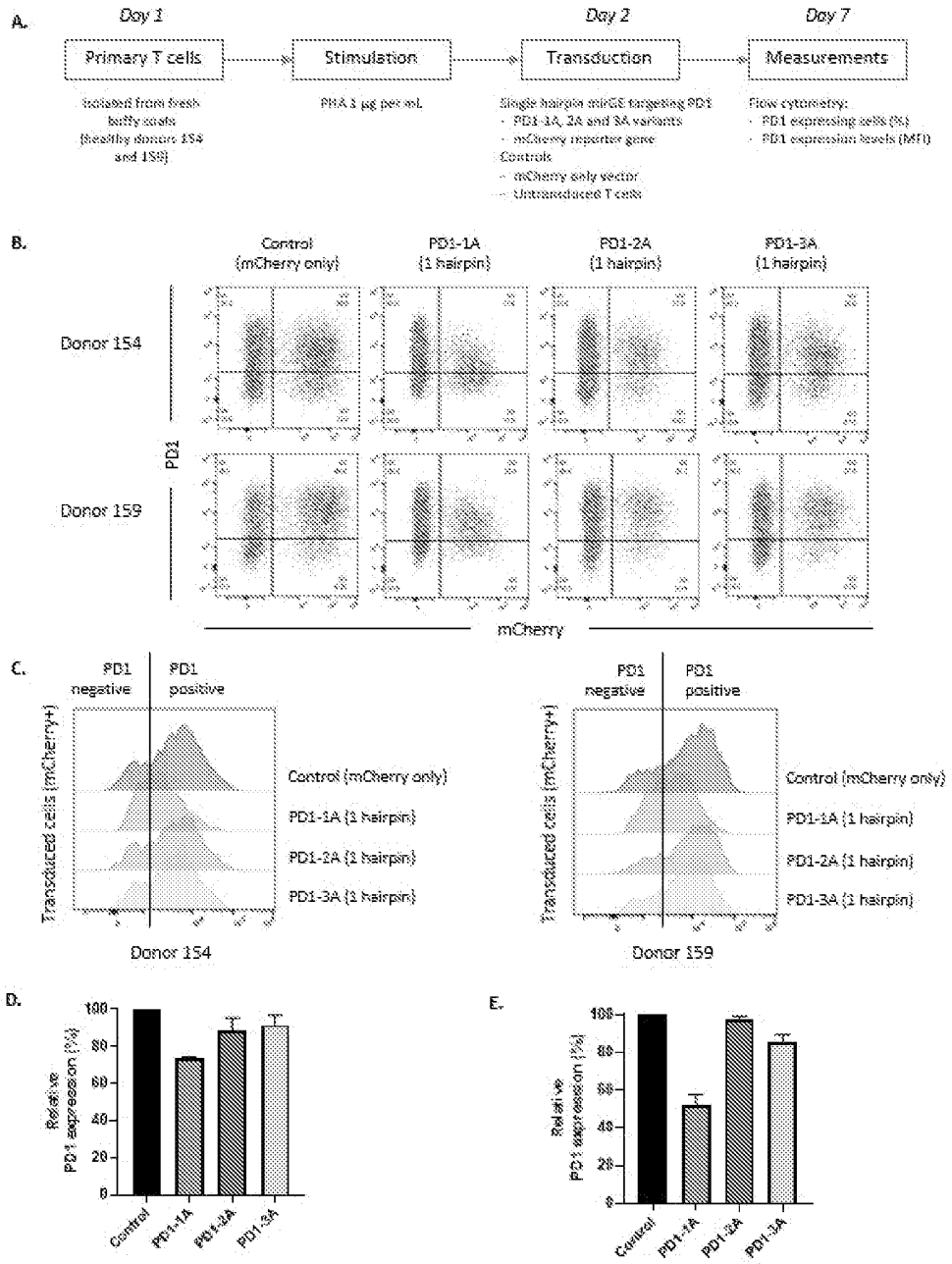

A.
Day 1
Day 2
Day 7
Primary T cells
Stimulation
Transduction
Measurements
Isolated from fresh buffy coats (healthy donors 154 and 159)
PHA 1 µg per mL
Single hairpin shRGE targeting PD1
- PD1-1A, 2A and 3A variants
- mCherry reporter gene
Controls
- mCherry only vector
- Untransduced T cells
Flow cytometry:
- PD1 expressing cells (%)
- PD1 expression levels (MFI)
B.
Control (mCherry only)
PD1-1A (1 hairpin)
PD1-2A (1 hairpin)
PD1-3A (1 hairpin)
Donor 154
Donor 159
PD1
mCherry
C.
PD1 negative
PD1 positive
Transduced cells (mCherry+)
Control (mCherry only)
PD1-1A (1 hairpin)
PD1-2A (1 hairpin)
PD1-3A (1 hairpin)
Donor 154
Donor 159
D.
Relative PD1 expression (%)
Control
PD1-1A
PD1-2A
PD1-3A
E.
Relative PD1 expression (%)
Control
PD1-1A
PD1-2A
PD1-3A

