A novel method for determining functional LDL receptor activity in familial hypercholesterolemia: Application of the CD3/CD28 assay in lymphocytes

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A Novel Method for Determining Functional LDL Receptor Activity in Familial Hypercholesterolemia: Application of the CD3/CD28 Assay in Lymphocytes

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Abbreviated title: An improved assay for LDL receptor activity using anti-CD3/CD28 beads

Abbreviations: DiI, 3,3”--dioctadecylindocarbocyanin; FH, familial hypercholesterolemia; NARC-1, neural apoptosis-regulated convertase 1; FITC, fluorescein isothiocyanate; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; LDLR, low-density lipoprotein receptor; LPDS, lipoprotein deficient serum; rIL-2, recombinant interleukin-2; MF, mean fluorescence; TC, total cholesterol; CV, coefficient of variation.

Abstract

Background: The objective of this study was to develop a new and simple method for measuring low-density lipoprotein receptor (LDLR) activity using peripheral lymphocytes enabling us to clinically diagnose familial hypercholesterolemia (FH) and ascertain the involved mutations (such as K790X mutation), that might not be clearly detected in the conventional method.

Methods: Our method comprised the following 2 features: First, we used anti-CD3/CD28 beads to stimulate T-lymphocytes to obtain a uniform fraction of lymphocytes and maximum up-regulation of LDLR. Second, we excluded the possibility of overestimation of lymphocyte signals bound only to its surface, by adding heparin to the cultured lymphocytes used for the LDLR assay.

Results: Based on the genetic mutation, the FH subjects were divided into two groups, K790X, (n = 20) and P664L, (n = 5), and their LDLR activities was measured by this method, which was found to be 55.3 ± 8.9% and 63.9 ± 13.8%, respectively, of that of the control group (n = 15). In comparison, the LDLR activity was 86.1 ± 11.6% (K790X) and 73.3 ± 6.3% (P664L) of that of the control group when measured by the conventional method, indicating that impairment of LDLR function in FH K790X subjects was much more clearly differenciated with our method than with the conventional method (paired t-test, $p < 0.0001$). The levels of LDLR expression also showed similar tendencies, that is, 89.4 ± 13.2% (K790X) and 76.9 ± 17.4% (P664L) of that of the control group when measured by the conventional method, and 78.1 ± 9.7% (K790X) and 70.3 ± 26.5% (P664L) when measured by our new method. In addition, we confirmed that there was little influence of statin treatment on LDLR activity among the study subjects when our method was used.

Conclusion: These results demonstrate that our new method is applicable for measuring LDLR

activity, even in subjects with an internally defective allele, and that T-lymphocytes of the FH K790X mutation possess characteristics of that allele.

Supplementary key words: familial hypercholesterolemia, K790X, P664L, low-density lipoprotein cholesterol receptor activity, anti-CD3/CD28 antibody, T-lymphocyte, heparin

1. Introduction

Familial hypercholesterolemia (FH), a genetic defect that causes marked elevation of plasma low-density lipoprotein cholesterol (LDL-C), tendinous xanthomas, and premature coronary artery disease, is a result of genetic abnormalities of the LDL receptor (LDLR), apolipoprotein B, and neural apoptosis-regulated convertase 1 (NARC-1) (1, 2). Although all of these abnormalities create disturbances in the metabolism of LDL, the LDLR defect is the most important and frequent cause of FH. The LDLR, which is located on the surfaces of hepatocytes and other organs, binds to LDL and facilitates both its uptake by receptor-mediated endocytosis and its delivery to lysosomes, where the LDL particle is degraded (3).

The LDLR gene comprises 18 exons that span 45 kb, and encodes a single-chain glycoprotein containing 839 amino acids in its mature form (4, 5). Currently, more than 800 different mutations have been identified worldwide (6). These mutations can be divided into 5 classes, based on their phenotypic effects: 1) null alleles; 2) transport-defective alleles; 3) binding-defective alleles; 4) internalization-defective alleles; and 5) recycling-defective alleles.

Confirmation of the diagnosis of FH requires either documentation of an LDLR gene mutation, or demonstration of a decrease in LDLR activity (1). However, clinically diagnosed FH is genetically more heterogeneous than conventionally expected (7), and approximately 40% of those diagnosed in the Japanese population do not exhibit these defects by genetic analysis (8). This proportion is similar in other countries (9). Therefore, a method that can estimate LDLR activity accurately and is complementary to genetic analysis is needed. Evaluation of LDLR activity using measurement of 3,3”-dioctadecylindocarbocyanin (DiI)-labeled LDL uptake in peripheral blood lymphocytes is conventionally used in the clinical setting (10). Although highly

sensitive, some studies have shown that FH can be distinguished from hypercholesterolemic non-FH by stimulation of T-lymphocytes (11, 12). Unfortunately, conventional methods currently available overestimate the LDLR activity of some types of defective LDLR, such as FH internalization-defective alleles, not because of the internalization but rather due to binding of DiI-LDL to the surface of lymphocytes (13). The K790X mutation, which belongs to the class of internalization-defective alleles (14), is one of the most common mutations among Japanese FH subjects with a frequency of occurrence is nearly 19.5% (8). Therefore, it is essential to develop a new and simple method to detect FH with this type of defective LDLR. If we can measure functional LDLR activity accurately, it would be easy to differenciate carriers of apolipoprotein B and NARC-1 mutations from carriers of LDLR mutations among clinically diagnosed FH subjects, as LDLR activity of the former would be in the normal range.

