



Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcεRI or FcγRI

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Abstract

Two novel stem cell factor (SCF) dependent human mast cell lines, designated LAD 1 and 2, were established from bone marrow aspirates from a patient with mast cell sarcoma/leukemia. LAD 1 and 2 cells have the ultrastructural features of human mast cells, and express FcεRI, CD4, 9, 13, 14, 22, 31, 32, 45, 64, 71, 103, 117, 132, CXCR4 (CD184), CCR5 (CD195); and intracytoplasmic histamine, tryptase and chymase. LAD 1 and 2 do not exhibit activating mutations at codon 816 of *c-kit*. Both LAD 1 and 2 release β-hexosaminidase following FcεRI or FcγRI aggregation. The availability of these cell lines offers an unparalleled circumstance to examine the biology of human mast cells. Published by Elsevier Science Ltd.

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1. Introduction

Mast cells are now documented to have a major role in innate and acquired immunity, and disorders of mast cell proliferation are well known. The ability, however, to study human mast cells has been limited by the absence of suitable long term human mast cell cultures. For the past 13 years, the only cell culture available to researchers and bearing any resemblance to human mast cells has been “human mast cell leukemia cells”, designated HMC-1 [1]. But HMC-1 cells have two deficiencies which limit usefulness: HMC-1 cells are growth factor independent [1], and inconsistently degranulate to IgE-dependent signals, presumably due to variable expression of the FcεRI α-subunit [2].

During routine study of cells from bone marrow aspirates from a patient with mast cell sarcoma/leukemia in SCF-containing serum-free media, we observed cultures of mast cells with functional FcεRI and FcγRI receptors which continued to proliferate. These unique cell cultures, now designated LAD 1 and 2, have been separately maintained in culture for over 24 months, frozen and recovered successfully, and characterized at the cellular and molecular levels. As will be shown, these mast cells resemble CD34⁺-derived human mast cells.

2. Materials and methods

2.1. Genesis of LAD cultures

Human bone marrow cells were obtained from a 44-year-old male during the diagnosis of, and assessment for, mastocytosis. Examination of bone marrow biopsies and aspirates were consistent with an aggressive form of mast cell sarcoma/leukemia [3]. Five different bone marrow aspirates and biopsies were performed, beginning in

Abbreviations: SCF, stem cell factor; LAD, laboratory of allergic diseases; IL, interleukin; SFM, serum-free media; FBS, fetal bovine serum; RT-PCR, reverse transcriptase polymerase chain reaction; IFN-γ, interferon gamma

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April 2000. Despite treatment, the patient's condition deteriorated and he expired. Mononuclear cells from the aspirates were cultured in serum-free media (StemPro-34; Life Technologies, Grand Island, NY) supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 50 μ g/ml streptomycin (complete SFM), 100 ng/ml rhSCF, with or without 100 ng/ml rhIL-6, and 30 ng/ml rhIL-3 (first week only) (Peprotech, Rocky Hill, NJ) [4]. Mast cells from these five separate cultures persisted and were separately maintained and passed in media containing rhSCF. Mast cells were established in culture as cell lines 24 months later, following characterization, demonstration of SCF dependence, and the demonstrated ability to freeze and recover these cells. Cultures were designated LAD 1–5. LAD 1 and 2 are the subject of this report. LAD 3–5 cells are still in development. For optimal cell growth, LAD cell density was maintained between 0.5×10^6 and 1×10^6 cells/ml. Cells were split 1:1 every 2–3 weeks. No mycoplasma was detected in any LAD cell lines by American type culture collection (ATCC). LAD 2 is available and now has been distributed to more than six research groups. Researchers must complete an MTA in accordance

with the NIH Office of Technology Development. Drs. Kirshenbaum, Akin and Metcalfe may distribute the cell line.