FIG. 15

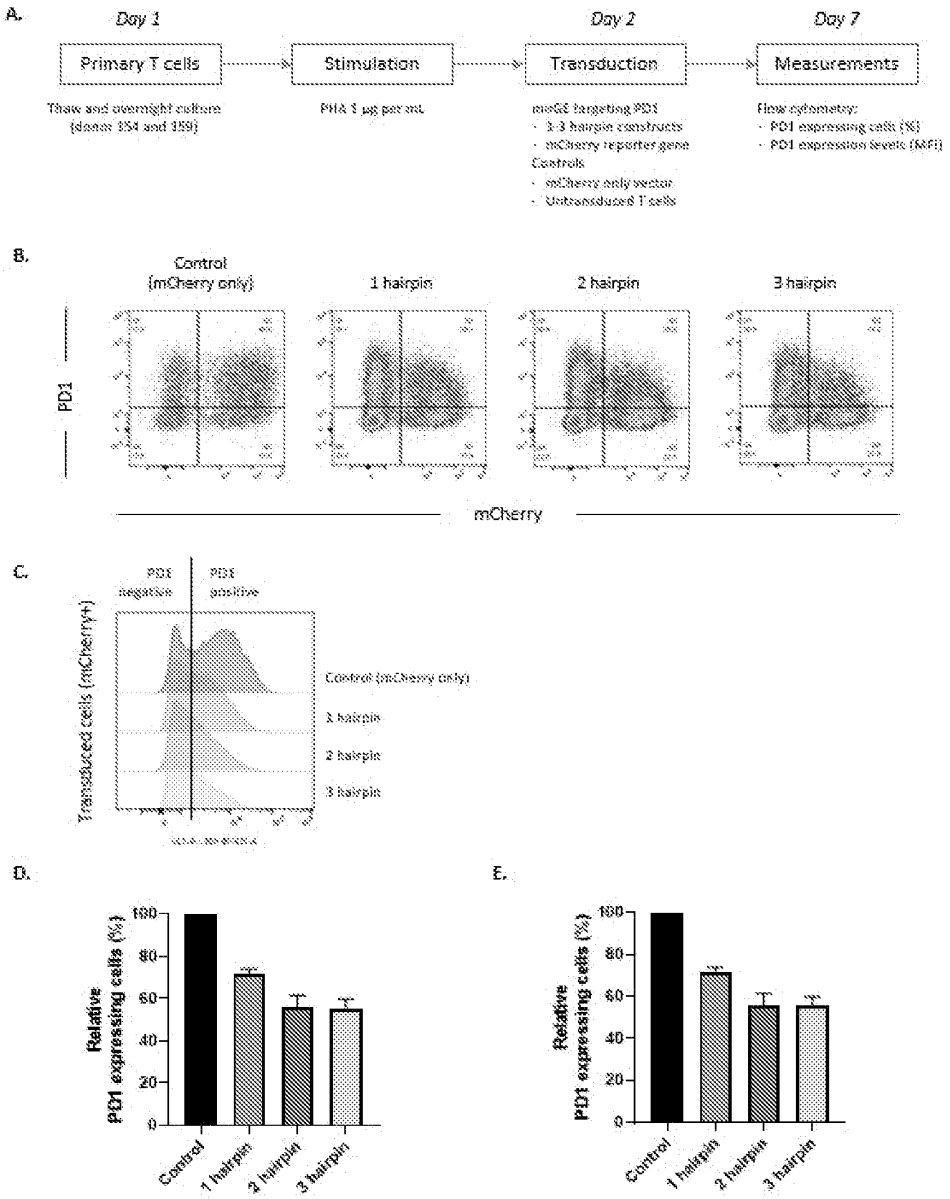

A.
Day 1
Day 2
Day 7
Primary T cells
Stimulation
Transduction
Measurements
Thaw and overnight culture (donor 354 and 359)
PHA 5 µg per mL
miGE targeting PD1
· 1-3 hairpin constructs
· mCherry reporter gene
Controls
· mCherry only vector
· Untransduced T cells
Flow cytometry:
· PD1 expressing cells (%)
· PD1 expression levels (MFI)
B.
Control (mCherry only)
1 hairpin
2 hairpin
3 hairpin
PD1
mCherry
C.
PD1 negative
PD1 positive
