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Normal Development of Mice Deficient in β_2M , MHC Class I Proteins, and CD8⁺ T Cells

BEVERLY H. KOLLER, PHILIPPA MARRACK, JOHN W. KAPPLER, OLIVER SMITHIES*

Major histocompatibility class I proteins display viral and self antigens to potentially responsive cells and are important for the maturation of T cells; β_2 -microglobulin (β_2M) is required for their normal expression. Mouse chimeras derived from embryonic stem cells with a disrupted β_2M gene transmitted the inactivated gene to their progeny. Animals homozygous for the mutated β_2M gene were obtained at expected frequencies after further breeding. The homozygotes appeared normal, although no class I antigens could be detected on their cells and the animals are grossly deficient in CD4⁻CD8⁺ T cells, which normally mediate cytotoxic T cell function.

AT LEAST 26 CLASS I HEAVY CHAIN genes are encoded in the classic major histocompatibility complex (MHC) and the adjacent Qa/Tla region of the mouse genome (1). On cell surfaces these heavy chains are noncovalently associated with a 99-amino acid light chain, β_2M (2). There have been reports suggesting that β_2M has additional functions that are independent of its association with the class I heavy chains (3); most notably, β_2M is identical to thymotaxin and may be involved in migration of hemopoietic stem cells from the bone marrow (4). In cell lines that fail to express β_2M , class I heavy chains accumulate in the endoplasmic reticulum and are not further processed to give cell surface proteins (5). Beginning at the midsonite stage of embryogenesis (6), the classical class I antigens (H-2K and H-2D/L in the mouse) are expressed on the cell surfaces of almost all somatic tissues where, upon binding fragments of self and foreign antigens, they become potential ligands for $\alpha\beta$ T cell receptors (7). During development, T cells possessing receptors that bind strongly to complexes of MHC proteins and self antigens are eliminated by the thymus (8). T cells possessing receptors with low affinity for self MHC are allowed to mature by a process known as positive selection, and subsequently they migrate to the periphery. Positive selection is also thought to determine

the phenotypes of the mature T cells. Interaction of the $\alpha\beta$ receptors on thymocytes with class I MHC leads to a mature T cell bearing CD8; interaction of the thymocyte $\alpha\beta$ receptor with class II MHC molecules leads to a mature T cell bearing CD4 (9).

A great deal has been learned about the selection of class I restricted T cells, that is, T cells that recognize antigens associated with specific class I proteins. Less is known about the involvement of class I restricted T cells in the immune response to various pathogens, in immune surveillance, and in the regulation of the immune system. Furthermore, although the structure and

expression of the nonpolymorphic class I related proteins (Qa/Tla) has been well characterized, their function has remained elusive. Some of these nonpolymorphic class I genes are expressed during early stages of embryogenesis (10), and the suggestion has been made that they may be involved in cell-cell interactions during development. Their early expression and their restriction to specific lymphoid populations in the mature animal has led to their designation as differentiation antigens. In addition, there have been reports that the interaction of class I molecules with a number of cell surface proteins may be of biological significance (11) and that class I molecules secreted in urine may provide olfactory signals that influence mating strategies (12).

The various functions that have been ascribed to class I proteins, and vicariously to β_2M , suggested to us the value of studying the effects in embryos and mature animals of an absence of β_2M and its dependent proteins. We therefore disrupted the β_2M gene in mouse embryonic stem (ES) cells by homologous recombination (13), as did Zijlstra *et al.* (14). ES cells are pluripotent and, when introduced into mouse blastocysts, can contribute to all tissues of the resulting chimeric animals, including the gonads (15). Alterations made in the ES cell genome can therefore be passed on to subsequent generations (14, 16). Two independent ES cell lines, E1439B and E1422A, carrying a disrupted β_2M gene were isolated (13), but only chimeras generated with cell line E1422A showed transmission (Table 1). The five male and two female (17) transmitting chimeras were mated to

Table 1. Generation of chimeric offspring. Chimeras were scored by the presence of agouti, chinchilla, or cream coat color contributed by the E14TG2a-derived ES cells (129/Ola, A^w, c^{ch}, p) on the black background of C57BL/6 mice.

Cell line	Blastocysts transferred	Progeny born (% of blastocysts transferred)	Chimeras (% of progeny born)	Female	Male	Transmitting chimeras
E1439A	144	51(35)	39(76)	17	22	0
E1422A	108	38(35)	30(79)	17	13	7

Table 2. Breeding data from transmitting chimeras. The percent transmission of the ES genome was scored by the presence of agouti offspring after breeding with either C57BL/6 or B6/D2 mice.