2.2. Growth and phenotypic characteristics and flow cytometric analysis

Cell growth characteristics of these cultures were examined in rhSCF and either rhIL-3, rhIL-4, rhIL-5 or rhIL-6. Kimura's stain was used to visualize mast cells on a hemocytometer, and acidic toluidine blue, Wright–Giemsa, or anti-tryptase were used for cytopreparations (Cytospin 3; Shandon, Pittsburgh, PA) [5]. Cells were also fixed in 2% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, and sectioned for electron microscopy (Hitachi 7100) [4]. To demonstrate the presence of CD surface markers, LAD 1 and 2 cells were incubated with monoclonal antibodies for CD2, 3, 4, 5, 8, 9, 13, 14, 15, 16, 19, 20, 22, 25, 31, 32, 34, 35, 37, 41a, 45, 56, 64, 72, 103, 117, 124, 125, 132, 184, 195, glycophorin A (CD235a) and Fc ϵ RI. Cells were also analyzed for intracellular tryptase and chymase by flow cytometry [4,5]. Histamine was determined

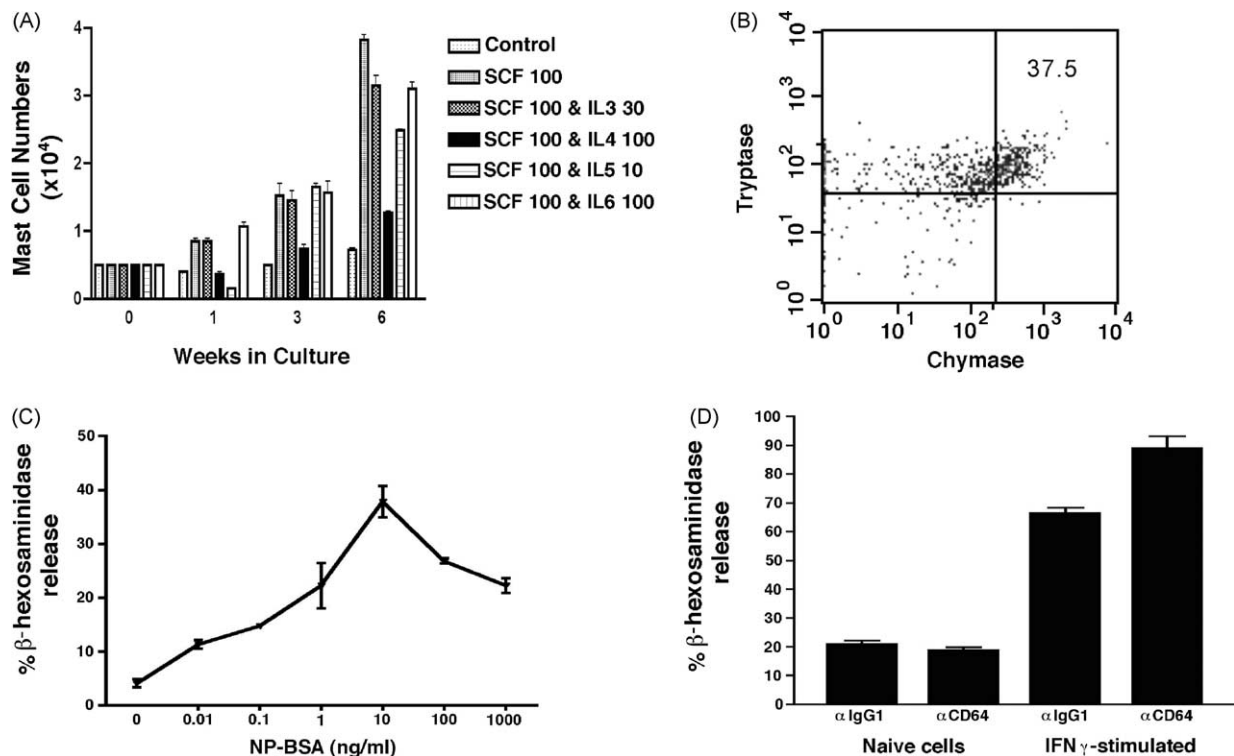


Fig. 1. Demonstration of LAD 1 mast cell growth, tryptase and chymase expression, and Fc ϵ RI and Fc γ RI induced β -hexosaminidase release: (A) mast cells cultured in 100 ng/ml rhSCF alone or in combination with 30 ng/ml rhIL-3, 100 ng/ml rhIL-4, 10 ng/ml rhIL-5, or 100 ng/ml rhIL-6. Results shown are mast cell numbers per ml and expressed as the mean $\pm$ S.E. of $n = 3$ separate experiments; (B) FACS analyses of intracytoplasmic tryptase and chymase expression. Isotype controls are shown as filled histograms. Results shown are representative of three experiments separately performed; (C) mast cells were incubated at 37°C overnight with 1 μ g/ml of IgE-NP followed by incubation with increasing concentrations of NP-BSA. Results are expressed as percentage of β -hexosaminidase release, basal release was 2–3%. Each data point is the mean $\pm$ S.E. of $n = 3$ experiments performed in duplicate; (D) cells were pre-incubated with or without 15 ng/ml of IFN- γ overnight. LAD cells sensitized with IgG1 followed by cross-linking with anti-IgG1 released 65% β -hexosaminidase. Cells activated with F(ab')₂ anti-CD64 fragments released 87% β -hexosaminidase. Results are expressed as percentage of β -hexosaminidase release. Basal release was 2–3%. Each data point is the mean $\pm$ S.E. of $n = 3$ experiments performed in duplicate.