On the other hand, P664L mutation, which is classified as a transport-defective allele (15), is also one of the common mutations among Japanese FH subjects, and its frequency of occurrence is 6% (8).

Binding, internalization, and degradation were measured in the presence of sulfated glycosaminoglycans, such as heparin, to remove LDL from the surface of LDLR when skin fibroblasts were used (16). Suzuki et al used rIL-2 and anti-CD3 monoclonal antibodies to stimulate lymphocytes for proliferation (17). Recently, an anti-CD3/CD28 monoclonal antibody with functional properties that allow easy stimulation of T-lymphocytes with high specificity became available (18). In this study, we have developed a novel and simple method for detecting internalization-defective LDLR activity, especially in FH with an internalization-defective allele, by application of anti-CD3/CD28 beads stimulation and heparin-mediated assay of lymphocytes.

2. Materials and Methods

Subjects

The study subjects were 25 genetically determined heterozygous FH subjects and 15 normal controls. The FH subjects were divided into 2 groups, based on their LDLR gene mutation: K790X (n = 20) and P664L (n = 5) (Table 1). Informed consent was obtained from all the subjects.

Lipid Measurements

Fasting blood samples were drawn for assays. Concentrations of serum total cholesterol (TC), triglyceride, and high-density lipoprotein cholesterol (HDL-C) were determined enzymatically. Low-density lipoprotein cholesterol concentrations were calculated using the Friedewald formula (19).

Molecular Analysis

Genomic DNA was isolated from the buffy coat of a centrifuged 5-ml blood sample anticoagulated with disodium EDTA, in accordance with the standard method. PCR-denaturing gradient gel electrophoresis, DNA sequencing, and Southern blot analysis were performed as descrived in our previous paper (8).

Isolation and culture of T-lymphocytes

Peripheral blood lymphocytes were isolated at room temperature using a density gradient

method (Ficoll-Paque; GE Healthcare UK, Little Chalfont, UK) from EDTA-anticoagulated blood samples. Cells were then washed 3 times in PBS and cultured at 37°C in 5% CO_2 at a concentration of 1×10^6 cells/mL in a lipoprotein-deficient serum (LPDS), 100 U/mL penicillin/streptomycin, 20 U/mL rIL-2 (COSMO BIO, Tokyo, Japan), and with or without the anti-CD3/CD28 beads (Dynal Biotech, Oslo, Norway), the number of which is equal to that of lymphocytes. A culture period of 72h was determined for 2 reasons. First, the increase of LDLR expression plateaued at 72h in our preliminary examination, and second, lymphocytes are usually cultured for 72h in conventionally available methods.

T-lymphocyte subpopulations were identified by labeling specific differenciated surface antigens with fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies (CD3-FITC; BECKMAN COULTER, Fullerton, CA).

Flow Cytometry

LDLR activity.

Lymphocytes (2×10^5) cultured in either LPDS or anti-CD3/CD28 beads were resuspended in PBS-1% BSA-containing $CaCl_2$. A wash step with PBS-1% BSA was repeated twice, and the cells were incubated at 37°C in 5% CO_2 for 2 h with 10 μg/mL DiI-LDL (Molecular Probes, Eugene, OR). Subsequently, the samples were washed 3 times with PBS-1% BSA and incubated at 4°C for 1 h in the dark, with or without 10 mg/mL heparin (Sigma-Aldrich, St. Louis, MO), to release the surface-binding DiI-LDL. Finally, the samples were incubated at 4°C for 30 min in the dark with CD3-FITC, re-washed 3 times with PBS-1% BSA, and resuspended in cold PBS. Measurements were performed on a FACS flow cytometer (BD Biosciences, San Jose, CA). For each sample,

fluorescence intensity of 10,000 events was recorded for data analysis. The results were expressed as the mean fluorescence (MF) of CD3-positive gated cells, and we subtracted the fluorescence of a sample incubated with 20 mM EGTA to exclude non-specific bindings.

LDLR expression.