Mouse number	Sex	Number of litters	ES cell-derived progeny/total progeny (% transmission)
28.2	M	6	3/28 (11)
29.2	M	6	13/50 (26)
30.1	F	2	3/6 (50)
31.2	M	2	8/8 (100)
31.2	F	1	3/3 (100)
33.1	M	4	10/23 (43)
35.2	M	4	10/21 (48)

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C57BL/6 animals or, in a few instances, to B6/D2 (Charles River), and transmission of the ES cell genome was scored by the birth of agouti offspring (Table 2). (The ES cell line is homozygous for the dominant agouti allele A_w , whereas C57BL/6 and B6/D2 are homozygous for the non-agouti allele.) Southern blot DNA analysis of tail DNA indicated that the agouti offspring inherited a copy of the mutated β_2M allele at the expected frequency of 50%. Heterozygous animals with the mutated β_2M allele were mated to each other, and DNA from the tails of their offspring was analyzed for the presence of the mutant gene. Animals homozygous for the mutant allele were present at the expected frequency of one in four. The animals appeared healthy and could not be distinguished visually from their litter mates. Thus the absence of β_2M , and therefore presumably the absence of cell surface expression of native class I proteins, did not appear to affect normal development.

To verify that the disruption of the β_2M gene resulted in loss of cell surface expression of class I genes, animals homozygous for the mutated gene were sacrificed, and their lymph nodes, spleen, and thymus were examined. The lymphoid organs from the $\beta_2M^{-/-}$ animals were of normal size and contained normal numbers of cells. Cells isolated from each of the tissues were exam-

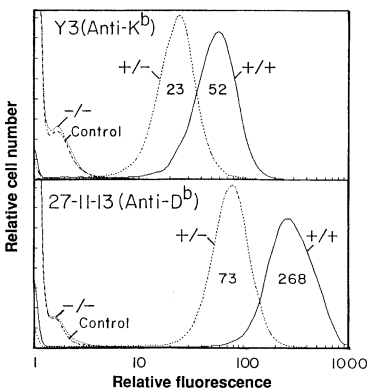


Fig. 1. Mice lacking a functional β_2M gene fail to express surface class I MHC proteins. Cells were isolated at 5 weeks of age from the lymph nodes of a normal mouse (+/+) and one heterozygous (+/-) and one homozygous (-/-) for the disrupted β_2M gene. These cells were stained with fluoresceinated MAb Y3 (anti- K^b) or biotinylated 27-11-13 (anti- $H-2D^b$) followed by phycoerythrin-coupled streptavidin (PE-streptavidin). Control staining reflects the background with no fluoresceinated antibody, or with PE-streptavidin alone. Staining profiles were measured on an EPICS C cytofluorograph using a three-log board. Identical results were obtained with a second group of animals 4 weeks of age.

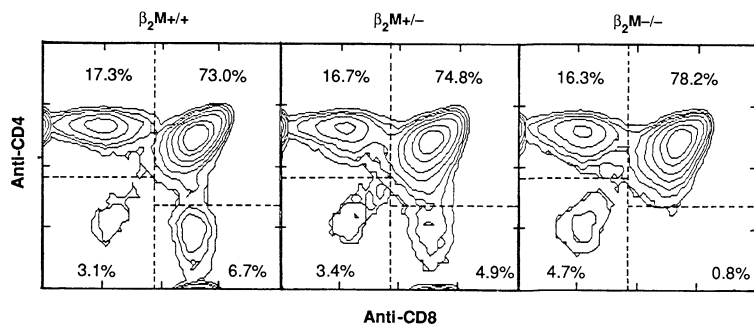


Fig. 2. Thymocytes were isolated from a normal mouse ($\beta_2M^{+/+}$) or from a mouse heterozygous ($\beta_2M^{+/-}$) or homozygous ($\beta_2M^{-/-}$) for the disrupted β_2M gene. These cells were stained with fluoresceinated 53-6 (rat MAb to mouse CD8) and biotinylated GK1.5 (rat MAb to mouse CD4) followed by PE-streptavidin and 3×10^4 of each type were analyzed on a Coulter Profile cytofluorograph using a four-log board.

ined with monoclonal antibodies (MAbs) to the class I antigens $H-2K^b$ and $H-2D^b$. Both 129/O1a, the mouse strain from which the ES cell line was derived, and C57BL/6, the strain with which the chimeras giving rise to these animals had been mated, express the $H-2^b$ haplotype. No staining above background was seen with cells obtained from the lymph nodes of homozygous $\beta_2M^{-/-}$ mice with antibodies against $H-2K^b$ or $H-2D^b$, whereas mice heterozygous for the mutation expressed reduced levels of class I antigens when compared to their normal littermates (Fig. 1). Similar patterns were seen upon examination of spleen and thymus cells with the same MAbs, as well as with a rat antibody that recognizes most mouse class I molecules. Thus, disruption of the β_2M genes results in animals with no detectable cell surface expression of native heavy chain complex. Allen *et al.* have reported that $H-2D^b$ can reach the cell surface in the absence of β_2M (18). In this instance, however, its conformation is significantly altered, as it fails to bind most antibodies specific for $H-2D^b$ or to function as a ligand for cytotoxic T cells. Our experiments would probably not have detected $H-2D^b$ in such an altered configuration.

