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13. ABSTRACT *(Maximum 200 Words)*

Muc1 is a cell-associated mucin glycoprotein that is highly over-expressed in greater than 90% of mammary gland tumors. Our studies have focused on elucidating the effect of Muc1 on tumor development, metastasis, and immune function. Muc1-deficient mice were bred with mice expressing the c-neu protooncogene under control of the MMTV promoter to generate spontaneous mammary tumors. These mice developed unifocal tumors between 7 and 18 months of age. Tumor onset, progression, metastasis, and immune function were analyzed in the Muc1-deficient and Muc1-expressing mice. Tumors developed in 93% (51/55) $Muc1^{+/+}$ mice and in 91% (51/56) of $Muc1^{-/-}$ mice. Age of tumor onset was seven weeks lower for mice lacking Muc1, and these mice were 1.5-times as likely to have lung metastasis as $Muc1^{+/+}$ mice. We found that the immune system of the Muc1-deficient mice was severely compromised, as both T cells and natural killer cells failed to be activated and dendritic cells were non-functional. Thus, the lack of Muc1 protein had a dramatic effect on tumor latency, metastasis and general immune competence. These results add a new dimension to the function of this important tumor antigen.

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(4) Introduction

MUC1 is a cell-associated mucin glycoprotein that is highly over expressed in greater than 90% of mammary gland carcinomas. The high level of expression in carcinomas and metastatic lesions suggests an important role in tumor progression and metastasis. Our hypothesis is that MUC1 is a multi-functional protein and that several structural features contribute to its ability to modulate tumor progression and metastasis. These structural features include the large rodlike extracellular domain that extends far out from the cell surface and in all likelihood modulates the adhesiveness of cells (cell-cell and cell-matrix) and the vulnerability of the tumor cells to immune effector cells. The second structural feature of significance is the cytoplasmic tail domain that is phosphorylated and interacts with other phosphorylated proteins and with cytoplasmic proteins that may be involved in signal transduction. Our aims, as modified in the first annual report (August 1998), were to induce tumors in Muc1-deficient and wild type mice by mating them with mice carrying the unactivated c-neu protooncogene driven by the mouse mammary tumor virus long terminal repeat (MMTV- LTR). We further proposed to examine the effects of T cells and NK cells on tumor development and metastasis in these mice and to examine the characteristics of mammary tumor cells derived from the $Muc1^{-/-}$ and $Muc1^{+/+}$ mice.

(5) Body

In order to study the effect of the Muc1 gene on mammary tumor development and progression, 56 female $Muc1^{-/-}$ and 55 $Muc1^{+/+}$ mice were enrolled in this study. All mice were virgin females positive for the c-neu protooncogene, which was transcriptionally regulated by the mouse mammary tumor virus long terminal repeat (MMTV-LTR) [1]. All mice were congenic with the FVB strain, the strain in which the MMTV-c-neu transgenic mice were generated, to eliminate the need to consider strain-specific effects from genes that modify tumor onset and progression [2]. Mice were monitored for tumor development until 78 weeks of age. Mammary gland tumors developed in 93% (51/55) of $Muc1^{+/+}$ (called WT) mice and in 91% (51/56) $Muc1^{-/-}$ (called KO). The average age of onset (defined as a tumor burden of 0.005 g size) of the mammary tumors in the $Muc1^{-/-}$ mice was 39 weeks compared with 46 weeks in $Muc1^{+/+}$ mice. This temporal difference was maintained in the times

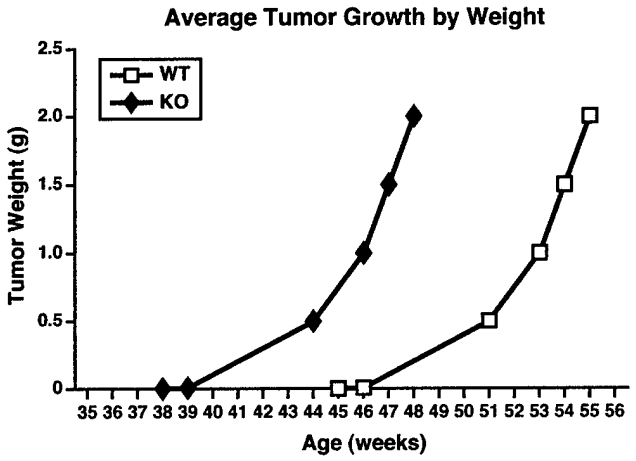

Fig 1. Age at onset of mammary tumors induced by the c-neu protooncogene is accelerated in mice lacking Muc1. Error bars are not shown, as tumor onset is extremely variable in these mice, occurring from about 7 months until 18 months. Tumors were palpated weekly and tumor size determined by the formula weight (g) $=(l \times w^2)/2$.

required to reach the 2 g size (48 weeks for Muc1$^{-/-}$ mice and 55 weeks for Muc1$^{+/+}$ mice (p = 0.08 from the log-rank test) (Fig. 1). Although a p value of 0.08 is not considered highly significant, there is a definite trend for earlier onset of tumors in the Muc1-deficient mice. Since c-neu is a protooncogene and tumor development is reliant upon naturally occurring mutations, tumor onset is slow and highly variable. The highly variable onset of tumors makes it difficult to reach statistical significance, even using an n of 55 animals. Tumor progression correlates with the presence of activating mutations in the juxta-transmembrane region of Neu, resulting in constitutive dimerization of the Neu receptor [3]. The mean duration of observation was 7 weeks significantly shorter on average (95% CI = 2 to 13 weeks) for the KO mice than for the WT mice (KO group: mean = 55 weeks, SD = 14 weeks, N = 60. WT group: mean = 62 weeks, SD = 16 weeks, N = 56. P = 0.013 from the two-sample t test). The mean age at sacrifice was also 7 weeks lower (95% CI = 1 to 13 weeks) for the KO mice than for the WT mice (KO group: mean = 55 weeks, SD = 14 weeks, N = 54. WT group: mean = 62 weeks, SD = 16 weeks, N = 54. P = 0.024 from the two-sample t test).