Lymphocytes (2×10^5) cultured in either LPDS or anti-CD3/CD28 beads, were resuspended in PBS containing 10 g/L BSA (PBS-1% BSA). A wash step with PBS-1% BSA was repeated twice, and the cells were incubated at 4°C for 1 h with monoclonal antibody specific for the LDLR IgG-12D10 (BML, Saitama, Japan) diluted in PBS-1% BSA (14). The samples were subsequently washed 3 times with PBS-1% BSA and incubated at 4°C for 30 min in the dark with a secondary antibody (Dako, Glostrup, Denmark). Finally, the samples were incubated at 4°C for 30 min in the dark with CD3-FITC, re-washed 3 times with PBS-1% BSA, and resuspended in cold PBS. Measurements were performed as described above. The results were expressed as the MF of the CD3-positive gated cells, and we subtracted the fluorescence of a sample incubated only with a secondary antibody to exclude non-specific bindings.

Statistical analyses

Values are expressed as mean ± SD unless otherwise stated. Differences in changes were compared using a paired t-test. The level of statistical significance was set at $p < 0.05$. Statistical analysis was performed using StatView 5.0 (SAS Institute Japan, Tokyo, Japan). To evaluate the precision of the assay, we estimated the coefficient of variation (CV), in terms of within-run and between-day precision, by making repeated measurements of blood samples from one study

subject using both the methods.

3. Results

Lymphoblastogenesis patterns and LDLR up-regulation by LPDS and anti-CD3/CD28 beads

Light microscopic observation revealed that, compared to the lymphocytes cultured with LPDS, lymphocytes cultured with anti-CD3/CD28 beads transformed more effectively into lymphoblasts. Stimulated lymphoblasts could be separated from the unstimulated ones according to areas in flow cytometric analysis (Fig.1). When the cells were cultured with LPDS, no significant increase in LDLR expression was observed during the culture period and the increase plateaued by 72 h (Fig. 2A). In contrast, LDLR expression significantly increased by 24 h, and peaked at 48 to 72 h in beads with anti-CD3/CD28 (Fig. 2B). LDLR expression increased 2- and 6.5-fold from the baseline value after co-culture with LPDS and anti-CD3/CD28 beads, respectively. The rate of increase did not differ between a normolipidemic subject and heterozygous FH subjects, but differed between heterozygous FH subjects and a compound heterozygote subject. Most importantly, however, the levels of LDLR expression in all subject categories plateaued by 72h. The subjects' clinical profiles are displayed in Table 2.

LDLR activity and expression in cultured lymphocytes obtained from heterozygous FH subjects with K790X and P664L mutations

MF was examined in each method; results were expressed as percentages of the mean control MF in each experiment. When lymphocytes were cultured with LPDS and washed with PBS alone according to the conventional method, LDLR activity of the K790X and P664L subjects were 86.1 ± 11.6% and 73.3 ± 6.3%, respectively (Fig. 3A). When lymphocytes were cultured with anti-CD3/CD28 beads and washed with PBS-containing heparin, the LDLR activity was 55.3 ±

8.9% and 63.9 ± 13.8%, respectively (Fig. 3B). Compared with the values obtained from the conventional method, our method allowed improved discrimination between FH and non-FH subjects with both mutations of different phenotypic classes.

Our method showed an LDL-R activity of lower than 80% of the normal control values in all of the K790X subjects, whereas in the conventional method, most of those subjects (almost 75% of the subjects) surpassed this value. Furthermore, there was a significant difference in LDLR activity between these 2 methods ($p < 0.0001$). In contrast, P664L subjects showed almost similar LDLR activity with both the methods.

The LDLR expression in the K790X and the P664L subjects was 89.4 ± 13.2% and 76.9 ± 17.4%, respectively, of that of control group when measured by the conventional method (Fig. 4A), but it was 78.1 ± 9.7% and 70.3 ± 26.5%, respectively, when measured by our new method (Fig. 4B). The differences in the LDLR expression of K790X subjects from that of the normal control group were more pronounced ($p < 0.05$) than those of the P664L group, when comparing the values obtained by our new method with those of the conventional method.

Confirmation of the effect of statins on LDLR activity

We examined the effect of statins (atorvastatin, 10 mg or 20 mg) on the values obtained from the conventional method, and the new method among some study subjects. There was little influence of statin treatment on LDLR activity measured by either method (Table 3).

Precision

Within-run precision (CV) for our assay and the conventional assay of LDLR activity was

9.1% and 8.6%, and that of LDLR expression was 3.4% and 8.6%, respectively. Between-day precision (CV) for both assays of LDLR activities was 5.5% and 8.5%, and that of LDLR expression was 3.4% and 8.5%, respectively.

4. Discussion

The three main findings of the study are as follows: 1) We developed a new and simple method to measure LDLR activity and expression in cultured peripheral lymphocytes, using anti-CD3/CD28 antibody for culturing and heparin for washing. 2) Heterozygous FH subjects with a K790X mutation showed much more pronounced reductions in LDLR activity and expression with our new method compared with the conventional method. 3) In FH subjects with a P664L mutation, the degree of reduction detected with our new method was almost equal to that detected with the conventional method.