using an enzyme immunoassay kit (Immunotech, Marseille, France) [6].

2.3. Storage of cells

Cells were suspended in non-DMSO, non-FBS containing pZerve cryopreservative (Celox Laboratories, St. Paul, MN) (10×10^6 cells per 1.5 ml), incubated for 30 min at 22 °C, transferred to –20 °C for 1 h, then –70 °C for 1 h, and placed in liquid nitrogen. For thawing, cells were warmed and resuspended with an additional 1.5 ml of complete SFM supplemented with rhSCF. Cells were equilibrated and gently agitated in small flasks for 6 h at 22 °C on shaker platforms at 40 rpm. Flasks were then incubated at 37 °C in 95% air, 5% CO₂. After 24 h, 3 ml of complete SFM with rhSCF was added.

2.4. β -Hexosaminidase release, codon 816 and telomerase assays

β -hexosaminidase release was measured following crosslinking of Fc ϵ RI or Fc γ RI [7]. For Fc γ RI-dependent activation, LAD cells were pre-incubated with 15 ng/ml interferon gamma (IFN- γ) overnight. *c-kit* activating mu-

tation analysis was assayed using RT-PCR and direct sequencing [8,9]. Telomerase was assayed using a telomerase polymerase reaction/ELISA kit [10].

2.5. Karyotypic analysis

To determine the karyotype of LAD 2, metaphases were harvested from 5×10^6 to 10×10^6 cells that were arrested in metaphase using colcemid overnight, placed in hypotonic potassium chloride, and fixed in methanol and acetic acid. Analysis using spectralkaryotyping was performed for studying chromosomal aberrations including aneuploidy and translocations.

2.6. Authentication of LAD 1 and 2 cell lines

To prove the derivation of LAD 1 and 2 cells lines from the patient described herein, DNA fingerprinting comparing primary patient bone marrow cells with LAD 1 and 2 cells was performed. DNA isolated from primary bone marrow, LAD 1 and 2 cells was analyzed employing six primer pairs and using the AmpFISTR Cofiler PCR amplification kit (Applied Biosystems, Foster City, CA).