The rate of tumor progression, once the tumor reached palpable size, was similar in both groups. The median time to progression from a palpable tumor (0.005 g) to 2.0g size was approximately equal between the two groups (Fig. 1. KO group: mean = 10.6 weeks, SD = 3.2 weeks, N = 50. WT group: mean = 11.0 weeks, SD = 4.7 weeks, N = 51, P = 0.57 from the two-sample t test). Within 95% confidence, the means of the two types differ by no more than 2 weeks (95% CI = -2.0 to 1.1 weeks). This similar growth rate was surprising, as we had shown previously, using a different mouse tumor model (MMTV-polyoma middle T antigen), that tumors in Muc1$^{-/-}$ mice grew more slowly than tumors in Muc1$^{+/+}$ mice. However, further analysis of the tumors that arose in the WT mice showed the Muc1 expression had been down-regulated (discussed in greater detail below). Thus, the similar growth rates may occur because once tumors have formed, Muc1 is not longer expressed in this particular tumor model.

The Muc1$^{-/-}$ mice are 1.5 fold as likely to have lung metastasis than Muc1$^{+/+}$ mice. However, the sample size was not large enough to make a conclusive comparison of the incidence of metastasis between the two groups. The sample incidence of metastasis was 38% (21/55) for the Muc1$^{-/-}$ group versus 25% (13/53) for the WT group. The incidence was not statistically significant (P = 0.13 from the chi-square test of homogeneity of proportions).

One possible interpretation of these results is that the immune system may have an effect on tumor progression at a time when the numbers of tumor cells were small, but once the tumors reached a sufficiently large size to be palpable, tumor proliferation overcame the capacity of the immune system to respond effectively. To determine the general immunocompetent state of the immune system in these two groups of mice, T cell blastogenesis assays were performed. Spleen, thymus, peripheral blood lymphocytes and tumor infiltrating lymphocytes were prepared and analyzed for ^{3}H-thymidine incorporation following stimulation with a limiting dilution of Concanavalin A ((ConA) 1 μg/ml down to 0.0625 μg/ml) (Fig. 2). Whereas the T cells from the Muc1-expressing mice were fully capable of proliferating following a six-day stimulation with ConA, the T cells from the Muc1-deficient mice showed virtually no proliferation. Similar results were obtained when T cells were stimulated more specifically with antibodies for CD3ε and CD28 (data not shown). Representative data for four animals is shown. Assays were

performed on 6 animals from each group (P <0.001 by unpaired two-sample t-test).

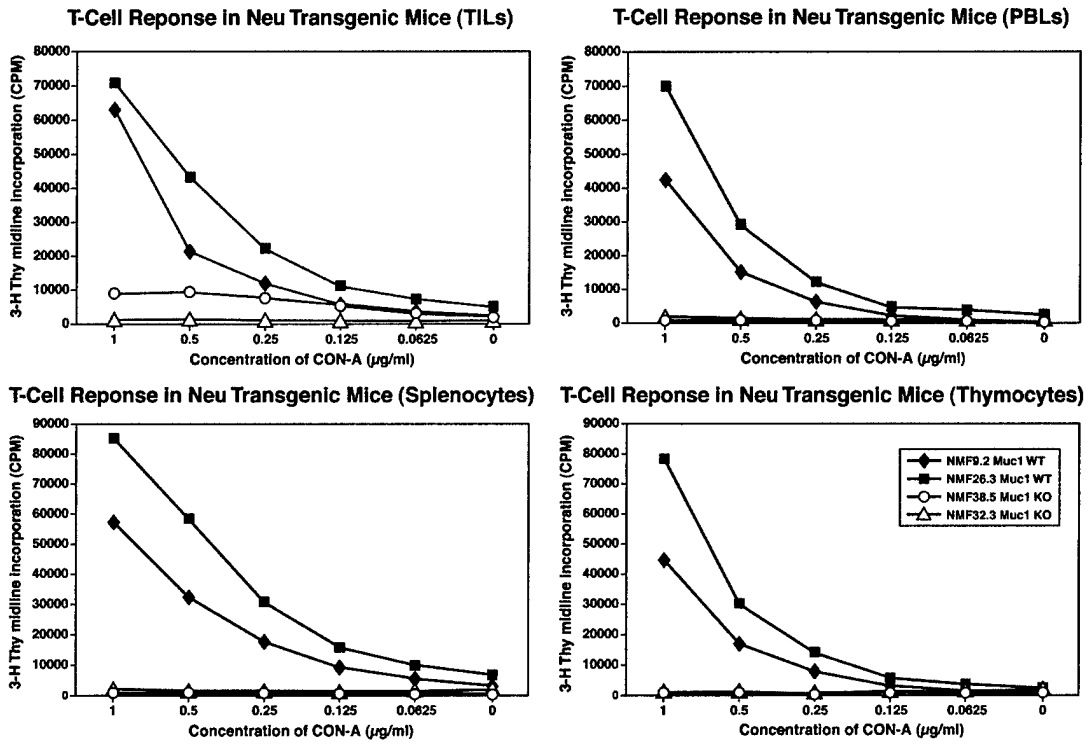

Fig. 2. T-cell blastogenic response is minimal in neu-expressing Muc1$^{-/-}$ mice. Proliferation in CD3+ T cells was determined by ^{3}H-thymidine incorporation in response to varying concentrations of concavalin A in vitro for five days.

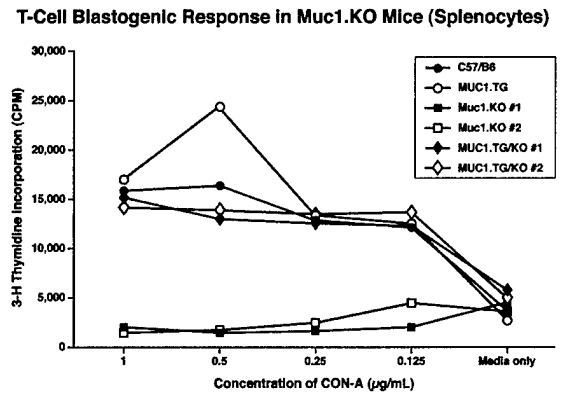

Fig. 3. T cell proliferation in Muc1$^{-/-}$, wt, and MUC1.Tg on Muc1$^{-/-}$ background. Proliferation in CD3+ T cells was determined by ^{3}H-thymidine incorporation in response to varying concentrations of concavalin A in vitro for five days.