To our knowledge, only few studies have investigated the relationship between LDLR genotype and its activity (20). Although there have been a number of studies on LDLR activity measurements in FH using DiI-LDL uptake in blood peripheral lymphocytes, some causes of defective LDLR activity, such as the K790X mutation, often appear to have been overlooked.

The 50-residue cytoplasmic domain of LDLR (amino acids 790 to 839) directs the receptors to coated pits, thereby facilitating rapid endocytosis of bound LDL (21). Therefore, any mutations encoded in this region are considered to be internalization-defective alleles. As for the K790X mutation, Iwasaki et al showed that Chinese hamster ovary (CHO) cells, which expressed the LDLR-K790X mutation, showed defective endocytosis of LDL and indicated that this mutation could be classified as an internalization-defective allele (14). In addition, LDL, which is bound to

the LDLR, cannot be released solely by washing with PBS; therefore, the conventional method, which only uses PBS, overestimates the values of defective LDLR.

In this study, MF of DiI greatly decreased in the K790X subjects, unlike the P664L subjects, when receptor assays were performed by washing lymphocytes with heparin (Fig. 3). These results indicate that T-lymphocytes of the K790X mutation also possess a characteristic similar to the internalization-defective allele; therefore, a conventional assay using lymphocytes would fail to measure their values accurately. On the other hand, the P664L mutation is classified as a transport-defective allele, meaning that conventional assays can measure mostly accurate values (15).

A series of trials have shown the efficacy of cholesterol-lowering therapy using HMG-CoA reductase inhibitors, statins, for primary and secondary prevention of coronary artery disease (22, 23). It has also been observed that statins up-regulate hepatic LDLR expression and inhibition of HMG-CoA reductase results in a secondary increase in LDLR activity (3, 24, 25). Therefore, it is possible that statin therapy might affect the results even though peripheral lymphocytes, and not hepatocytes, were used in this study (26, 27). There have been some conflicting reports about the relationship between statin therapy and LDLR activity of lymphocytes (12).

We confirmed whether or not statin therapy affected the results in this study. We compared the LDLR activity using 2 different samples, either under statin treatment or not, from the same study subject. However, we failed to find any effects of statin treatment on lymphocyte LDLR activity measured by our method (Table 3). One explanation for this could be that stimulation of lymphocytes by anti-CD3/CD28 beads have far greater influence on LDLR expression of peripheral lymphocytes than statin therapy; the second is that the effects of statin therapy on

peripheral lymphocytes are too small to affect the results during the culture period. Consequently, there is no need to discontinue statin therapy to examine LDLR activity.

This study has several limitations. First, a relatively small number of subjects was included in this study. However, our subjects were comparatively uniform—there were no statistically significant differences in age, BMI, and lipid profiles between the K790X and the P664L group. Second, we confirmed the influence of statins on LDLR activity in only some subjects included in this study (Table 3), since FH is extremely high-risk to the patients. And third, only two kinds of mutations were examined because FH is too genetically heterogeneous to study a consistent number of patients with the same LDLR mutation (8). In addition to genetic heterozygosity, LDLR internalization-defective alleles are relatively rare among LDLR mutations. Therefore, we were unable to perform this assay for LDLR internalization-defective allele other than the K790X mutation.

In conclusion, our new method enables us to estimate the function of LDLR more accurately and to diagnose FH with higher sensitivity, particularly in LDLR class 4 mutations (internalization-defective type), compared with the conventional method. We suggest that this method is applicable for the diagnosis of hyperlipidemic patients who cannot be accurately diagnosed by conventional methods.

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Figure Legends

Figure 1. Pattern of lymphoblastogenesis by LPDS and anti-CD3/CD28 beads

A: Light microscopic findings of lymphoblastogenesis cultured with LPDS for 72 h. Magnification 400×. B: Light microscopic findings of lymphoblastogenesis cultured with anti-CD3/CD28 beads for 72 h. Beads are round (indicated by yellow arrows) while lymphocytes have a distorted shape. Magnification 400×. C: Forward-scatter (FSC) versus side-scatter (SSC) plots for lymphoblastgenesis cultured with LPDS for 72 h. D: FSC versus SSC plots for culture with anti-CD3/CD28 beads for 72 h. Stimulated lymphoblasts can be distinguished from unstimulated lymphoblasts according to their areas (surrounded by red circles).

Figure 2. LDLR up-regulation by culture with LPDS and anti-CD3/CD28 beads for a period of 5 days

This experiment was performed with a subset of the study subjects, whose profiles and clinical data are provided in Table 2.

A: LDLR expression (MF) of 4 subjects was measured for 5 days. Lymphocytes (2×10^5) cultured with LPDS.

B: LDLR expression (MF) measured in the same way except lymphocytes were cultured with anti-CD3/CD28 beads, the number of which is equal to that of lymphocytes.

Measurements were performed for CD3-positive T-lymphocytes only.