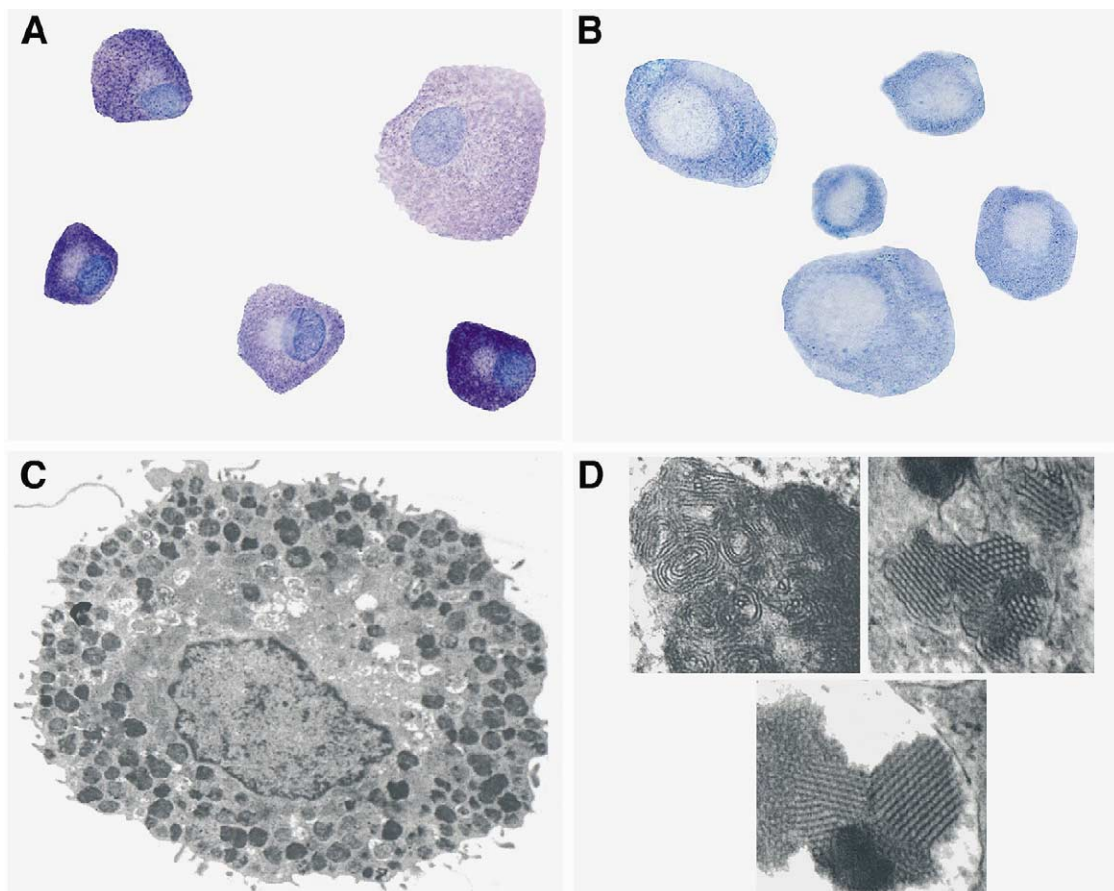


Fig. 2. Light and electron microscopy of LAD 1 human mast cells: (A) acid toluidine blue staining of five mast cells (630 $\times$); (B) tryptase staining of five mast cells (630 $\times$); (C) electron microscopy of a human mast cell showing numerous cytoplasmic projections (8000 $\times$); (D) ultrastructural examination of cytoplasmic granules with scroll patterns, lattice formation, and homogeneous material (150,000 $\times$).

3. Results

3.1. Growth and intracytoplasmic tryptase and chymase studies

LAD 1 and 2 mast cells were observed to double in number every 2–3 weeks in rhSCF (Fig. 1A). Addition of rhIL-3, rhIL-5 or rhIL-6, did not increase cell numbers. The addition of rhIL-4 reduced cell numbers (Fig. 1A). No apoptosis or necrosis was observed in the presence of rhIL-4. FACS analysis of LAD 1 and 2 cells demonstrated that greater than 98% were positive for tryptase and approximately 37% were positive for both tryptase and chymase (Fig. 1B).

3.2. Histamine and β -hexosaminidase Fc ϵ RI and Fc γ RI release studies

Histamine per cell averaged 6.5 pg per LAD 1 cell and 3.1 pg per LAD 2 cell, compared to 0.9 pg per HMC-1.1 cell. Aggregation of Fc ϵ RI on LAD 1 cells resulted in β -hexosaminidase release (Fig. 1C) of approximately 40%. LAD 1 cells were next incubated with IFN- γ to up-regulate Fc γ RI expression [7]. β -Hexosaminidase release increased from 20%, due to baseline expression of Fc γ RI receptors, to 87% when naïve cells were pre-incubated with IFN- γ and activated with F(ab')₂ anti-CD64 fragments (Fig. 1D). β -hexosaminidase data for LAD 1 was similar to LAD 2.

3.3. Morphological and ultrastructural characteristics

Acid toluidine blue and tryptase staining of LAD 1 and 2 cells (Fig. 2A and B) showed oval or round nucleated cells with metachromatically staining granules. By electron microscopy, LAD cells measured 8–15 μ m in diameter with rough surfaces and cytoplasmic projections (Fig. 2C). Cytoplasmic granules of consistent size were noted with scroll, lattice and homogeneous patterns (Fig. 2D). Analysis of aspirated bone marrow mast cells from the patient also revealed atypical spherical or oval-shaped, non-fusiform cells with pleomorphic nuclei, occasional nucleoli, numerous small granules (>100 per cell), and few if any promastocytes.

3.4. Surface phenotype of LAD cells

LAD 1 and 2 cells expressed surface Fc ϵ RI, CD4, 9, 13, 22, 45, 64, 71, 103, 117, 132, 184 and 195 markers, and to a lesser degree, CD14, 31 and 32. Aspirated bone marrow mast cells from the patient stained positive for CD13, 117, but were negative for CD2, 25, 34 and 35.