To determine that this effect was due to the lack of the Muc1 gene and not to a mutation that occurred elsewhere in the genome during the generation of the Muc1$^{-/-}$ animals, we tested proliferation in human MUC1 transgenic mice on the Muc1$^{-/-}$ background. The MUC1 transgenic line of mice was created using a 10 kb piece of human DNA containing the entire human MUC1 gene using its own endogenous promoter (called MUC1.Tg). We have shown that expression is tissue specific and copy number-dependent, i.e., the transgenic mouse does not overexpress human MUC1, but rather expresses it in a physiological manner [4]. When T-cell blastogenesis was assayed in MUC1.Tg mice

on the Muc1-deficient background, the human MUC1 gene was found to compensate for the loss of mouse Muc1. Uptake of ^{3}H-thymidine was observed in Muc1$^{+/+}$ and in MUC1.Tg mice lacking mouse Muc1 but not in Muc1-deficient mice (Fig. 3).

Muc1 gene may be affecting hematopoietic cell lineages. We and others had recently detected Muc1 expression in activated T cells, NK cells, and dendritic cells. B cells failed to

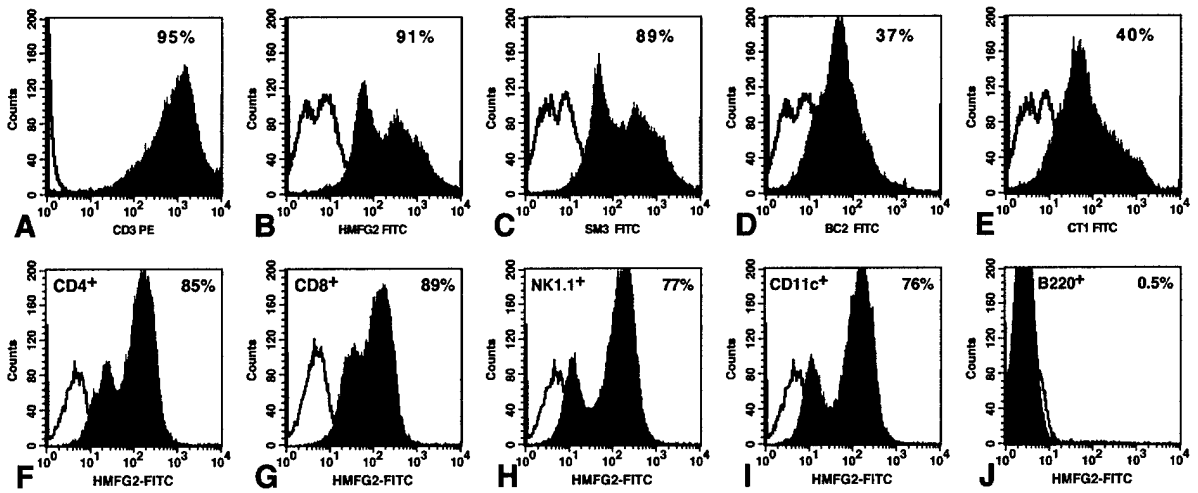

Fig. 4. FACs analysis showing MUC1 expression on T cells, NK cells and dendritic cells. Top Panel. A) Flow cytometric analysis to determine purity of CD3^{+} cells isolated from total thymic cell population of MUC1.Tg mice by MACs. (B-E) Flow cytometric analysis of MUC1 expression on purified CD3^{+} cells isolated from thymocytes of MUC1.Tg mice and stimulated *in vitro* for 6 days with anti-CD3ε and anti-CD28 (1 ug of each antibody for 10^{6} cells). Bottom Panel. Flow cytometric analysis of MUC1 expression on CD4^{+} cells (F), CD8^{+} cells (G) NK1.1^{+} cells (H), CD11c^{+} cells (I) and B220^{+} cells (J). CD4^{+} and CD8^{+} cells were derived from spleen of MUC1.Tg mice and activated *in vitro* for 6 days. These experiments have been repeated 4 times with similar results. Monoclonal antibodies to the MUC1 tandem repeat domain were: HMFG-2, SM-3, BC2 [5]. A polyclonal antiserum, CT1, directed to the MUC1 cytoplasmic tail was used following permeabilization of the cells [6]. Isotype controls are represented by open histograms, stained cells are represented by solid histograms.

express Muc1. Shown in figure 4 is Muc1 expression of FACs scan analyses using a variety of antibodies directed to Muc1. Results in the MUC1.Tg and WT mice have been confirmed by studies in humans, showing a similar pattern of reactivity [7-10].

The failure of the T cells from the Muc1-deficient mice to proliferate was a very surprising result. To more fully assess this phenotype, T cell development was examined. T cell development is characterized by sequential steps that can be easily defined by the expression of particular cell surface proteins. Most T cells develop in the thymus through an ordered maturation process, giving rise to mature T cells with antigen-specific T cell receptors (TCRs). The earliest immature T cells lack expression of CD4 or CD8 and are called double negative

(DN). DN thymocytes express the TCR signaling subunits TCRζ and the CD3 chains, but remain negative for mature TCR until rearrangement of the TCR (γ,δ,β) genes begins. Rearrangement of the β chain allows the TCRβ chain to associate with the invariant α chain to form a pre-TCR complex. Signaling through the pre-TCR allows the differentiation of DN cells to the CD4+CD8+ (double positive [DP]) stage, where rearrangement of TCRα then leads to expression of the mature form of the αβTCR. DP, αβTCR+ thymocytes undergo a number of TCR-mediated selection events and ultimately differentiate into either CD4+ or CD8+ mature single-positive (SP) T cells.

The size and the overall numbers of cells in the thymus, spleen and PBLs of $Muc1^{-/-}$ mice are not dramatically different from their age and sex matched wt control mice. However, FACs analysis of cell populations in the thymus revealed less than 5% $CD4^+/CD8^+$ double positive (DP) population as compared to 76% in wt mice (Fig 5A). In absolute terms, we observed a dramatic decrease in the numbers of DP cells (10^7 down to 10^6 cells) and a 10-fold increase in DN cells in mice that lack Muc1 (Table I). Though there is lack of proliferation in the splenic and peripheral blood lymphocytes, the percent and numbers of single positive (SP) $CD8^+$ or $CD4^+$ cells were not significantly altered in the $Muc1^{-/-}$ mice as compared to their wt counterparts (Fig 5B, C, and Table I). However, these cells expressed significantly lower levels of CD3ε, CD3ζ and TCRαβ (spleen, Fig 5D; PBLs, Fig 5E). In addition, after TCR complex engagement, the SP $CD8^+$ and $CD4^+$ cells failed to express the early and late cell surface activation markers (CD69 and IL2R) (Fig 5F and G). Taken together, these data suggest a profound defect in T cell development and maturation.