Figure 3. LDLR activity measured by two methods

The MF was examined in each method, and the results were expressed as percentages of

the mean fluorescence of the control group in each experiment. A: Internalized DiI-LDL (%) measured by the conventional method. Normal: 100 ± 15.8%, K790X: 86.1 ± 11.6%, P664L: 73.3 ± 6.3%, B: Internalized DiI-LDL (%) measured by our new method. Normal: 100 ± 23.5%, K790X: 55.3 ± 8.9%, P664L: 63.9 ± 13.8%. Differences in value between the K790X group and the control group measured using the new method were significant compared with the difference in the values obtained using the conventional method. Values are expressed as mean ± SD. *$p < 0.05$, **$p < 0.0001$.

Figure 4. LDLR expression measured by two methods

The MF was examined in each method, and the results were expressed as percentages of the MF of the control group in each experiment. A: LDLR expression (%) measured by the conventional method. Normal: 100 ± 20.4%, K790X: 89.4 ± 13.2%, P664L: 76.9 ± 17.4%, B: LDLR expression (%) measured by our new method. Normal: 100 ± 21.5%, K790X: 78.1 ± 9.7%, P664L: 70.3 ± 2.6%. Differences in the value between the K790X group and the control group were significant. Values are expressed as mean ± SD. *$p < 0.05$.