Transduced cells (mCherry+)
Control (mCherry only)
1 hairpin
2 hairpin
3 hairpin
D.
Relative PD1 expressing cells (%)
Control
1 hairpin
2 hairpin
3 hairpin
E.
Relative PD1 expressing cells (%)
Control
1 hairpin
2 hairpin
3 hairpin

FIG. 16

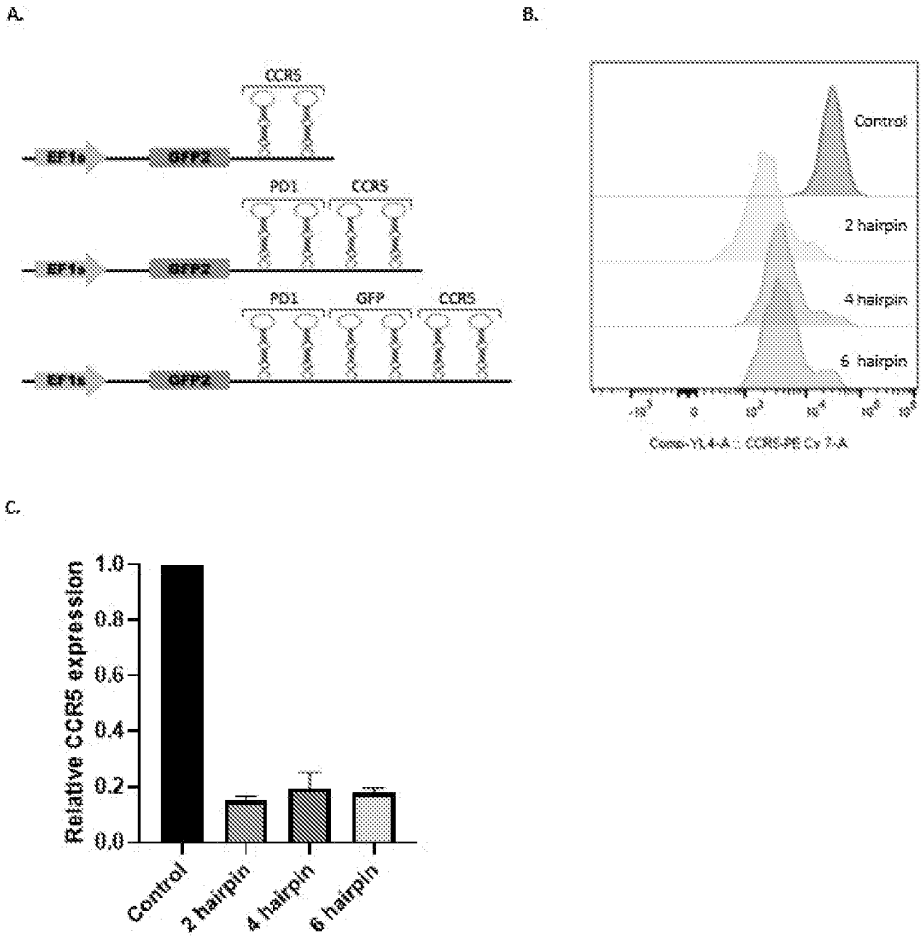

A.
CCR5
EF1α
GFP
PD1
CCR5
EF1α
GFP
PD1
GFP
CCR5
EF1α
GFP
B.
Control
2 hairpin
4 hairpin
6 hairpin
C.
Relative CCR5 expression
1.0
0.8
0.6
0.4
0.2
0.0
Control
2 hairpin
4 hairpin
6 hairpin