Table I. Total cell numbers in thymus and spleen of $Muc^{-/-}$ and $Muc1^{+/+}$ mice.

	$Muc1^{-/-}$	WT
Thymus		
Total cells	$1.7 \times 10^8 \pm 1.7 \times 10^7$ *	$2.3 \times 10^8 \pm 4.7 \times 10^7$
CD8	$1.1 \times 10^7 \pm 1.6 \times 10^6$	$1.1 \times 10^7 \pm 1.8 \times 10^6$
CD4	$1.6 \times 10^7 \pm 3.0 \times 10^6$	$1.1 \times 10^7 \pm 1.7 \times 10^6$
DP	$7.3 \times 10^5 \pm 1.7 \times 10^5$ **	$2.1 \times 10^8 \pm 7.0 \times 10^7$
DN	$1.4 \times 10^8 \pm 1.8 \times 10^7$ **	$1.1 \times 10^7 \pm 1.7 \times 10^6$
Spleen		
Total cells	$1.0 \times 10^8 \pm 2.5 \times 10^6$	$8.6 \times 10^7 \pm 7.8 \times 10^6$
CD8	$9.5 \times 10^6 \pm 6.5 \times 10^5$	$8.5 \times 10^6 \pm 9.6 \times 10^5$
CD4	$1.5 \times 10^7 \pm 8.5 \times 10^5$	$1.5 \times 10^7 \pm 4.7 \times 10^6$
DP	$9.5 \times 10^5 \pm 5.9 \times 10^4$	$7.6 \times 10^5 \pm 1.9 \times 10^5$
DN	$7.9 \times 10^7 \pm 8.9 \times 10^6$	$6.1 \times 10^7 \pm 1.2 \times 10^7$

Table I Legend. Thymocytes from 3 week old C57BL/6 mice were analyzed for their CD4+ and CD8+ T cells by two-color flow cytometric analysis. Average and standard deviation of the meant for five mice/group is shown. *$p<0.005$, **$p<0.001$