Fig. 1

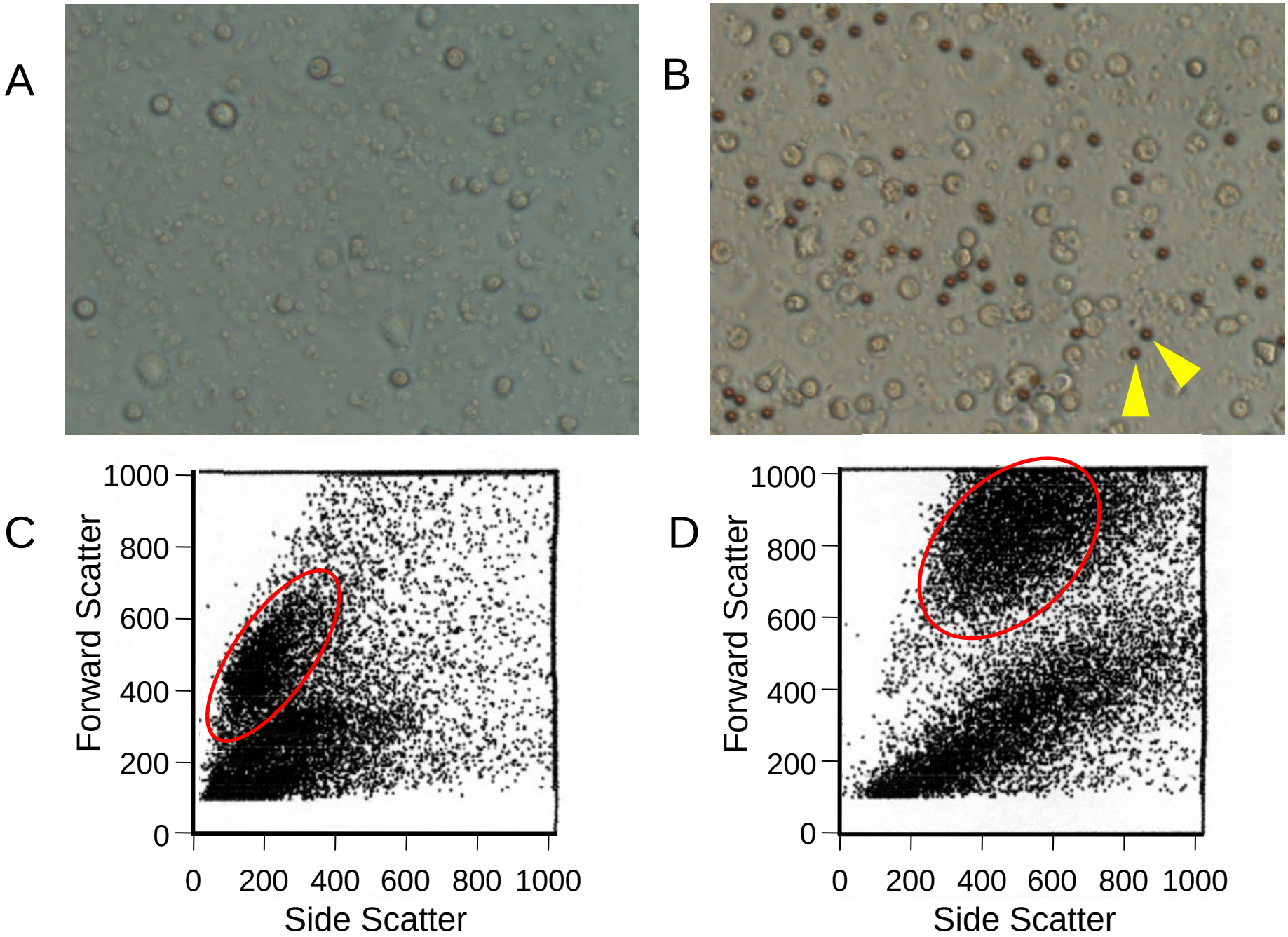

Fig. 2

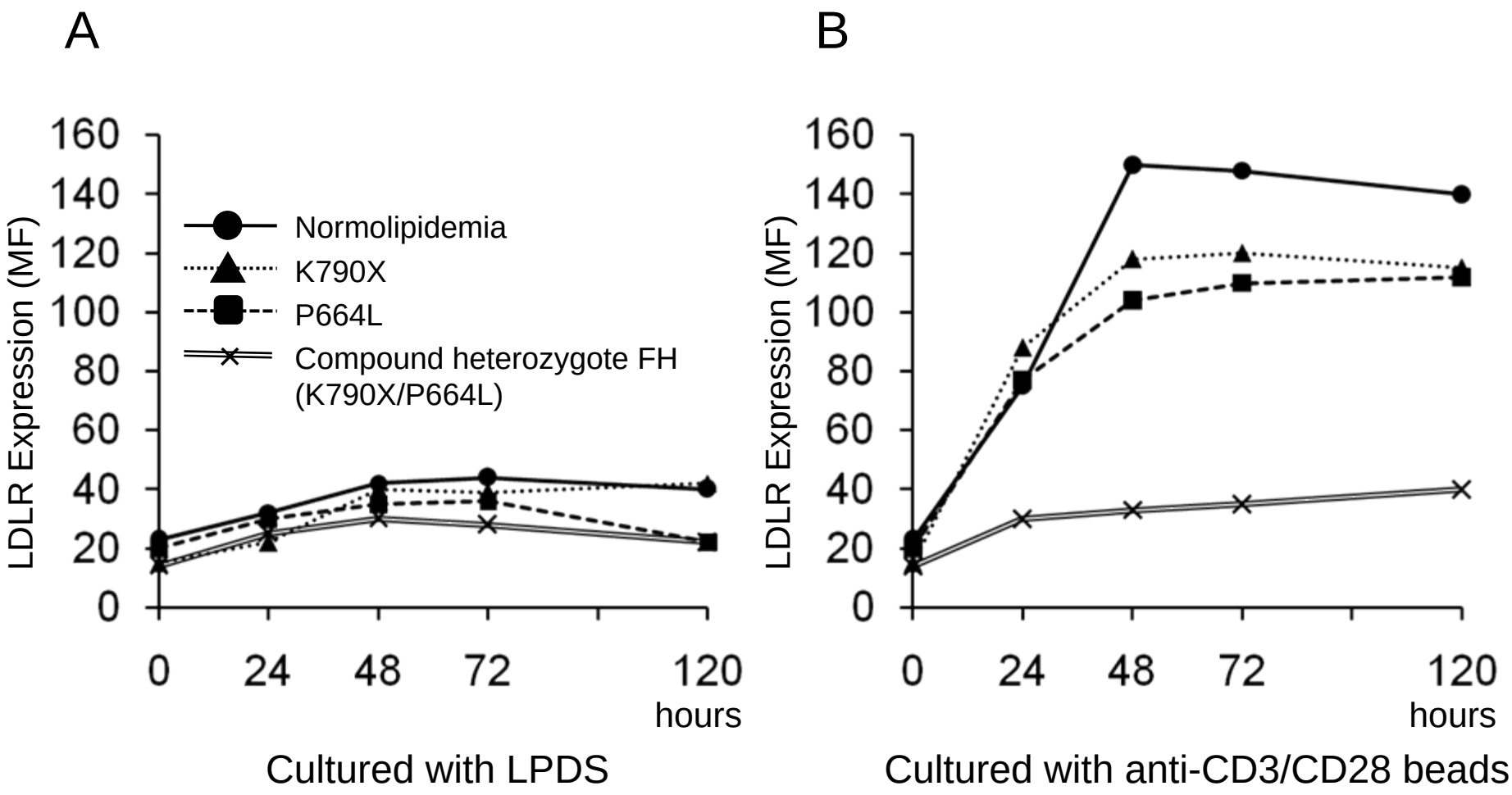
A
B
LDLR Expression (MF)
160
140
120
100
80
60
40
20
0
Normolipidemia
K790X
P664L
Compound heterozygote FH
(K790X/P664L)
0
24
48
72
120
hours
Cultured with LPDS
Cultured with anti-CD3/CD28 beads