FIG. 17

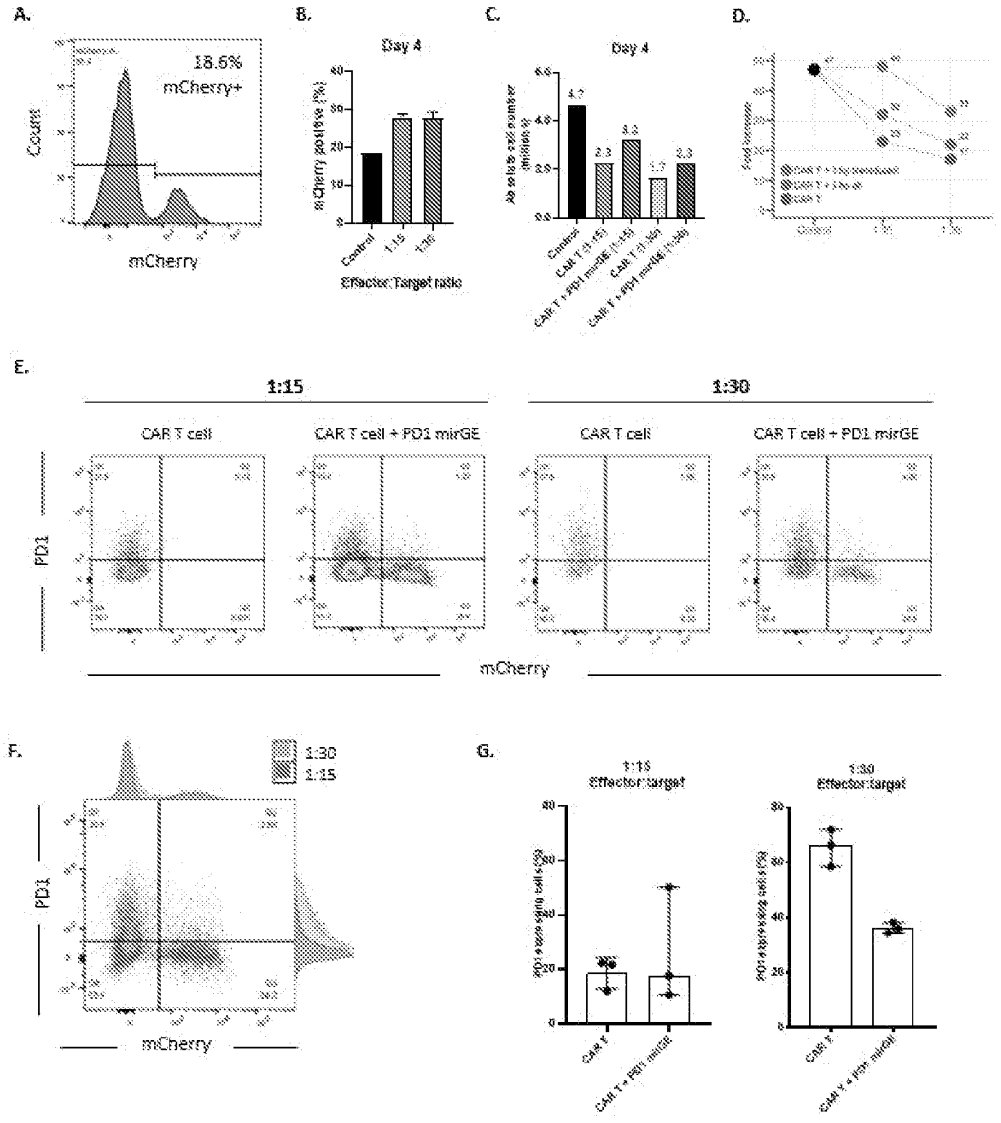

A.
18.6%
mCherry+
Count
mCherry
B.
Day 4
mCherry positive (%)
Control
1:15
1:30
Effector:Target ratio
C.
Day 4
Control
CAR T (1:45)
CAR T + PD1 miRGE (1:45)
CAR T (1:30)
CAR T + PD1 miRGE (1:30)
D.
E.
1:15
1:30
CAR T cell
CAR T cell + PD1 miRGE
CAR T cell
CAR T cell + PD1 miRGE
PD1
mCherry
F.
1:30
1:15
PD1
mCherry
G.
1:15
Effector:target
1:30
Effector:target
CAR T
CAR T + PD1 miRGE