The interaction of maturing T cells with MHC-antigen complexes in the thymus is important both for the deletion of undesirable self-reactive T cells and for the positive selection of T cells able to recognize foreign antigens. We therefore examined the effect of the lack of class I antigens on the maturation of T cells by isolating and staining thymocytes with antibodies that delineate various stages of T cell differentiation. Lymphoblasts initially lack CD4 and CD8 proteins, but begin to express both of these molecules on their surfaces after entering the thymus. Subsequently these cells also begin to express the T cell receptor (TCR) (19).

Thymocytes expressing TCRs with high affinity for self-MHC molecules, either alone or in association with self peptides, are deleted in the cortex of the thymus (8). At the same time, positive selection occurs for cells bearing TCRs with presumably low affinity for these antigens, but with the potential for recognizing foreign antigens presented by self MHC. Although the mechanism of this positive selection is not known, interaction of the TCR receptor either with class I or with class II MHC antigens will direct further differentiation of the $CD4^+CD8^+$ cells into $CD4^+CD8^+$ or $CD4^+CD8^-$ T cells, respectively (9). CD4 and CD8 serve as accessory molecules in the interaction between the T cell receptor and MHC antigens. CD8 stabilizes interaction of the T cell with class I antigens, whereas CD4 stabilizes interactions with class II antigens (20).

Thymocytes obtained from 5-week-old animals were stained with MAb to CD4 and CD8 (Fig. 2). The data show that the $CD4^-CD8^+$, $CD4^+CD8^+$, and $CD4^+CD8^-$ cell populations in the thymuses of normal, $\beta_2M^{-/-}$, and heterozygous animals are identical. In contrast, the $CD4^+CD8^+$ populations differ between animals of the different genotypes. $CD4^+CD8^+$ cells represent 10% of the cells of the normal thymus, but less than 1% of the cells in the thymus of the $\beta_2M^{-/-}$ mice. These results are consistent with other work that showed that blocking of class I epitopes with MAbs affected development of $CD4^-CD8^+$ T cell populations (21). The number of $CD4^+CD8^+$ T cells in the heterozygote is also somewhat reduced. The same results were obtained with cells from 4-week-old $\beta_2M^{-/-}$ and $\beta_2M^{-/+}$ animals. The few CD8-staining cells in the $\beta_2M^{-/-}$ mice may include $\alpha\beta^-CD8^+$ thymocytes. If, as has been suggested, these cells are precursors to the $CD4^+CD8^+$ pop-

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ulation, their frequency would not be expected to be altered in the $\beta_2M^{-/-}$ animals. Experiments with thymocytes enriched for this population are necessary to address this question.

To determine whether the absence of the class I genes affected the maturation of T cells, as indicated by the expression of the T cell receptor genes, thymocytes were stained with antibodies directed against either the TCR $\alpha\beta$ or TCR $\gamma\delta$ receptor. No significant difference in the profile of $\alpha\beta$ T cell receptor positive cells was seen in $\beta_2M^{-/-}$ animals compared to normal, indicating that class I antigens are not needed for the maturation of thymocytes to TCR bearing CD4 $^+$ 8 $^+$ or CD4 $^+$ 8 $^-$ cells.

We next examined peripheral T cells for expression of $\alpha\beta$ TCR and CD4 and CD8. The yields of T cells bearing $\alpha\beta$ TCRs from the spleen and lymph nodes of animals lacking β_2M were not significantly different from those of normal littermate controls. As expected, between 20 and 32% of all T cells bearing $\alpha\beta$ TCRs also bore CD8 in $\beta_2M^{+/+}$ and $\beta_2M^{+/-}$ animals (Table 3). Interestingly, although CD4 $^-$ CD8 $^+$ thymocytes were somewhat depleted in β_2M heterozygous animals, the level of CD4 $^-$ CD8 $^+$ T cells in the peripheral lymphoid tissues of these mice were comparable to those of normal littermates. This is perhaps the consequence of some unknown homeostatic mechanism for maintenance of particular CD4:CD8 ratios. In contrast, virtually none of the $\alpha\beta$ T cells expressed CD8 in animals homozygous for the β_2M mutation. A preliminary experiment was done to find out whether the few $\alpha\beta$ T cells that appeared CD8 $^+$ in mutant mice were due to noise in the staining procedures. T cells from these animals were therefore grown for several days on plastic coated with MAb to CD3 and interleukin-2, a procedure that often stimulates the proliferation of CD8 $^+$ T cells preferentially. CD8 bearing $\alpha\beta$ T cells did not appear in greater numbers after such treatment, although $\gamma\delta$ bearing T cells did grow out (below), so we must at present conclude that CD8 $^+$, $\alpha\beta$ cells are virtually absent in animals that lack class I MHC expression. This class of cells normally includes the majority of cytotoxic T cells.