Fig. 3

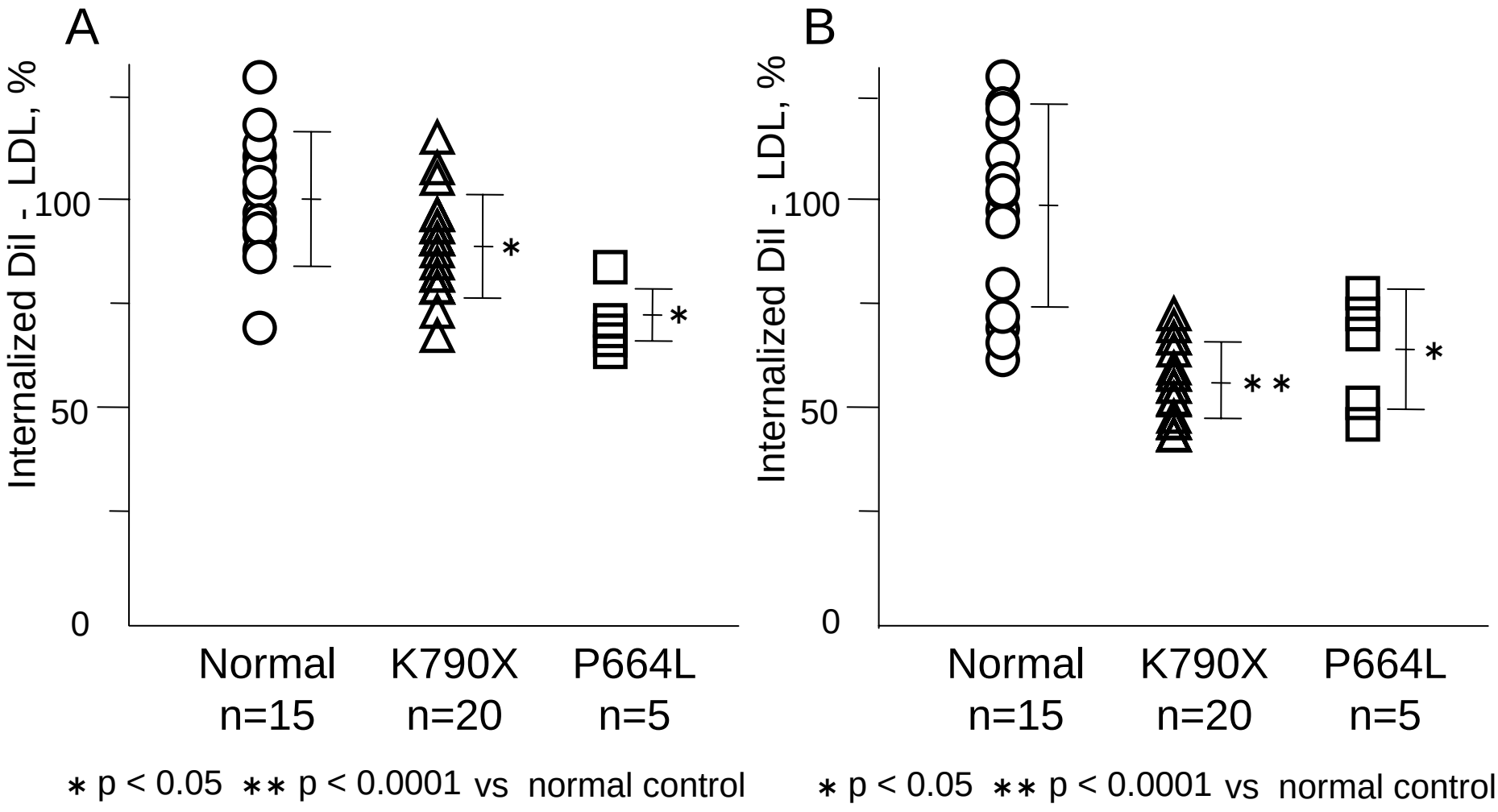
A
Internalized DiI - LDL, %
100
50
0
*
*
Normal
n=15
K790X
n=20
P664L
n=5
* p < 0.05 ** p < 0.0001 vs normal control
B
Internalized DiI - LDL, %
100
50
0
**
*
Normal
n=15
K790X
n=20
P664L
n=5
* p < 0.05 ** p < 0.0001 vs normal control