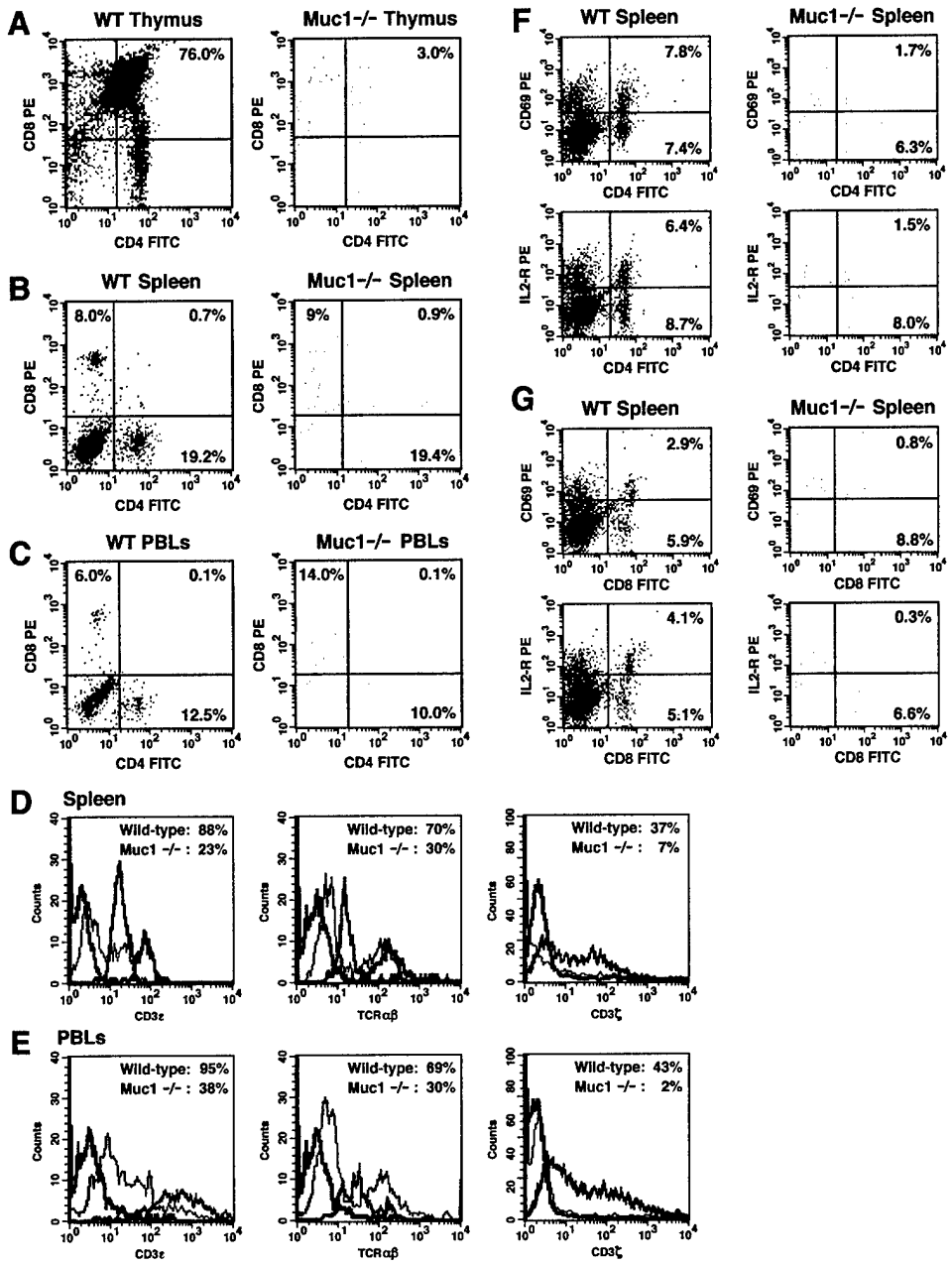

Fig. 5. Flow cytometric analyses of lymphocytes show decreased double positive (DP) population and altered levels of T cell receptor components in Muc1$^{-/-}$ mice. CD4/CD8 profile of thymocytes from 6 to 8 week old adult mice (A), splenocytes (B) and peripheral blood lymphocytes (PBLs) (C) were prepared, surface stained, and analyzed by standard flow cytometry. Panel A shows DP thymocytes make up 76% of the cell population in Muc1$^{+/+}$ mice, whereas in the Muc1$^{-/-}$ mice the DP population is only 3%. Single color staining for CD3ε, TCRαβ and CD3ζ profiles on T cells isolated from spleen (D) or peripheral blood lymphocytes (E) of wt (purple) versus Muc1$^{-/-}$ (yellow) mice versus isotype controls (black) shows alterations in CD3 and TCR levels. Expression of early and late activation markers, CD69 and IL2R on CD4^{+} (F) or CD8^{+} (G) splenic T cells activated *in vitro* for 24h. These experiments have been repeated 6 times with similar results. All mice represented are on the C57BL/6 background.

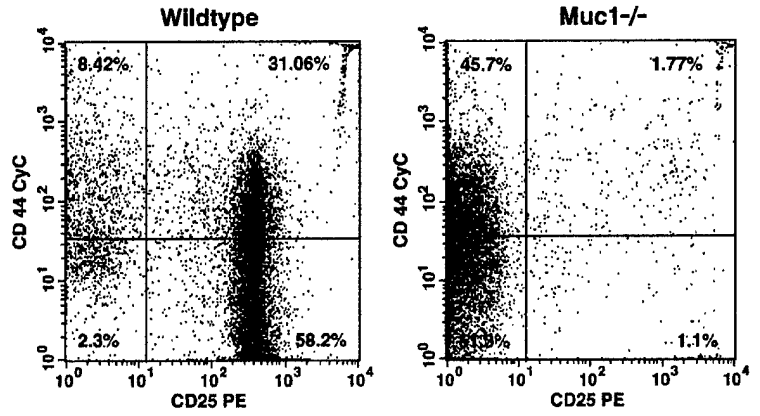

Fig. 6. CD44/CD25 profile of lineage negative thymocytes from Muc$^{-/-}$ versus Muc1$^{+/+}$ mice show blockage at an early developmental stage. Thymocytes from 3-4 week old Muc1$^{-/-}$ mice and wt mice were selected for triple negative populations (TN) and stained with CD25 and CD44. Profiles of these cells are shown with percent positive cells indicated by the number in each quadrant. This experiment has been repeated three times with similar results. All mice represented are on the C57BL/6 background.

By examining the lineage negative population, we were able to determine more precisely at what stage of thymocyte development the block occurred in the Muc1$^{-/-}$ mice. Thymocytes lacking markers CD8, CD4, and CD3 were analyzed for the expression of CD44 and CD25, which define distinct immature T cell populations [11]. Muc1$^{-/-}$ mice showed a block at the most immature T cell stage, as most lineage negative thymocytes were CD44^{+}CD25^{-} (Fig 6). The block appears to be at the developmental step that requires expression and function of a competent pre-TCR signaling complex [12, 13]. The maturational arrest in Muc1$^{-/-}$ mice could be either due to a lack of TCR Vβ chain rearrangement and subsequent pre-TCR expression or defective signaling initiated by the pre-TCR complex.

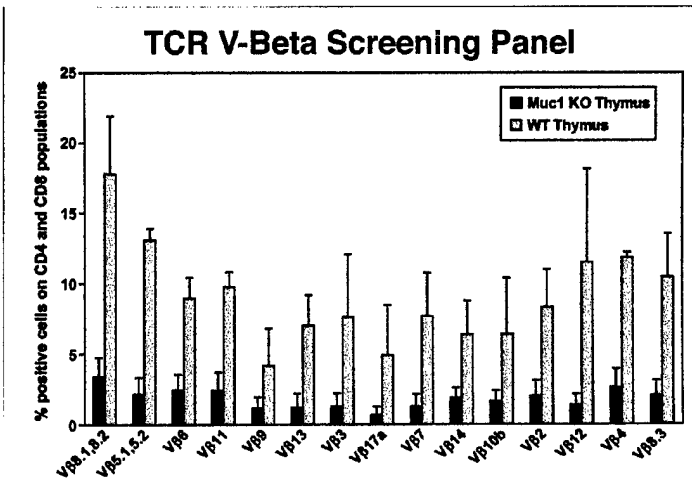

Fig. 7. TCR Vβ chain is expressed at low levels in KO mice. Flow cytometric analysis of Vβ chain expression among CD4+ and CD8+ thymocytes. Error bars indicate the standard deviation.

To determine if the immature thymocytes isolated from Muc1$^{-/-}$ mice had a defect in their TCR Vβ chain gene segment rearrangements, we performed a FACs analysis using a panel of 15 TCR Vβ-specific monoclonal antibodies. We detected a significantly lower percent of thymocytes isolated from Muc1$^{-/-}$ mice with TCR Vβ expression as compared to their wt counterparts (Fig 7), suggestive of improper or incomplete rearrangements. Thus, the absence of Muc1 expression may affect the TCR Vβ chain rearrangement and in turn affect the pre-TCR complex expression and maturational signals.

The ability of Muc1$^{-/-}$ mice to reject allogeneic tumors in the absence of a functional T cell immune response was analyzed by injecting Muc1$^{-/-}$ and wt mice with several allogeneic tumor cells. Muc1$^{-/-}$ mice on the FVB background were injected with B16 melanoma cells derived from C57BL/6 mice of a different haplotype, and Muc1$^{-/-}$ mice on the C57BL/6 background were injected with human breast cancer cell line, T47D. We found that the recipient mice rejected the tumors and remained tumor free (Table II). As expected, these same mice developed tumors when injected with tumor cells derived from mice with the same haplotype. Similar results have shown that mice that lack both CD4 and CD8 are capable of rejecting allogeneic tumors [14]. Nevertheless, these data suggest that Muc1 is not indispensable for allogeneic tumor rejection and that other immune regulatory mechanisms compensate for the lack of T cell responses.

Table II. Muc1 KO mice reject allogeneic and human tumor cells.