3.5. PCR analysis for codon 816 activating mutations and telomerase activity

Nucleic acid analysis determined that neither LAD 1 nor 2 cells have an activating *c-kit* mutation (Fig. 3). Tumori-

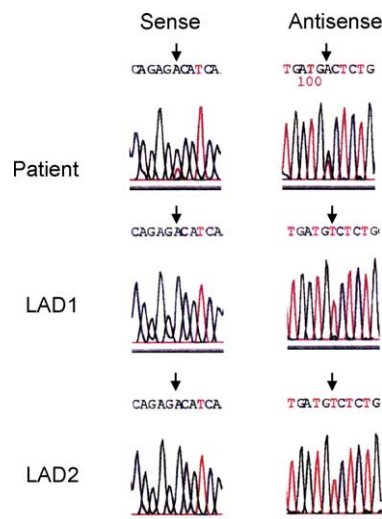


Fig. 3. Detection of *c-kit* cDNA codon 816 mutation by DNA sequencing. Total RNA was extracted from LAD cells and the peripheral blood mononuclear cells from a patient with systemic mastocytosis. The RNA was reverse transcribed with oligo (dT) primers and the resulting cDNA was used to amplify a 373 bp PCR product flanking codon 816 of *c-kit*. This PCR product was purified and sequenced from both sense and antisense directions using the BigDye terminator v3.0 chemistry and an ABI-3100 genetic analyzer according to standard protocols. The Asp816Val *c-kit* mutation (arrows) was detected in the cells from the patient with systemic mastocytosis but not in LAD 1 and 2 cells.

genic mast cells, such as RBL-2H3 and HMC-1, express persistently high telomerase activity throughout the cell cycle [10]. LAD 2 telomerase activity (arbitrary units) averaged 2200, comparable with 2000 for RBL-2H3 and 2300 for HMC-1 cells.

3.6. Karyotypic analysis

Karyotypic analysis using spectralkaryotyping of stimulated LAD 2 cells revealed a 48, XXY, +1, +5, –16 karyotype. Significant loss of material from chromosome 1 could be suggestive of –1p (Fig. 4).

3.7. Authentication of LAD 1 and 2 cell lines

Qualitative band analysis of LAD 1 and 2 cells and primary bone marrow cells employing six primer pairs showed identical band patterns, establishing the derivation of LAD 1 and 2 cells from the patient herein described.

4. Discussion

LAD 1 and 2 cells are the first reported human mast cell lines which closely resemble primary cultures of CD34⁺-derived human mast cells, respond to rhSCF (Fig. 1A), and have functional Fc ϵ RI and Fc γ RI receptors (Fig. 1B and C). Both LAD 1 and 2 cells survive in culture