Fig. 4

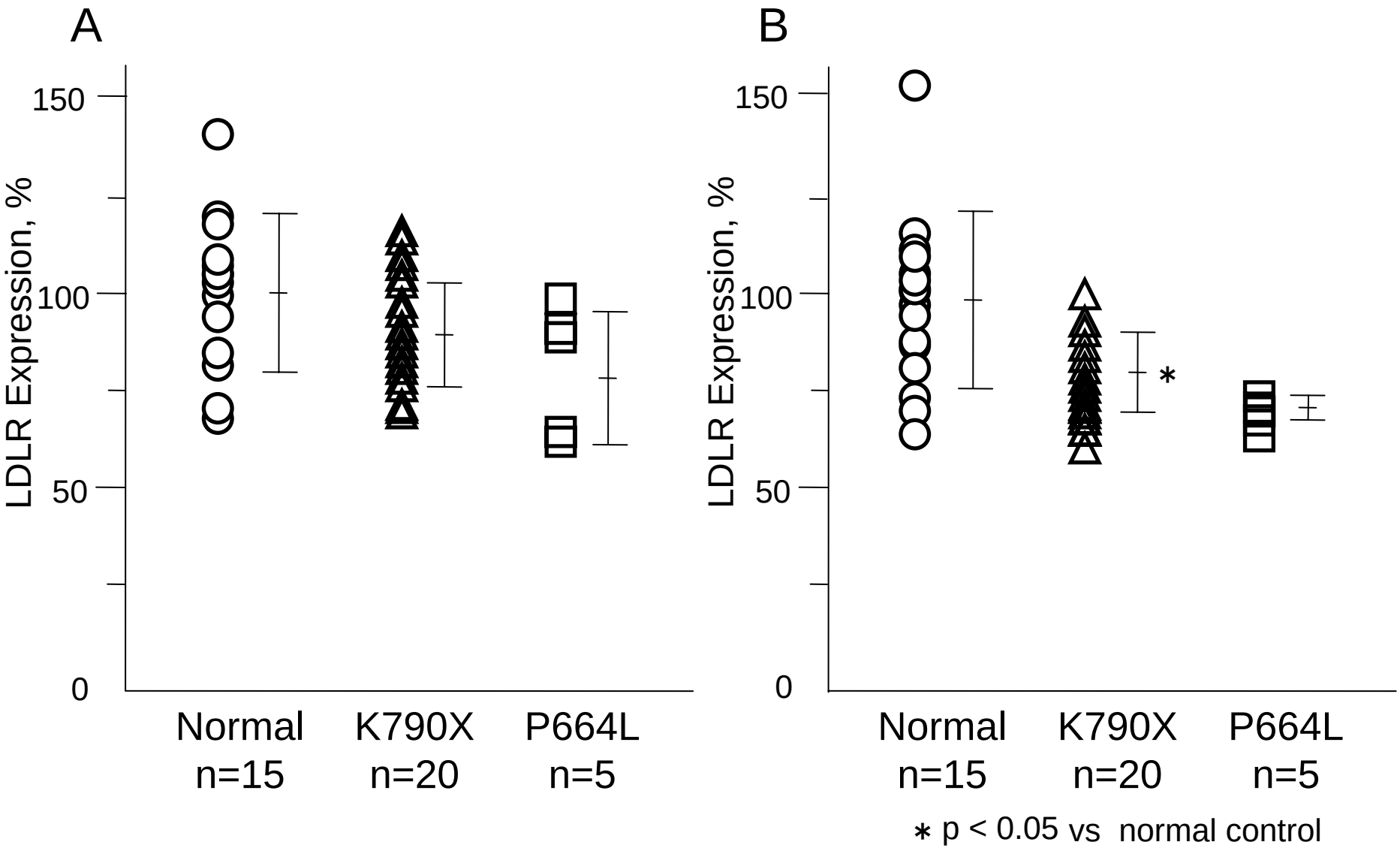
A
B
150
100
50
0
LDLR Expression, %
Normal
n=15
K790X
n=20
P664L
n=5
*
* p < 0.05 vs normal control

TABLE 1. Plasma lipid concentrations in study subjects

subjects	gender (m/f)	age (yr.)	BMI (kg/m^2)	TC (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)	TG (mg/dl)
normolipidemia (n=15)	6/9	67 ± 16	23.5 ± 3.0	184 ± 29	53 ± 17	114 ± 28	89 ± 30
FH heterozygous K790X	10/10	52 ± 16	23.6 ± 2.8	342 ± 59 *	47 ± 14	274 ± 54 *	95 ± 50
FH heterozygous P664L	4/1	51 ± 23	21.3 ± 3	366 ± 50 *	42 ± 7	283 ± 67 *	114 ± 47

Values are shown as mean±SD. Lipid concentrations were measured before any medication was prescribed.
FH, familial hypercholesterolemia; BMI, body mass index; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride;
* $p<0.0001$, data compared with normolipidemia

TABLE 2. Plasma lipid concentrations in LDLR up-regulation study subjects

subjects	gender (m/f)	age (yr.)	BMI (kg/m^2)	TC (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)	TG (mg/dl)
normolipidemia	m	26	18	177	77	86	70
FH heterozygous K790X	f	28	21	307	47	249	55
FH heterozygous P664L	f	23	22	428	42	358	139
FH compound heterozygote K790X/P664L	m	37	21	589	26	544	96

FH, familial hypercholesterolemia; BMI, body mass index; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride;

TABLE 3. Examination for the influences of statin therapy on LDLR activity measured by T-Lymphocytes

Method	statin on	statin off	p
new method (%)	53 ± 9	56 ± 8	0.7219
conventional method (%)	87 ± 8	94 ± 23	0.5799

Values were collected from 5 subjects whose lipid-lowering agents had been discontinued at least for 2 weeks