Recipient Strain	Donor	Tumors Formed
FVB Muc1$^{-/-}$ (n=5)	C57BL/6 B16 melanoma cells (10^6 cells/mouse)	No
C57BL/6 Muc1$^{-/-}$ (n=5)	T47D human breast tumor cells (10^6 cells/mouse)	No
C57BL/6 Muc1$^{-/-}$ (n=5)	C57BL/6 B16 melanoma cells (10^6 cells/mouse)	Yes

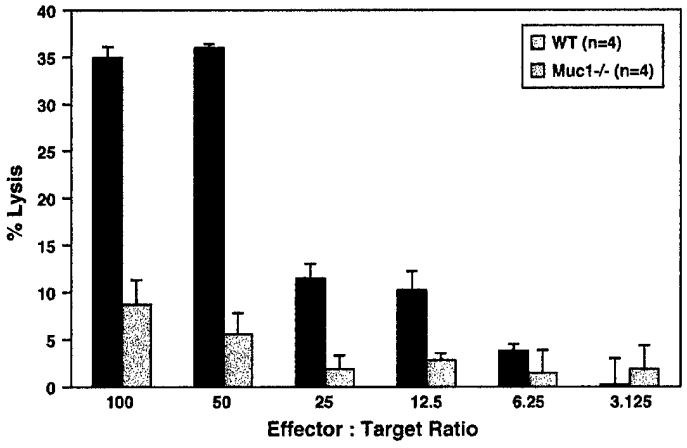

Fig. 8. Lytic activity of NK cells is decreased in Muc1$^{-/-}$ mice. FVB mice, both Muc1$^{-/-}$ and WT mice, were injected with allogeneic tumor cells three days prior to testing splenic NK cells for cytolytic activity against YAC cells. An average of 5 experiments is shown.

Given the observation that NK cells express Muc1, and that these cells may participate in the innate immune response against tumor cells *in vivo*, we tested the NK cell activity from these mice *in vitro*. We injected wt and Muc1$^{-/-}$ mice with allogeneic tumors three days prior to testing the lytic activity of NK cells against the target YAC cells. We observed that the NK cells isolated from Muc1$^{-/-}$ mice had significantly ($p<0.001$) lower lytic activity against YAC cells as compared to the wt mice (Fig 8). Although it is still unclear as to how Muc1$^{-/-}$ mice reject allogeneic tumors, these results show that lack of

Muc1 affects NK cell function *in vitro* and in turn the innate immune response in the Muc1$^{-/-}$ mice *in vivo*. More studies are needed to fully evaluate the activation of resting NK cells *in vivo* and some of these studies are presently under way.

Recent studies from other laboratories have suggested that dendritic cell (DC)-derived cytokines such as type I IFNs or IL-12 are essential in initiating the activation of resting NK cells, thus triggering NK cytolytic activity and γ-IFN production [15]. Because DCs are critical for NK as well as T cell functions [16] and because DCs express MUC1, we examined the functionality of DCs from the Muc1$^{-/-}$ mice. To generate DCs, we cultured bone marrow-derived mononuclear cells from Muc1 KO mice with GM-CSF and IL-4 and found that these cells did not survive in culture nor did they develop into functional DCs (data not shown). Therefore, lack of Muc1 not only alters T and NK cell function, but also affects differentiation and maturation of DCs from pluripotential stem cells.

The data presented here suggest that Muc1 plays an essential role at an early developmental branch point when lymphoid, myeloid and erythroid cell lineages are induced. It remains unclear in which compartment the absence of Muc1 affects this cell fate decision. Muc1-deficient lymphoid precursors could fail to respond to instructive signals within the stromal microenvironment of the bone marrow and/or fail to home to the thymus. Alternatively, these precursors may reach the thymus but may fail to activate one of the essential signal transduction pathways that lead to differentiation and maturation.

Given the observation that B cells from MUC1.Tg mice are negative for MUC1 expression, we hypothesized that B cell function would be least affected by lack of Muc1. B cells seemed to develop normally in the absence of Muc1, since normal B cell phenotype was observed in the Muc1$^{-/-}$ mice (data not shown). We also determined the proliferative capacity of B cells isolated from Muc1$^{-/-}$ mice in response to a polyclonal stimulus, lipopolysaccharide (LPS). No differences in the levels of immunoglobulin isotypes secreted from B cells (Fig 9) were observed between Muc1$^{-/-}$ mice and the wt control mice, demonstrating that the lack of Muc1 does not interfere with T-independent B cell function. T-dependent B cell functional studies are currently under investigation. All of our studies, with the exception of DCs, have examined the cells of the lymphoid lineage. Therefore, the characterization of cells from the myeloid and erythroid lineage is necessary and is underway.

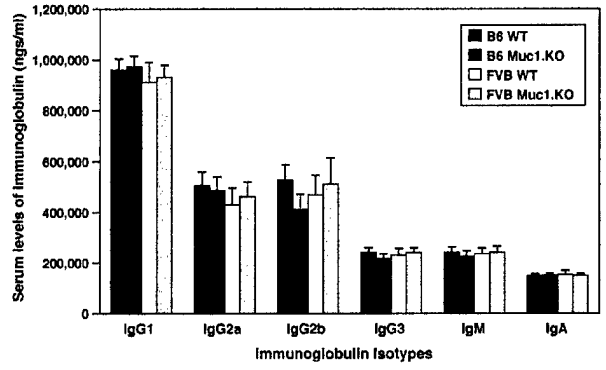

Fig. 9. Proliferation of B cells in Muc1-/- versus WT mice. Mice were injected intraperitoneally with LPS. Serum was collected from four bleeds starting 3 days after the first injection and weekly thereafter and was analyzed for immunoglobulin isotypes by ELISA. Both C57BL/6 and FVB mice are represented. An average of 5 experiments is shown.