FIG. 18

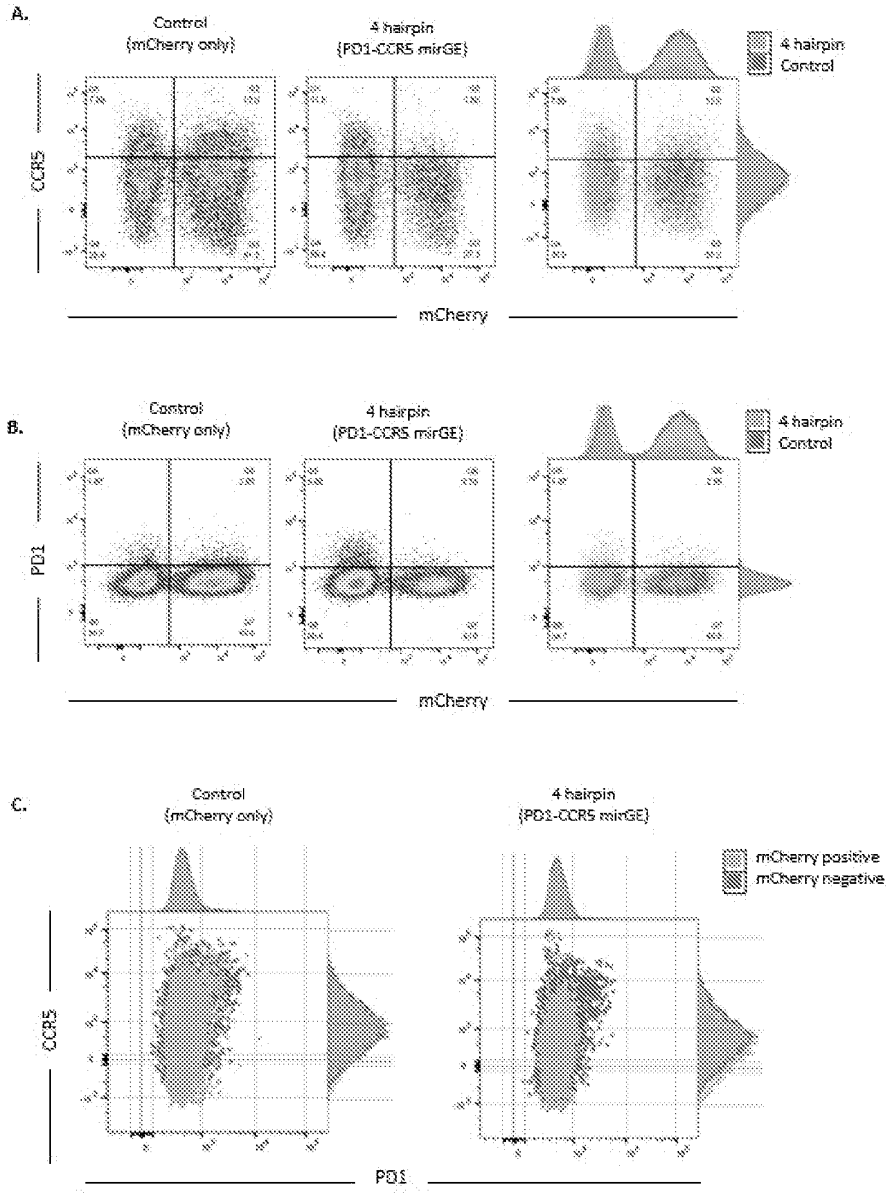

A.
Control
(mCherry only)
4 hairpin
(PD1-CCR5 mirGE)
4 hairpin
Control
CCR5
mCherry
B.
Control
(mCherry only)
4 hairpin
(PD1-CCR5 mirGE)
4 hairpin
Control
PD1
mCherry
C.
Control
(mCherry only)
4 hairpin
(PD1-CCR5 mirGE)
mCherry positive
mCherry negative
CCR5
PD1

FIG. 19