Thymocytes and T cells from spleen and lymph node were also examined for expression of $\gamma\delta$ TCRs (Table 4). The numbers of these cells were similar in $\beta_2M^{-/-}$ mice and controls. The outgrowth experiment (above) showed that the $\gamma\delta$ -bearing cells from $\beta_2M^{-/-}$ animals could proliferate and ~25% were CD8 $^+$. Therefore these studies suggest that $\gamma\delta$ T cells may not require class I expression for their existence, even if they also bear CD8.

Table 3. Mice lacking β_2M are depleted in CD8 $^+$, $\alpha\beta$ TCR $^+$ T cells. T cells were isolated at 5 weeks of age from a normal mouse ($\beta_2M^{+/+}$) and from one heterozygous ($\beta_2M^{+/-}$) and one homozygous ($\beta_2M^{-/-}$) for the deleted β_2M gene, by passage over nylon wool (22). The cells were stained with biotinylated-597 [hamster anti-mouse $\alpha\beta$ TCRs (23)], then with PE-streptavidin and fluoresceinated anti-mouse CD4 or anti-mouse CD8 as in Fig. 2. Staining profiles were analyzed on a Coulter Profile cytofluorograph. Identical results were obtained in a second set of experiments carried out on 4-week-old animals.

Mouse	% $\alpha\beta^+$ T cells bearing			
	CD4		CD8	
	Spleen	Lymph node	Spleen	Lymph node
$\beta_2M^{+/+}$	74.8	75.5	22.4	21.7
$\beta_2M^{+/-}$	64.3	76.9	31.3	23.9
$\beta_2M^{-/-}$	96.7	98.9	2.2	0.6

Table 4. $\gamma\delta$ TCR-bearing T cells appear normal in animals lacking β_2M . Thymocytes and T cells were isolated from animals as in Table 3. Cells were stained with biotinylated GL3 (anti-mouse δ) (24), then with PE-streptavidin and analyzed on a Coulter Profile cytofluorograph.

Mouse	% Cells bearing $\gamma\delta$ TCRs		
	In thymus	In NW spleen*	In NW lymph node*
	$\beta_2M^{+/+}$	0.69	3.57
$\beta_2M^{+/-}$	0.60	3.55	1.83
$\beta_2M^{-/-}$	0.57	3.22	1.62

*T cells purified by passage over nylon wool (NW) columns (22).

The construction of a mouse lacking β_2M and, consequently, also lacking cell surface class I molecules has allowed us for the first time to ask directly a number of questions concerning the functions of these proteins in living animals. The fact that $\beta_2M^{-/-}$ develop normally shows that neither β_2M nor the class I heavy chains associated with it at the cell surface play a vital role in embryogenesis, although we cannot exclude the possibility that some subtle functions may have been disrupted. We also cannot exclude the remote possibility that class I heavy chains not associated with β_2M play some role in development. The normal size of the thymus in the $\beta_2M^{-/-}$ animals and the normal numbers of CD4 $^-$ 8 $^-$ thymocytes in these animals make it unlikely that β_2M is responsible for the migration of bone marrow cells to the thymus. The only lesion found in the animals to date is the virtual absence of CD8 $^+$ TCR $\alpha\beta$ T cells. This indicates that positive selection of CD8 $^+$ class I restricted mature T cells requires their interaction with class I antigens in the thymus. The majority of the cytotoxic T cells in the normal immune system are CD8 $^+$ and recognize foreign antigen when bound by MHC class I molecules. It will be of interest to determine what effects the severe deficiency of these effector cells in the $\beta_2M^{-/-}$ animals, as well as the concurrent absence of class I molecules on all somatic cells, have on immune competence. Our $\beta_2M^{-/-}$ mice, although not germ-free, have been housed in isolator cages and are serologically negative for all

viruses tested. Exposure of these animals to specific infectious agents will allow us to assess the role of cytotoxic T cells in the immune response to a number of pathogens. It will also be possible for the first time to evaluate the role these cells play in the development of neoplasms. In addition, it will be possible to determine whether the absence of expressed class I antigens allows these animals to be universal donors or acceptors of tissue grafts. The ability to create mice with specific genetic lesions provides a powerful means of examining in vivo various components of the complex biological systems of mammals.

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