Although the lack of a functional immune system in Muc1-/- mice could account for the earlier tumor onset and increased incidence of metastasis, it was difficult to explain the similar rate of tumor progression in $Muc1^{-/-}$ and $Muc1^{+/+}$ mice (Fig. 1). We had shown previously in MMTV-driven polyoma middle T antigen (MTag) mammary tumor mice that tumors lacking Muc1 grew more slowly than tumors expressing Muc1 [17]. However, our analysis of neu-induced tumors in $Muc1^{+/+}$ mice showed virtually no expression of Muc1 (Fig. 10A,D; Table III), although apparently normal mammary tissue around the tumors expressed Muc1 on the apical surfaces of ducts and glands at levels that would be expected (Fig. 10B). It appears that Muc1 protein was down-regulated in tumors expressing large amounts of neu protein. This result was surprising, as Muc1 has been found to be expressed in more than 90% of mammary tumors in both humans and mice [9, 17]. Tumors showed strong expression of neu protein by immunohistochemistry (Fig. 10C) and western blot analysis (data not shown). Western blot analysis using antibodies specific for Muc1 (CT2) and neu (Santa Cruz Biotechnology) confirmed the immuno-histochemical results (Fig. 11). Our hypothesis is that neu negatively regulates Muc1 expression in the neu tumors and that downmodulation of Muc1 expression gives the neu-expressing neoplastic cells a selective advantage during tumor evolution. Studies are underway to test this hypothesis. Interestingly, a discordant pattern of expression of MUC1 and erbB2/neu has also been noted in renal cell carcinomas in humans [18].

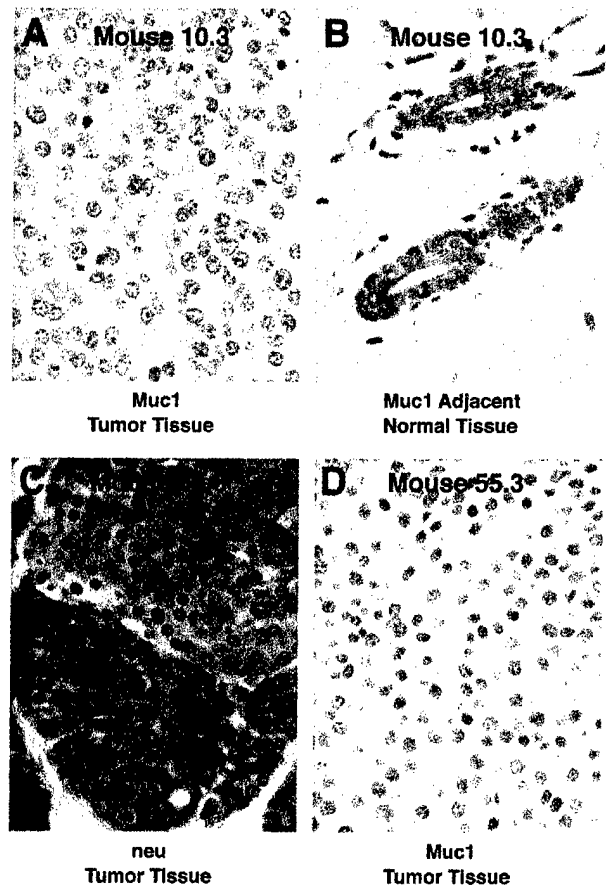

Fig. 10. Immunohistochemical analysis of neu-induced tumors in $Muc1^{+/+}$ mice lacked Muc1 expression. Tumors were fixed in methacarn, embedded in paraffin, and 5 micron sections stained with a hamster monoclonal antibody to the MUC1 cytoplasmic tail or to neu protein (Santa Cruz Biotechnology).

The lack of Muc1 expression in neu tumors in $Muc1^{+/+}$ mice made less relevant our previous plans to measure tumor cell binding to extracellular matrix components. Since the tumors in both groups of mice lacked Muc1 expression, it would be difficult to reach a definitive answer regarding Muc1 influence on cell-cell or cell-matrix binding.

Table III.

Immunohistochemistry	Muc1$^{+/+}$ (n = 47)
Positive for Muc1 staining	3
Negative for Muc1 staining	44

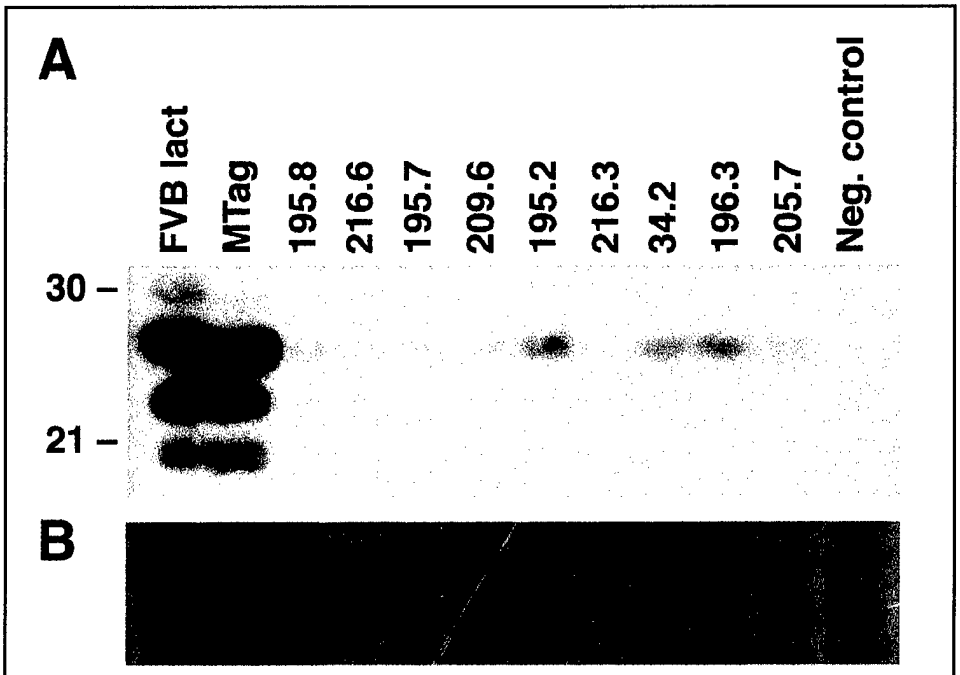

Fig. 11. Western blot analysis for Muc1 protein shows non-detectable to low levels of expression. A. Lysates (200 ug) from neu-induced tumors, lactating mammary gland (positive control), an MTag tumor (positive control) and Muc1$^{-/-}$ neu tumor (negative control) were run on SDS-PAGE and blotted with CT2, a hamster monoclonal antibody that specifically recognizes the Muc1 cytoplasmic domain. The three bands represent the band pattern for Muc1. The lactating mammary gland and MTag tumor lanes show the expected levels of Muc1 protein. B. Poinceau S staining shows approximately equal loading of protein in each lane.