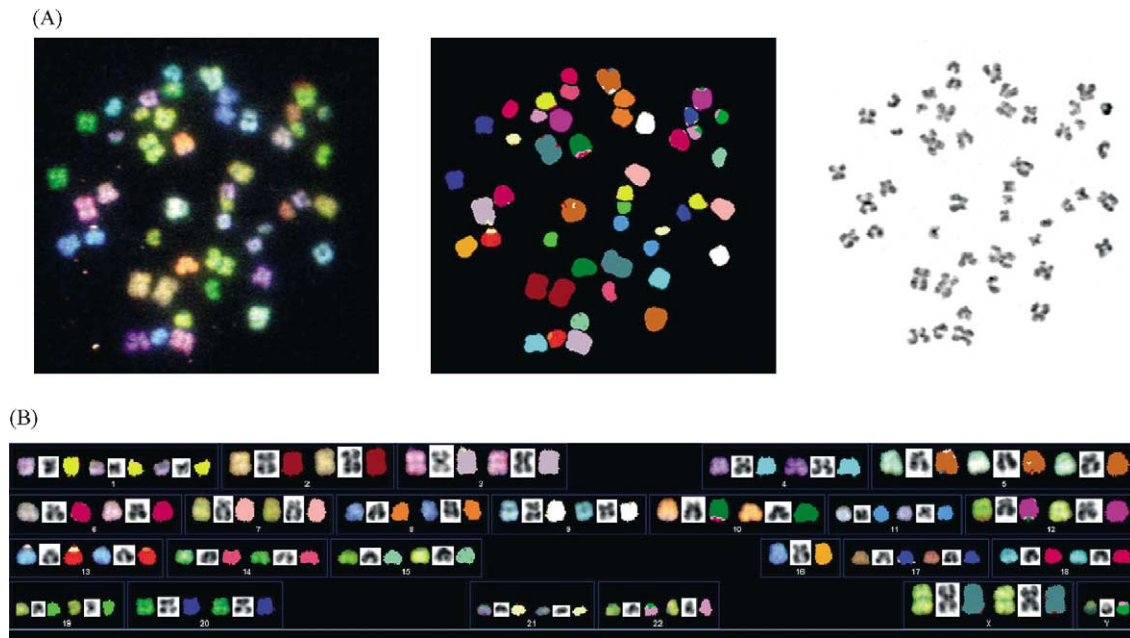


Fig. 4. Spectralkaryotyping images of a metaphase from LAD 2: (A) (left to right) hybridization, classification and DAPI images; (B) karyotype of the same metaphase showing the cell to be 48, XXY, +1, +5, -16. Significant loss of material from chromosome 1 could be suggestive of -1p. A more definitive band assignment for the deletion on chromosome 1 using longer chromosomes for conventional Giemsa banding was not possible due to the slower doubling time of the cells.

without SCF, and double in numbers in approximately 3 weeks in the presence of 100 ng/ml SCF (Fig. 1A). The slower doubling time reflecting cell proliferation and death, in contrast to 3–5 days for some tumorigenic cell cultures, may allow LAD cells to exhibit a more mature phenotype (Fig. 2C and D). The addition of rhIL-3, rhIL-5, or rhIL-6 did not influence LAD cell numbers, while rhIL-4 reduced cell numbers (Fig. 1A).

LAD cells by all criteria were mast cells (Fig. 2) and expressed surface FcεRI, CD4, 9, 13, 22, 45, 64, 71, 103, 117, 132, CCR5 and CXCR4, and to a lesser degree, CD14, 31 and 32 [11–13]. The presence of functional FcεRI receptors is an important feature of the LAD mast cell cultures, with IgE-mediated β-hexosaminidase release of approximately 40% (Fig. 1C). Naïve LAD cells also express FcγRI (CD64), which is up-regulated in the presence of IFN-γ. LAD cells sensitized with IgG1 followed by anti-human IgG1, or F(ab')₂ anti-CD64 fragments released 65 and 87% β-hexosaminidase, respectively (Fig. 1D).

HMC-1.2 cells have the *c-kit* Asp816Val activating mutation, which is known to cause SCF-independent phosphorylation of CD117, and may contribute to the proliferation of these cells. LAD 1 and 2 cells do not exhibit an *c-kit* activating mutation at codon 816 (Fig. 3) [14]. The lack of a mutation at codon 816 in LAD 1 and 2 may explain the requirement for SCF for proliferation of these cells, but does not, however, explain why these cells may be maintained in long term culture.

Studies are underway to determine why LAD 1 and 2 cells persist. One contribution may be that LAD cells ex-

hibit increased levels of telomerase, as in other established tumor cell cultures, in addition to changes in spectralkaryotyping. LAD cells are thus novel human mast cell lines which should facilitate research in human mast cell survival and proliferation studies, and in mast cell activation through FcεRI and FcγRI.

Acknowledgements

Contributions. Arnold S. Kirshenbaum cultured the LAD 1 cell line, developed the freeze/thaw protocol for LAD cell lines, performed growth studies, FACS analysis, activation studies following FcεRI aggregation, and assumed the lead in preparing this report; Cem Akin cultured the LAD 2–5 cell lines, performed growth studies, FACS analysis and mutational analyses; Yalin Wu examined cell lines for intracytoplasmic and *c-kit* activating mutations; Menachem Rottem examined cells for IL-4 induced necrosis and apoptosis; Julie P. Goff performed electron microscopy; Michael A. Beaven examined telomerase expression; V. Koneti Rao karyotyped cells using spectralkaryotyping; and Dean D. Metcalfe was principal investigator on one of the protocols under which these studies were performed, provided resources, funding, and guidance for all research, and assisted in preparation of this report. We wish to thank Darawalee Wangsa for her assistance in karyotyping, and Mitchell E. Horwitz for his assistance in establishing authentication of LAD cells.

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