(6) Key Research Accomplishments:

c-neu-induced mammary gland tumors in $Muc1^{-/-}$ mice show:

- Seven week decrease in tumor latency (39 weeks vs. 46 weeks)
- Growth rate similar in $Muc1^{-/-}$ and $Muc1^{+/+}$ mice once tumors are of palpable size
- 1.5-fold increased risk of lung metastasis
- Severely compromised immune system
- Failure of T cells to proliferate in response to ConA or TCR stimulation
- Failure of NK cells to activate properly
- Failure of dendritic cells to form
  - T cell receptor expressed at extremely low levels on T cells
  - Thymocyte development arrested in CD4/CD8 double negative stage
- Human MUC1 transgene compensates for the deletion of mouse Muc1 gene in T cell proliferation assay

(7) Reportable Outcomes

Manuscripts – none

Abstracts

Mukherjee, P., Canales, N., Sterner, C.J., and Gendler, S.J. Expression of MUC1 mucin on activated T-cells from human MUC1 transgenic mice (MUC1.Tg) : Implications in the development of breast cancer. *American Association for Cancer Research,* April, 1999.

Mukherjee, P., Madsen, C.S., Sterner, C. and Gendler, S.J. MUC1, a cell-associated mucin, is essential for normal functioning of the immune system. *Keystone Symposia: T Lymphocyte Activation, Differentiation and Death,* January, 2000.

Gendler, S.J., Mukherjee, P., Ginardi, A.R., Madsen, C., Sterner C., Adriance, M.C., Schroeder, J., Rowles, J. Canales, N., Reid, B., Thompson, M., and Hinojosa-Kurtzberg, M. Role of MUC1 in Tumor Progression and Immune Regulation. *Mucins in Health and Disease,* July, 2000.

Sterner, C., Mukherjee, P., Ginardi, A.R., Adriance, M., Hentz, J. and Gendler, S.J. Mammary tumors in Muc1 deficient mice show a significant decrease in tumor latency, increase in metastasis, and a defective immune system. *Mucins in Health and Disease,* July, 2000.

Mukherjee, P., Ginardi, A.R., Sterner, C.J. and Gendler, S.J. MUC1 plays a critical role in T cell activation and development. *Mucins in Health and Disease,* July, 2000.

Gendler, S.J., Sterner, C. and Mukherjee, P. Mammary tumors in Muc1-deficient mice show a significant decrease in tumor latency, an increased risk of metastasis, and a compromised immune system. *Era of Hope, June, 2000.*

Presentations

Lerner Research Institute, Cleveland Clinic, June 1999.
Mammary Gland Biology Conference, Il Ciocco, Italy, May 2000.
Sixth International Workshop on Carcinoma Associated Mucins, Cambridge, U.K., July 2000.

Patents/Licenses – none

Degrees obtained – none

Cell lines developed from $Muc1^{+/+}$ c-neu tumors and from $Muc1^{-/-}$ c-neu tumors.

Animal models – Muc1-deficient c-neu mice developed.

Funding Applied For

Source:	NIH
Investigator:	Melissa Adriance
Mentor:	Dr. Sandra J. Gendler
Title:	Minority Predoctoral Fellowship Program
Amount:	$17,900 first year
Dates:	09/01/2000 – 08/31/2003

Source:	U.S. Army Medical Research and Materiel Command
Investigator:	Melissa Adriance
Mentor:	Dr. Sandra J. Gendler
Title:	ErbB2 mediated downmodulation of Muc1 in the MMTV-c-neu transgenic mouse model of breast cancer
Amount:	$22,000 first year
Dates:	03/01/01 – 02/28/04

Employment Opportunities – none

(8) Conclusions

In summary, our studies have focused on elucidating the effect of the Muc1 mucin on tumor development and metastasis. We bred Muc1-deficient mice with mice expressing the c-neu protooncogene under control of the MMTV promoter to generate spontaneous mammary

tumors. Tumor onset in the Muc1$^{-/-}$ mice occurred seven weeks earlier than in Muc1$^{+/+}$ mice and progressed at equivalent rates to the 2 gram size ($p = 0.08$). Risk of metastasis was about 1.5-fold higher in the Muc1-deficient mice. The early onset and increased incidence of metastasis in the Muc1 knockout mice suggested an immune system defect. We hypothesized that small numbers of tumor cells could be effectively eradicated by an effective immune system. However, once the tumor number reached a critical size, the immune system may be less effective. We found that immune function was severely compromised in Muc1-deficient neu mice. T cells from Muc1-deficient splenocytes or tumor infiltrating lymphocytes failed to proliferate following ConA or T cell receptor stimulation. Muc1-deficient mice also had defective NK cell killing activity and non-functional dendritic cells. These immune system defects could account for the early onset of tumors and increased metastasis in the Muc1$^{-/-}$ mice.

Muc1 protein is expressed at fairly high levels on T cells, NK cells, and DCs. Our results demonstrated that Muc1 is critically required for (i) the differentiation of DN to DP cells in the thymus, (ii) T cell proliferation in response to CD3ε and TCR crosslinking, (iii) NK cell function, and (iv) DC maturation. In addition, we demonstrated that Muc1 is not indispensable for T cell-independent B cell responses and allogeneic tumor rejection. The profound perturbation of thymocyte development in Muc1-deficient mice underlines the crucial role of Muc1 in T cell ontogeny, because other mucins or proteins are unable to compensate for the absence of Muc1. The molecular consequences of the absence of Muc1 in lymphoid cells remain elusive. One can speculate a signal transduction role as an adaptor protein in T cell fate induction and/or differentiation and maturation of T cells in the thymus. Our evidence that Muc1 is phosphorylated and interacts with a number of T cell kinases supports this theory (unpublished data). While Muc1 is uniquely structured to act as a receptor, its ligand is presently unknown. The significance of a transmembrane protein with an extended mucin domain that is heavily O-glycosylated should be considered, as O-linked oligosaccharides are just beginning to be appreciated as signaling molecules [19, 20]. These structural features of Muc1 argue that it is involved in transmitting signals from the extracellular environment to the cell cytoplasm in both epithelial and non-epithelial cell types. Thus, the lack of Muc1 protein had a dramatic effect on tumor latency, metastasis and general immune competence. These results add a new dimension to the function of this important tumor antigen.

(9) References

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(10) Appendices

None

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